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Cephalopod research and EU Directive 2010/63/EU: Requirements, impacts and ethical review☆,☆☆

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ABSTRACT

For the first time, European Union legislation on animal research and testing has extended its scope to include invertebrate species—the Class Cephalopoda. EU Directive 2010/63/EU, which was due to be implemented in Member States 1 January 2013, covers all "live cephalopods" used in scientific procedures that are likely to cause the animals adverse effects such as "pain, suffering, distress or lasting harm".
This paper examines practical implications of the new EU law for cephalopod research. It evolved from a meeting of European cephalopod researchers held in Naples in 2011 (EuroCeph), which in turn was stimulated by discussions within The Boyd Group (a UK forum on animal experiments). This paper:

1. describes key requirements of Directive 2010/63/EU;
2. explains the project evaluation process that all regulated scientific projects involving animals must undergo before they can be authorised within Member States;
3. presents a series of hypothetical case studies, to illustrate how, in practice, the principles for project evaluation might be applied in cephalopod research and testing;
4. highlights the need for widely agreed guidance specific to cephalopods, to assist regulators, establishments and researchers in implementing the new law; and
5. concludes with a list of practical steps that researchers might take to ensure compliance with the Directive in the national legislation of all EU Member States.

1. Introduction

A new European Directive on the *Protection of Animals Used for Scientific Purposes* (Directive 2010/63/EU, European Parliament and Council of the European Union, 2010) covers all "live cephalopods" (both adults and juveniles). The Directive, which came into force on 9 November 2010, comprehensively revises and extends current European Union (EU) law on the use of animals in research and testing, bringing this whole Class of invertebrates under its scope—which, until now, has included only vertebrates.

EU member states had to transpose the requirements of the new Directive into their national laws by 10 November 2012, and the new regulations had to be implemented from 1 January 2013.

This paper and its companion in this volume (Andrews et al., 2013) aim to assist European researchers in complying with the new EU Directive, by highlighting key requirements of the new law and exploring their practical implications for the use of cephalopods in scientific procedures. Although primarily aimed at EU researchers, the papers also have wider relevance, as the new EU requirements (e.g. relating to ethical review and practical animal welfare) are likely to impact on standards required by journals and funding bodies for the conduct of research in this area.

1.1. Background to the new EU Directive

There is a long history of legislation on the use of laboratory animals in Europe. In a number of countries, current regulations have evolved

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from statutes first enacted in the 1970s or earlier, with some originating in the 19th century, e.g. Denmark in 1891; Germany in 1883; and UK, one of the first to regulate, in 1876 (European Science Foundation, 2001; US Congress—Office of Technology Assessment, 1986).

Pan-European rules, aiming to harmonise national regulations on the use of vertebrates in research and testing, were put in place in 1986 via EU Directive 86/609/EEC (Council of the European Communities, 1986) and European Convention 123 (Council of Europe, 1986).[1] Directive 2010/63/EU (European Parliament and Council of the European Union, 2010) is the first major revision of EU law on this matter.

Until now, very few rules and regulations on the use of animals in scientific procedures around the world have covered invertebrates (Box 1). Inclusion of cephalopods in the scope of Directive 2010/63/EU therefore marks a major change, which must be transposed into relevant national legislation in all EU countries.

However, although major, the change is not abrupt or unexpected, as work to revise Directive 86/609/EEC began nearly 10 years ago. The revision was prompted by: *i.* disparities in levels of protection of laboratory animals between Member States that it was thought could distort the EU internal market, *ii.* advances in experimental techniques, *iii.* new insights into animal welfare, and *iv.* the evolution of ethical approaches to animal experimentation. Taken together, these factors demanded revision and replacement of Directive 86/609/EEC "with more stringent and transparent measures" (Directive 2010/63/EU: Recital 4, European Parliament and Council of the European Union, 2010).

Progress towards the new Directive has included reports and opinions from European Commission expert committees and an advisory body (2002 and 2004), recommendations from a series of Technical Expert Working Groups (2003), a public consultation comprising questionnaires for both 'citizens' and 'experts' (2006), and an impact assessment (2006–2007), leading to adoption of a first-draft proposal for the revision in November 2008 (see http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). After two readings in the European Parliament, the revised Directive was adopted on 22 September 2010, and came into force on 9 November 2010.

1.2. Major requirements of the Directive relevant to the use of cephalopods

Cephalopods have been included within the scope of the new Directive on the grounds that, as for vertebrates, "there is scientific evidence of their ability to experience pain, suffering, distress and lasting harm" (Directive 2010/63/EU: Recital 8, European Parliament and Council of the European Union, 2010). It is clear that there are gaps in this scientific evidence, and need for better understanding of pain, suffering and distress in cephalopods (see Andrews et al., 2013 for further discussion). However, as far as the Directive is concerned, the decision to include cephalopods and the reasoning behind it are no longer matters for debate.

Cephalopods are "in" the Directive, with the same legal status as vertebrates—hence, like vertebrates, they "should always be treated as sentient creatures", and the animals' welfare "should be given the highest priority", in the context of "keeping, breeding and use" and across their "life-time experience" (Recitals 12 and 31, European Parliament and Council of the European Union, 2010).

The Directive brings a range of new mandatory requirements for research and testing involving cephalopods. Some of these are likely to be familiar to cephalopod researchers currently working within voluntary codes of practice (e.g. requirements for good practice in housing and care and minimising potential suffering throughout the animals' lives); but others will be new to most, if not all, cephalopod researchers within the EU (e.g. requirements for prior authorisation and reporting of regulated procedures).

Key requirements include the following:

I. Before they can begin, all 'projects' involving cephalopods must be authorised by a competent authority appointed by the Member State in which the research is to take place.

   A *competent authority* is a body responsible for implementing a specific task (or tasks), laid down by the Directive, within a Member State—e.g. project evaluation and/or project authorisation. Member States must designate one or more competent authorities to fulfil these tasks.[2]

   A *project* is "a programme of work with a defined scientific objective involving one or more *procedures*" (see below), which can run for a term of up to five years, after which authorisation must be renewed.

   A *procedure* is any use of an animal covered by the Directive for experimental or other scientific or educational purposes, which "may cause the animal pain, suffering, distress or lasting harm equivalent to or higher than that caused by the introduction of a needle in accordance with good veterinary practice". This can include procedures that do not involve any 'invasive' technical acts such as administration of substances or surgery, but which cause psychological distress (such as anxiety) above the threshold level of suffering defined above. Unless specifically justified as part of the authorisation process, procedures may only be carried out at authorised user establishments (see IV below).

   Authorisation is limited to the procedures and purposes described in the application. If, during the life of the project, there is need for any *amendments* to the project plans that may have a negative impact on animal welfare, these must also be authorised.

II. The authorisation process will involve an evaluation of the proposed project, which is discussed in detail below.

III. Personnel who design procedures and projects, carry out procedures, care for, and/or kill animals must be adequately educated and trained, and those in the last three categories must be supervised until they have demonstrated their competence.

   The Directive sets out a list of "required elements of education and training"; and, on this basis, Member States must publish minimum requirements for education and training and for obtaining, maintaining and demonstrating competence. It is also likely that there will be EU-wide guidance on these matters.

IV. Unless scientifically justified, procedures may only be carried out at an authorised user establishment. To be authorised, the establishment must:

   Comply with the requirements of Annex III of the Directive, on the care and accommodation of animals.

   Have sufficient staff on site, who must be adequately educated and trained, and supervised until they demonstrate their competence (see III above).

   Have a "designated veterinarian with expertise in laboratory animal medicine, or a suitably qualified expert[3] where more

[1] Note that: *i.* Council of Europe Conventions are not legally binding as such; *ii.* European Convention 123 is unlikely to be revised in line with the new Directive, as activities relating to animal welfare are currently on hold at the Council of Europe. A list of signatories to and ratifications of the Convention is available at: http://tinyurl.com/bd5w66z.

[2] Further information should be available from the National Contact Points for the Directive in each Member State. See the list published available at: http://ec.europa.eu/environment/chemicals/lab_animals/ms_en.htm.

[3] The phrase 'suitably qualified expert' is included to allow for the fact that general veterinarians may not have received training relating to some species (e.g. marine fish, cephalopods), and so may not have the requisite expertise to advise on health and well-being under certain circumstances. Decisions as to who is best qualified to fill this role at a user establishment will need to be agreed by the competent authority on a case by case basis.

Box 1
Countries that currently include invertebrates in regulations on animal experiments. Please note that apart from the UK two other European countries, Switzerland and Norway (not members of the EU), regulated scientific procedures involving invertebrate species before Directive 2010/63/EU.

Europe. In 1993 the UK extended its Animals (Scientific Procedures) Act 1986 to include one species of invertebrate, the cephalopod *Octopus vulgaris* (although, so far, no regulated use of these animals has been reported, Home Office 2011)[1].
Two other European countries that are not members of the EU also regulate scientific procedures involving invertebrate species: Switzerland covers cephalopods and decapod crustaceans (Swiss Federal Veterinary Office 2011)[2], and Norway covers "squid, octopi, decapod crustaceans and honey bees" (2011)[3].
Outside Europe. Examples include the Australian Government National Health and Medical Research Council's Code of Practice (2004 p.3)[4], which covers "cephalopods such as octopus and squid"; the New Zealand Animal Welfare Act (1999), which includes "octopus, squid, crab, lobster and crayfish" (New Zealand Ministry of Agriculture and Forestry 2000 p.8)[5]; and the Canadian Council on Animal Care's system of self regulation, which includes "cephalopods and some other higher invertebrates [that] have nervous systems as well developed as some vertebrates", inasmuch as they may "warrant inclusion" in requirements for protocol review, because of their capacity to experience pain, stress, distress or other suffering (CCAC p.1 1991)[6].
online resources; last visited, July 2012

1. Home Office, 2011. Statistics of Scientific Procedures on Living Animals Great Britain 2010: time series tables. Home Office, London, UK.
   Available at: http://www.homeoffice.gov.uk/publications/science-research-statistics/research-statistics/other-science-research/spanimals10/.
   Also available at: http://data.gov.uk/dataset/statistics-scientific-procedures-living-animals.
2. Swiss Federal Veterinary Office, 2011. 'Legislation' link to German & French versions of Swiss Animal Welfare Act (2008).
   Available at: http://www.bvet.admin.ch/themen/tierschutz/index.html?lang=en.
3. Norwegian Government, 2011. Norwegian Animal Welfare Act (2009). English translation.
   Available at: http://www.regjeringen.no/en/doc/laws/Acts/animal-welfare-act.html?id=571188.
4. Australian Government National Health and Medical Research Council, 2004. Australian Code of Practice for the Care and Use of Animals in Scientific Procedures. 7th Edition. Australian Government: 85 pp. Norwegian Government (2011). Norwegian Animal Welfare Act (2009). English translation.
   Available at: http://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/ea16.pdf.
5. New Zealand Ministry of Agriculture and Forestry, 2000. The Use of Animals in Research, Testing and Teaching: Users' Guide to Part 6 of the Animal Welfare Act 1999. MAF Policy Information Guide 33. Ministry of Agriculture and Forestry, New Zealand: 62 pp.
   Available at: http://www.biosecurity.govt.nz/regs/animal-welfare/pubs/animals-used-in-research.
   Also available at: http://www.biosecurity.govt.nz/files/regs/animal-welfare/pubs/guide-animal-welfare-act-1999.pdf.
6. CCAC—Canadian Council on Animal Care, 1991. Categories of Invasiveness in Animal Experiments. CCAC, Ottawa: 2 pp.
   Available at: http://www.ccac.ca/en_/standards/policies/policy-categories_of_invasiveness.

appropriate" to advise on the well-being and treatment of animals; and nominate one or more people to take responsibility for:
(a) overseeing the welfare and care of the animals;
(b) ensuring that the staff dealing with the animals have access to information specific to the species involved;
(c) ensuring that the staff are "adequately educated, competent and continuously trained", and supervised where necessary (see III above).

Set up an animal welfare body, which will "focus on giving advice on animal welfare issues", so as to give animal welfare "the highest priority in the context of animal breeding, supply and use", by carrying out the tasks listed in Box 2.

V. Animals "taken from the wild" may not be used in procedures, unless there is scientific and/or animal welfare justification that this is the only way to achieve the objective, and specially bred animals are not suitable.
   Captive breeding of cephalopods is not always straightforward and purpose-bred animals may not be readily available. However, the above provision is worded so as to enable researchers using wild species that are not normally bred for research to make a scientific and/or animal welfare justification for capture from the wild. Any use of wild caught animals must be specifically authorised, and the animals must be captured by appropriately trained and "competent persons, using methods which do not cause the animals avoidable pain, suffering, distress or lasting harm".
VI. Researchers will need to keep careful records of their use of animals and provide statistical information to their national competent authority—including information on the actual severity of procedures (see further discussion below).
   Member States must publish their national statistical information annually, and must also send data to the European Commission, which will "establish a common framework for submitting the information"[4]
VII. Member States should carry out "regular inspections" of establishments that breed, supply and use animals, including cephalopods, covered by the Directive. A "proportion" of these inspections will be without prior warning; and there should be "effective, proportionate and dissuasive" penalties for infringements of the regulations.

1.3. Impact assessment: scope of cephalopod research in the EU

In thinking about the impacts of the new Directive on the use of cephalopods in research, and needs for support and guidance for researchers, it is useful to have an idea of the scale of cephalopod research

[4] Expected to appear at: http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm.

Box 2
Tasks of an animal welfare body, following Article 26—Directive 2010/63/EU.

The animal welfare body shall as a minimum carry out the following tasks:

(a) advise the staff dealing with animals on matters related to the welfare of animals, in relation to their acquisition, accommodation, care and use;
(b) advise the staff on the application of the requirement of replacement, reduction and refinement [the Three Rs—see discussion below] and keep it informed of technical and scientific developments on the application of that requirement;
(c) establish and review internal operational processes as regards monitoring, reporting and follow-up in relation to the welfare of animals housed or used in the establishment;
(d) follow the development and outcome of projects, taking into account the effect on the animals used, and identify and advise as regards elements that further contribute to replacement, reduction and refinement;
(e) advise on re-homing schemes, including the appropriate socialisation of the animals to be re-homed.

in the EU, including the species involved, the type of work carried out, and where it is done.

The EU has for the past 20 years collected statistical data on the use of vertebrates in research and testing (Laboratory Animals: Statistical Reports; see http://ec.europa.eu/environment/chemicals/lab_animals/reports_en.htm). However, there is no equivalent information for cephalopods, because there has been no previous regulation of scientific research involving these animals, and therefore no requirement for reporting.

To attempt to fill this gap, one of us (GP) has carried out a short pilot survey of published research involving cephalopods linked to the Member States of the European Union, as part of a wider more in-depth analysis.

The pilot survey was based on full original papers (not reviews or abstracts) indexed by Web of Science, published between January 2005 and September 2011. The search term "cephalop*"[5] (topic field) combined with the names of all of the EU countries (address field) returned 1231 papers. Each record was then carefully examined, in order to select only papers reporting work likely to be regulated under the new EU Directive (i.e. involving procedures that could cause the animals pain, suffering, distress or lasting harm, at or above the threshold for regulation). This analysis led to the discarding of nearly two thirds of the initial sample, mostly publications concerning palaeontology or fisheries, leaving 432 papers.

In many papers, the country in which the work was carried out was not explicitly stated. However, the affiliations of corresponding authors were assumed to give a reasonable, though imperfect, basis for assigning papers to countries. In 370 of the papers involving cephalopod research that would likely be regulated under the new Directive, the corresponding author was from an EU country, and the number of papers per country is shown, as a map, in Fig. 1.

The original sample of 432 papers in which European authors were involved (regardless of whether they were first author or not) was then further examined in order to gain an overview of the fields of research and cephalopod species used in scientific studies with European involvement. Although some of these studies will not have been carried out in an EU country, there is an argument that, on ethical grounds, such studies should comply with the spirit of the new EU Directive.[6]

Table 1 summarises the major research areas covered by these 432 papers, and Fig. 2 shows the range of species involved. The most studied species were *Sepia officinalis* and *Octopus vulgaris* (23% and 16% of the sample respectively). An examination of species used by location of corresponding author reveals that *S. officinalis* appears to be the preferred species for studies in France, Germany and UK, whereas *O. vulgaris* is the preferred species in Spain and Greece; and that in Portugal and Italy the two species (*S. officinalis* and *O. vulgaris*) appear to be roughly equally studied (Fig. 3).

Overall, a very wide range of species of cephalopod was involved in the publications considered in this survey, including some which do not inhabit EU waters (e.g. genera: *Nautilus*, *Idiosepius*).

1.4. Ensuring compliance with the Directive

Fulfilling the requirements of the Directive will most likely be easier for cephalopod researchers working in establishments that also carry out projects involving regulated procedures on vertebrates. However, this document aims to provide information and guidance that will help all cephalopod researchers to comply with the Directive.

Following a meeting of cephalopod researchers (EuroCeph 2011, see Fiorito, 2011), at which there was a session on impacts of the new EU Directive, members of the cephalopod research community have come together under the umbrella of the newly established CephRes organisation, with a number of aims, including providing mutual support during transposition and implementation of the new Directive, and working (with others) to develop guidance in a number of the areas identified above. To assist in this process, the CephRes web-site (http://www.cephres.org/) presents a range of resources on its EU Directive pages, including an interactive on-line guide to the main provisions of the Directive, with links to further information; a list of action points for cephalopod researchers during the transposition process; and a place for discussion and sharing information about matters relevant to the Directive.

This paper and Andrews et al. (2013), appearing in this special issue, both evolved from EuroCeph 2011 (Fiorito, 2011). Discussion was

[5] This criterion may lead to an underestimation of the research effort, since, in the indexing process, the broad term "cephalopod" might not be included in the topic field, even though the research involves cephalopod species. Furthermore, it should be noted that Web of Science does not include 'grey' literature or published studies that are not peer reviewed (both of which might also include uses of cephalopods that would be regulated by the new Directive).

[6] This approach is increasingly taken by EU researchers and funding bodies operating overseas; for example, the UK Medical Research Council requires that, when collaborating with overseas laboratories, researchers and their local ethics committee in the UK should satisfy themselves that animal welfare standards are consistent with UK legislation covering animal experiments (see: MRC, BBSRC, Wellcome Trust, NERC and NC3Rs, 2008, updated 2010. *Responsibility in the Use of Animals in Bioscience Research: Expectations of the Major Research Council and Charitable Funding Bodies*. MRC, BBSRC, NERC, the Wellcome Trust and NC3Rs, London: 22 pp.).

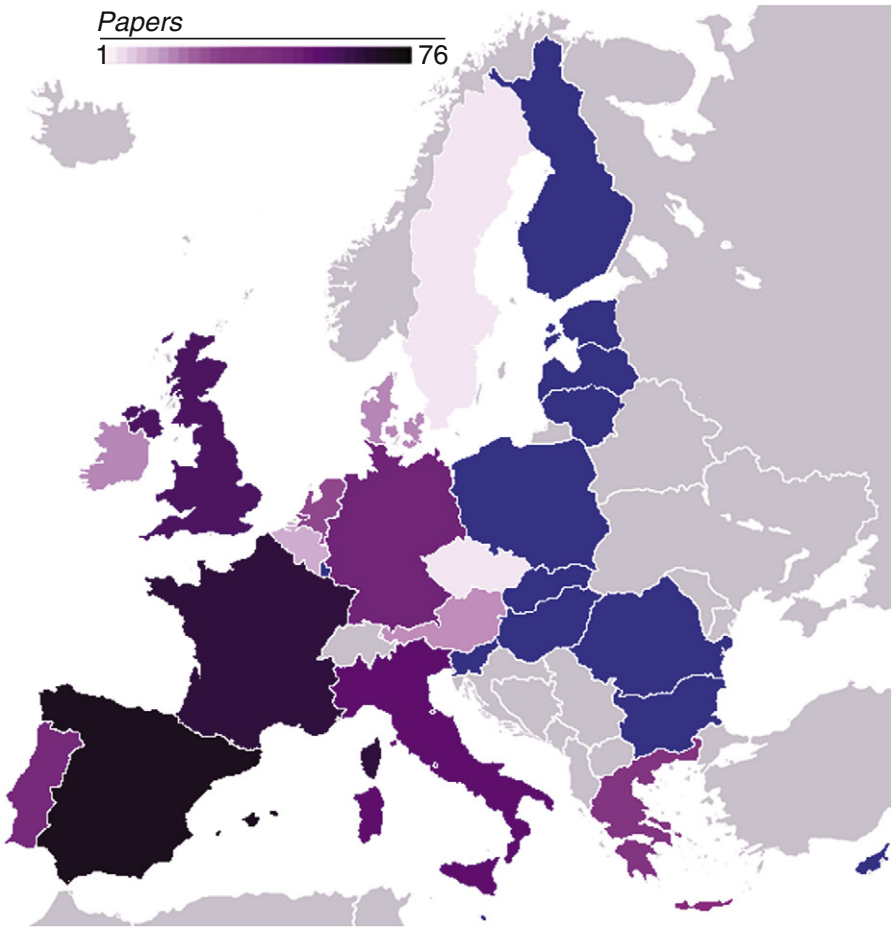

Fig. 1. A geographical overview of the research effort on cephalopod biology in the EU. Only published works that potentially fall within the framework of the Directive 2010/63/EU are included. The number of papers published (n = 370) was assigned to each country on the basis of the affiliation of the corresponding author. Member states of the European Union for which no relevant papers were published are in blue; European countries not in the EU and some surrounding countries are in grey.

originally stimulated by the work of a sub-group of the Boyd Group: a UK forum that brings together a wide range of perspectives on the use of animals in science [for further information refer to: http://boydgroup.wordpress.com/. Together, the papers look in more detail at practical aspects of meeting the requirements of the Directive. This paper discusses requirements for project authorisation and project evaluation, including ethical evaluation, and includes a selection of illustrative examples. Its companion explores the recognition, assessment and management of pain, suffering and distress in cephalopods and methods for anaesthesia and analgesia and humane killing.

Table 1
Major topics of research on cephalopods, in papers published between 2005 and 2011 with author(s) from EU countries.

Research topic	% papers in sample (n = 432)
Aquaculture	10
Behaviour	10
Environment and pollution	8
Genomics/development	5
Life history	29
Neuroscience	6
Physiology	15
Zoology/morphology	17

2. Project evaluation requirements in the EU Directive

2.1. Factors for consideration

The Directive requires that an "impartial project evaluation independent of those involved" is carried out as part of the authorisation process. This takes "ethical considerations" into account (Recitals 38 & 39, European Parliament and Council of the European Union, 2010), and must include the following (Article 38):

a. An evaluation of the objectives of the project
The objectives must fall into one of the categories listed as permissible purposes under the Directive (Box 3). The project must be "justified from a scientific or educational point of view", in terms of the "validity, usefulness and relevance of the expected result" (Article 38(2) (a); Recital 39, European Parliament and Council of the European Union, 2010).
b. An assessment of the project's compliance with the "Three Rs" principles of replacement, reduction and refinement of animal use (Article 38(2) (b) and Annex VI)
This means that, at the application stage and throughout a project, there must be on-going consideration and implementation of strategies for:

- *replacement* of the use of living animals with non-animal alternative methods wherever possible, e.g. by using in vitro methods or modelling studies; or by avoiding the use of animals in regulated

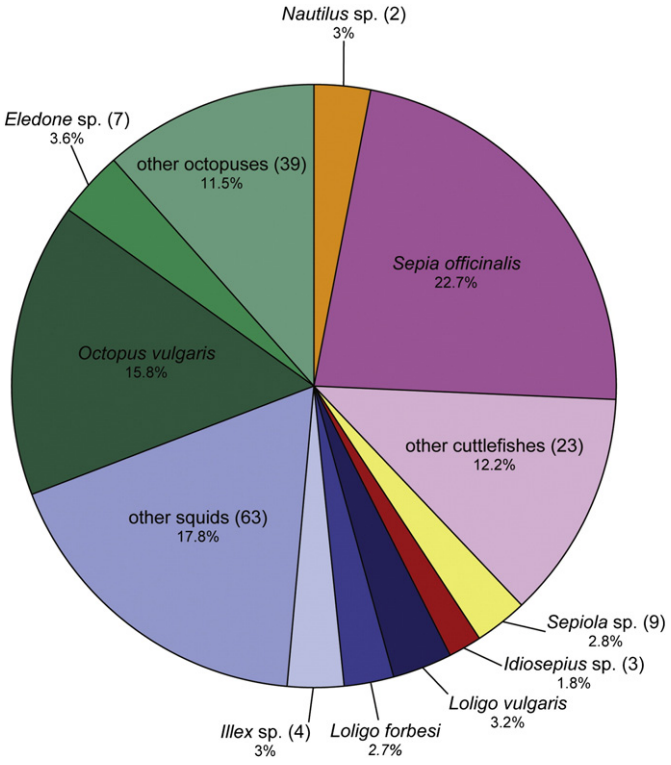

Fig. 2. Cephalopod species (and genera) used in studies published between 2005 and 2011 with author(s) from EU countries. Only species that appeared in at least five publications are included. Each slice shows the percentage of papers per species included in this sub-sample (154 species over the total of 163 counted). Where genera are reported, the number of species is indicated in parentheses.

Box 3
Purposes of procedures, permitted under the Directive 2010/63/EU (Article 5).

Procedures may be carried out for the following purposes only:

(a) basic research;
(b) translational or applied research with any of the following aims:
  (i) the avoidance, prevention, diagnosis or treatment of disease, ill-health or other abnormality or their effects in human beings, animals or plants.
  (ii) the assessment, detection, regulation or modification of physiological conditions in human beings, animals or plants.
  (iii) the welfare of animals and the improvement of the production conditions for animals reared for agricultural purposes
(c) for any of the aims in (b), in the development, manufacture or testing of the quality, effectiveness and safety of drugs, food- and feed-stuffs and other substances or products;
(d) protection of the natural environment in the interests of the health or welfare of human beings or animals;
(e) research aimed at preservation of the species;
(f) higher education, or training for the acquisition, maintenance or improvement of vocational skills;
(g) forensic inquiries.

procedures, e.g. by asking a different type of question; or by making use of existing data or literature;
- *reduction* of the number of animals used, to the minimum needed to achieve the scientific objectives—through use of optimal experimental and statistical design, employing expert statistical advice wherever appropriate; and
- *refinement* of experimental procedures and housing, husbandry and care, so as to cause those animals that *are* used the least possible pain, suffering, distress or lasting harm throughout their lives.

The Three Rs principles were first described by Russell and Burch (1959). The principles are now internationally accepted as an essential requirement for the ethical conduct of scientific procedures involving animals, and it is widely recognised that scientific quality is also improved by acceptance and effective implementation of the Three Rs (Directive, Recital 11, Article 4, European Parliament and Council of the European Union, 2010; also see: CIOMS and ICLAS, 2011; Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011; Ritskes-Hoitinga et al., 2006). Further discussion on the Three Rs is available in a number of publications (e.g.: Balls, 1994a, 1994b, 1994c; Buchanan-Smith et al., 2005; Festing, 1994; Festing et al., 2002; Flecknell, 1994) and web-based information sources such as the Canadian Council on Animal Care Three Rs microsite (CCAC), the UK National Centre for the Three Rs (NC3Rs), and US John Hopkins University ALWEB sites (ALTWEB).[7]

c. An assessment and assignment of the severity classification of the procedures (Article 38(2)(c))
All procedures outlined in a project application must be classified according to the likely severity of their adverse effects on the animals—the categories being "non-recovery" (for procedures carried out wholly under anaesthesia from which the animal does not recover consciousness), "mild", "moderate" and "severe" (Article 15(1) and Annex VIII explain these further and include examples of procedures falling into the different categories).
The severity classification of a procedure sets an upper limit on the level of suffering that an animal undergoing the procedure is allowed to experience (Article 40(1)(b); see LASA/APC "*Final report of a LASA/APC Working Group to examine the feasibility of reporting data on the severity of scientific procedures on animals*", 2008[8] for further discussion).
Before assigning a severity classification, potential adverse effects must first be identified, predicted and minimised as far as possible. Adverse effects covered by the Directive include pain, distress, lasting harm and other forms of suffering, such as hunger, anxiety, boredom and osmotic or thermal stress. These effects may occur at any time during the life of the animals—e.g. as a result of capture and transport to the laboratory, routine handling, housing and husbandry, or method of killing, as well the effects of the procedures themselves.
Anaesthesia must be used during procedures, unless it is judged more traumatic than the procedure itself and/or is incompatible with the purpose of the procedure. Analgesia, or another appropriate method, must be used to ensure that pain, suffering or distress is kept to a minimum (Article 14).

[7] CCAC: Canadian Council on Animal Care, CCAC Three Rs Microsite, available at: http://3rs.ccac.ca/en/. NC3Rs: National Centre for the Replacement, Refinement and Reduction of Animals in Research: 3Rs Information Portal, available at: http://www.nc3rs.org.uk/informationportal. ALTWEB: Alternatives to Animal Testing; John Hopkins University, Bloomberg; available at: http://altweb.jhsph.edu/.

[8] Available at: http://www.lasa.co.uk/publications.html.

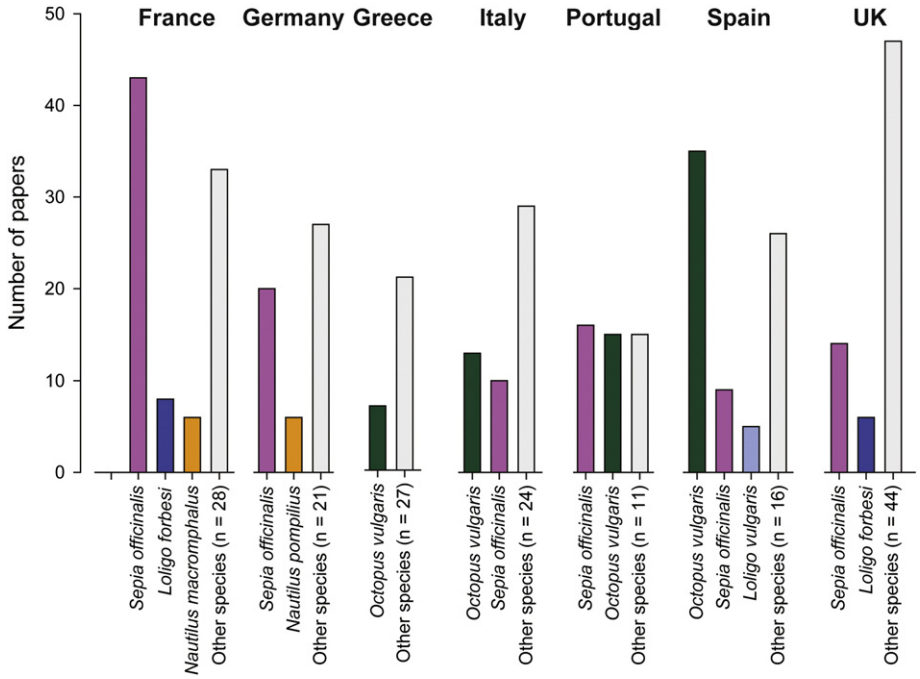

Fig. 3. Estimation of species of cephalopod most frequently used in research and testing in EU countries. Bars show number of papers. Species (colour coded according to Fig. 2) were assigned to countries by considering the affiliation of the corresponding author of papers published between 2005 and 2011. Only species appearing in at least five publications are shown. Data from Austria, Belgium, Denmark, Ireland, The Netherlands and Sweden are not included in this figure due to insufficient papers for any given species.

As part of the project evaluation, information must be provided on the use of humane end-points. Humane end-points are closely linked to the severity classification of a procedure, and describe the circumstances in which the procedure will be stopped for animal welfare reasons, so as to limit the level of suffering experienced by the animal, regardless of whether further scientific results could be achieved (see for example reference to NC3Rs[9] for further discussion and references). When the humane endpoint is reached, immediate action must be taken to reduce suffering, such as humanely killing the animal, taking the animal off the procedure, or administering analgesia. It is also important to note that the Directive states that animals must not be used in procedures that cause "severe pain, suffering or distress that is likely to be long-lasting and cannot be ameliorated"—unless there are exceptional and scientifically justifiable reasons, and then only with authorisation from the European Commission (Articles 15(2) and 55(3)). One intention of this wording is to encourage refinement of the procedure so that does not cause this level of suffering.

d. A harm–benefit analysis of the project (Article 38(2)(d))
The harm–benefit analysis should "assess whether the harm to the animals in terms of suffering, pain and distress is justified by the expected outcome, taking into account ethical considerations, and may ultimately benefit human beings, animals or the environment".
To inform the evaluation, applications for project authorisation must include, at least, the project proposal and the items listed in Box 4. A non-technical project summary may also be required. Requests for exemptions to specific requirements of the Directive (where permitted) must also be 'argued for' in the application (see further detail in Box 4).
The harm–benefit analysis will involve critical evaluation of:

- the potential benefits of the project (see also (a) above);
- the likelihood that these outcomes will be achieved in practice, given the experimental design, facilities and expertise;
- the harms likely to be caused to the animals and the possibility for reducing or avoiding these by further application of the Three Rs (see (b) and (c) above).

There are many specific factors and questions that might be considered within each of these three general areas, and these have been listed and discussed in a wide variety of publications from countries around the world. As an example, Smith et al. (2007) presents a summary 'scheme for assessment of benefits and harms' that draws on a number of these publications, which might serve as a useful starting point and aide-mémoire. The scheme is reproduced in Table 2, along with a list of sources of further information—see especially Animal Procedures Committee (2003) for a detailed examination of procedures for harm–benefit analysis in this area.

e. A determination as to whether and when the project should be assessed retrospectively (Article 38(2)(f))
Retrospective assessment will look back over the project—examining:

- whether and how far its objectives have been achieved;
- the harm caused to the animals, including numbers, species and severity of procedures; and
- elements that might contribute to further implementation of the Three Rs (Article 39.1).

Retrospective assessment is required for all projects involving procedures classified as 'severe', and when determined during project evaluation by the competent authority. Member states may decide to exempt, in advance, all projects involving only 'non-recovery' or 'mild' procedures (Article 39). However, it is good practice, and more in the spirit of the Directive, to retrospectively assess all projects—and researchers can also choose to carry out such reviews for themselves, whether or not the competent authority requires it. As the term 'retrospective' suggests, it is intended that assessment be done at the end of a project as a whole. However, depending on circumstances, it may also be valuable to 'retrospectively' assess at specific point(s) during the lifetime of a project—e.g. to assess the actual adverse effects of a new surgical procedure and review

[9] NC3Rs: National Centre for the Replacement, Refinement and Reduction of Animals in Research, Humane Endpoints. Available at: http://www.nc3rs.org.uk/humaneendpoints.

Box 4
Information to be provided in applications for project authorisation, following Annex VI and Article 38(2) (e)—Directive 2010/63/EU.

1. Relevance and justification of the following:
   (a) use of animals including their origin, estimated numbers, species and life stages;
   (b) procedures.
2. Application of methods to replace, reduce and refine the use of animals in procedures.
3. The planned use of anaesthesia, analgesia and other pain relieving methods.
4. Reduction, avoidance and alleviation of any form of animal suffering, from birth to death where appropriate.
5. Use of humane end-points.
6. Experimental or observational strategy and statistical design to minimise animal numbers, pain, suffering, distress and environmental impact where appropriate.
7. Re-use of animals and its accumulative effect on the animals.
8. The proposed severity classification of procedures.
9. Avoidance of unjustified duplication of procedures where appropriate.
10. Housing, husbandry and care conditions for the animals.
11. Methods of killing.
12. Competence of persons involved in the project.

Article 38(2)(e) also requires that justification is provided for exemptions to certain general requirements of the Directive. Where cephalopods are concerned, these may include requests for permission to:

- use an endangered cephalopod species or cephalopods taken from the wild;
- carry out procedures in a place that is not an authorised users' establishment (see point 4 above);
- re-use (in a different procedure) animals that have already undergone a procedure;
- use drugs such as neuromuscular blocking agents that could limit an animal's ability to show pain;
- depart from any of the general standards of animal care and accommodation outlined in Section A of Annex III of the Directive.

For other species, special justification will also be required for killing animals by a method not listed in Annex IV of the Directive or for departing from standards of animal care and accommodation outlined in Section B (species-specific section) of Annex III. However, at present, cephalopods are not included in either of these.

possibilities for refinement, once the first few surgeries have been completed.

The aim of retrospective review is to ensure that the experience gained in a project is used to inform the design and conduct of any future work, so as to "reduce the harms and increase the benefits… aiming to improve both animal welfare and the quality of science and to help inform future debate on these issues", as well as "to continue to apply the Three Rs throughout the project's duration" (see: Jennings and Howard, 2004; also see pp. 31–36 of RSPCA and LASA, 2010 for further discussion). For this reason, 'looking back' over a project, or parts of it, will also be beneficial as part of the animal welfare body's on-going role in ensuring the implementation of the Three Rs and following the development of projects.

Specific procedures for retrospective review will be determined by each member state during transposition of the new Directive, and, at the time of writing, it is uncertain how, exactly, the different EU countries will implement this requirement in practice. Jennings et al. (2007)[10] and RSPCA and LASA (2010) suggest principles for developing workable and useful retrospective review processes, which might help in implementing this requirement.

2.2. Need for guidance specific to the use of cephalopods

It is clear that, in order to meet the requirements of the Directive, guidelines will be needed on aspects of care and maintenance of cephalopods and the conduct of ethical and humane research involving these species, similar to those that have been developed for vertebrates over the course of many years.

In particular, widely agreed guidance specific to cephalopods will be needed in the following areas covered by the Directive:

i. *Signs of pain, suffering, and distress*. The Directive requires recognition, reduction, avoidance and alleviation of any form of animal suffering, from birth to death where appropriate, but there is uncertainty and debate about appropriate clinical signs indicative of pain, suffering and distress in cephalopods (Andrews et al., 2013).
ii. *Use of humane end-points* (Article 13). That is, the humane "stop-points" in experimental protocols. These are based on signs of suffering, and so, again, there is some uncertainty where cephalopods are concerned.
iii. *Appropriate methods of anaesthesia and analgesia* (Article 14). There is uncertainty about the efficacy of currently used methods in cephalopods.
iv. *Prospective classification of the severity of procedures involving cephalopods* (Article 40). Annex VIII of the Directive provides guidance, but does not include any examples that are specific to cephalopods.
v. *Housing, husbandry and care conditions*. Annex III of the Directive sets standards for housing and care, but does not mention cephalopods specifically.
vi. *Humane killing*. Annex IV of the Directive sets out "appropriate" methods of killing that must be used, unless there is specific scientific justification or it is shown that another method is more humane—but cephalopods are not included.
vii. *Supply and capture of cephalopods*. A range of capture methods is currently used for cephalopods, and there is uncertainty about their relative humaneness and how best to ensure competence in using them. There is also room for debate about the circumstances in which it is appropriate to use wild-caught, rather than purpose-bred, cephalopods in procedures; and under what circumstances it might be acceptable/desirable to re-home cephalopods or release them back into the wild when they are no longer being used in scientific procedures.
viii. *Competence of personnel*. Are the necessary skills and competencies, and therefore requirements for education and training and supervision, of researchers using cephalopods any different from those who use vertebrate animals? If so, there will be need for specific guidance on these matters.

[10] Available online at: http://www.lasa.co.uk/publications.html.

ix. Assessment of actual severity of procedures involving cephalopods. This is required for statistical reporting of animal use (Article 54) and useful guidance on day to day monitoring and the assessment of actual severity is available (European Commission, 2012). See http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm (click on 'severity assessment framework' and 'illustrative examples').

3. Practical processes for project evaluation and ethical review

Under the new Directive, project evaluation and authorisation are tasks for a competent authority (or authorities) designated by the Member State. Provided that *requirements* for project evaluation, including ethical considerations, are met (see above), the organisation of the *processes* by which the evaluations are carried out is likely to vary between different Member States.

At present, in most EU countries, national or regional committees bear overall responsibility for project evaluation of regulated projects involving vertebrate animals, which may or may not include ethical review in the form of a harm-benefit analysis. In addition, the majority of Member States have voluntary local, institutional ethical review processes in some or most establishments; and in four Member States (Belgium, Czech Republic, the Netherlands and the UK) institutional review processes are currently a mandatory part of the project evaluation and authorisation process, working together with national committees and/or inspectorates (Smith et al., 2007).

At the time of writing, Member States are still working to transpose the new Directive, and it remains to be seen whether the present arrangements will change and, if so, what form their competent authorities for project evaluation and authorisation (including a harm-benefit analysis) will take and at what 'levels' (national and/or regional and/or local) they will operate.

3.1. Competencies

In order to provide helpful advice to researchers, and make informed, balanced, sensitive decisions, project evaluation processes will need to include participants with a range of relevant expertise and perspectives. This should cover scientific, animal welfare and ethical aspects, including competencies in relevant scientific fields, animal husbandry, care and welfare, the Three Rs and statistics, and provide for non-technical/public input (see: RSPCA and LASA, 2010; Smith et al., 2007 for further discussion and additional competencies). Where cephalopods are concerned, it will be valuable to include participants with expertise in cephalopod biology, husbandry and care.

3.2. Animal welfare bodies and ethical review

Animal welfare bodies (AWBs) that include the competencies listed above can provide valuable support and advice to researchers and other relevant staff at all stages in a project, from initial idea to completion. As Box 2 shows, AWBs have specific roles in following "the development and outcome of projects" and advising staff on: the welfare of animals at all stages (including acquisition, accommodation, care and use), the application of the Three Rs, "monitoring, reporting and follow-up" of the welfare of animals, and on re-homing schemes.

As part of this work, AWBs can help to ensure that ethical issues arising in the care and use of animals are identified at a local level and addressed appropriately—by, for example:

i. advising on ways of minimising harms and maximising benefits in the design of studies;
ii. helping to prepare the best possible application for authorisation to go to the competent authority;

Table 2
Outline scheme for the assessment of benefits and harms in scientific projects involving animals (i.e. to encompass, at a minimum, all animals covered by current EU Directive 2010/63).
Reproduced after Smith et al. (2007) with permission.

Assessment of potential benefits of the project
How will the results add to existing scientific and/or clinical knowledge and how might they be used?
What practical applications, if any, are envisaged at this stage?
And what is the potential value of these insights and/or applications?

- Are the objectives of the project:
  - original, in relation to previous or on-going studies
  - timely, in relation to other studies that might be done (what is the need to do *this* study, *now*?)
  - realistic, in that they are achievable with the time and other resources available?
- If there is an element of replication of previous work, how strong is the case for this, and what efforts have been made to avoid mere duplication?
- If this is on-going work, how does the present proposal relate to what has gone before? What progress was made in previous studies, and what scientific or other benefits have resulted?
- What is the relevance of this project to other studies in this field of research and what might be the implications for other areas of research, if any?

Assessment of likelihood that the potential benefits will be achieved in practice
Is there is a reasonable expectation that the potential benefits will be achieved in practice, given the:
- choice of animal model and scientific approach
- validity of experimental design (e.g. use of appropriate number of animals involved, appropriate use of controls) and whether and how this has been informed by statistical or other advice
- competence of researchers and other staff, including their training, supervision, experience and expertise
- appropriateness and quality of facilities
- researchers' plans for communicating and using and/or building on the findings of the project?

Assessment of the harms caused to animals and possibilities for reducing these, in terms of
- the need to use animals *at all* (what efforts have been made to seek suitable alternatives to the use of animals in regulated procedures? Has as much information as possible already been gained from in vitro or other ex vivo work?)
- optimisation of the numbers of animals that will be involved (neither too many nor too few to achieve a meaningful scientific result) and quality of experimental design—again, what advice has been sought?
- the severity of the potential harms in the proposed studies, considering *all* potential adverse effects, psychological as well as physical, and their duration, in relation to:
  - the species and strain of animal used
  - the effects of the procedures themselves
  - wider factors, such as: the source of the animals (including, where relevant, their breeding conditions) and where relevant, the conditions of transport to the laboratory; and arrangements for their husbandry and care, including provision of environmental enrichment
  - the fate of the animals at the end of the experiments—will they be used in another procedure, killed (by what method?) or re-homed or released? And
  - how all of these factors will be influenced by the competence of researchers and other staff, and the quality of the facilities involved
- possibilities for refining the impact of the study on the animals so as to cause less harm to the animals whilst achieving a valid scientific outcome, e.g. by
  - using a different species or strain
  - obtaining animals from a different source
  - adapting or enriching animal housing and care
  - modifying the techniques involved
  - enhancing the monitoring of the animals and implementing humane end-points
  - better use of anaesthesia and analgesia and/or provision of other special care

iii. ensuring that all the necessary local facilities, expertise and other resources are available to support the work;
iv. providing a forum for on-going consideration of ethical issues arising in projects and husbandry and care of animals; and
v. providing information and advice to help in implementing the Three Rs more fully.

The minimum requirement for AWB membership set out in the Directive (Article 26(2)) comprises "the person or persons responsible for the welfare and care of the animals and, in the case of a user

[establishment], a scientific member". The AWB "shall also receive input from the designated veterinarian" or other suitable expert. This is a small number of people to achieve all of the tasks required of the AWB, so it is important to note that establishments are free to go beyond the minimum and include additional members.

The aim is to establish an AWB that is a valuable *resource* for the establishment, which engages with researchers and supports them in carrying out 'ethical' science with good standards of animal welfare.

3.3. Need for an on-going ethical review process

The more general advisory and supportive aims for ethical review/project evaluation processes, discussed above, are emphasised in FELASA's view that:

> "*Ethical review processes should not be merely 'committees for review of particular projects', but should aim to permeate and influence the ethos of every institution in which animals are used—creating an appropriate 'culture of care' and providing advice and resources to ensure proper consideration of ethical aspects and application of the Three Rs in all scientific work involving animals*" (Smith et al., 2007).

This means that ethical review should not be a one-off event that takes place at the application stage of a project, nor should it be something that is 'done to' researchers and their projects. Rather, it should be an on-going process—part of the culture of practising ethical science—for which researchers take responsibility, in dialogue with AWBs, the competent authority and others with appropriate expertise (RSPCA and LASA, 2010).

4. Examples for discussion

A series of brief case studies are presented below. The cases are intended to illustrate some of the principles of project evaluation and on-going ethical review within establishments, as described above. They cover a range of hypothetical scenarios relevant to cephalopod research, presented as generalised non-technical summaries.

Some of the cases include regulated procedures that would in practice form part of a 'project' requiring prior authorisation by the relevant competent authority, as per the requirements laid out in Section 3 of the Directive (Articles 36–45) and discussed above.

The case commentaries explore a wide range of questions, aiming to:

i. assist researchers preparing applications for authorisation, by highlighting areas in which further information and discussion would be needed, so as to cover all the elements that project evaluation must address (including the items listed in Annex VI of the Directive); and
ii. illustrate points that might be considered during (ethical) evaluation of projects or on-going work by the competent authority and/or by institutional AWBs.

Application of the principles for evaluation, authorisation and on-going review of projects outlined in the new Directive (and summarised in Section 2 above) requires sensitive *ethical judgement*. Because judgement is involved, the outcomes of project/ethical evaluation processes are likely to vary both between and within Member States—just as similar kinds of judgement do in other contexts such as clinical research and medical practice.

For this reason, in the examples we discuss factors relevant to authorisation, but leave the question of whether or not the work should be authorised open for further consideration by readers—who we hope will include members of ethical review processes/animal welfare bodies and regulators, as well as researchers and others with an interest.

The cases are intended to provoke discussion.

4.1. Example 1: investigating the effects of a parasitic infection on adult O. vulgaris

A research team wishes to investigate the effect of a diet of crabs contaminated with a sporozoan parasite on the behaviour and growth of *O. vulgaris* in the laboratory over a period of three months. This research is required because animals that have ingested this parasite in the wild are unsaleable owing to their appearance (growth stunted and development of cysts on the gills and the arms). Crabs are considered to be the natural vector but the parasite has no effect on the crabs. The aim of the research is to understand the effect of the parasite on the octopus and to devise strategies to prevent and treat such infections.

The main practical aspects of the project involve the following:

- injection of the isolated live or dead sporozoites into the carapace of small live crabs, using five different doses of live parasite;
- feeding the live crabs to groups of *O. vulgaris* (one 'dead sporozoite' group and five 'live parasite' groups);
- daily monitoring of food intake and body weight and observation of skin condition;
- weekly testing of behaviour using an established behavioural battery including visual and tactile discrimination;
- weekly sampling of blood (from branchial heart) and inspection of gills;
- finally, one week after the development of either macroscopic gill or skin lesions, animals will be killed and used for a detailed study of the pathology.

4.1.1. Opportunities for implementing the Three Rs

Replacement (partial or complete)

Have there been any in vitro studies of the effects of the parasite on isolated tissues or organs?

Have there been studies of pathology in animals already suffering effects of exposure to the parasite in the wild? Could such in vitro tests or studies of animals naturally exposed to the parasite in the wild partially or completely replace the proposed work? If such studies have already been carried out, how have they helped inform the design of the proposed study, and what new information will the study bring?

Reduction: study design

What is the scientific justification for using five different dose groups and could the number be reduced? Is evidence available or will there be a pilot study, to help decide scientifically relevant and humane dose levels? Could the dosing protocol begin with a low dose and progress to higher doses, so that higher levels of exposure can be avoided where possible? How many animals will be in each group, and has statistical advice been taken to ensure that the optimum number will be used (neither too many nor too few animals to achieve statistically valid results)?

Refinement

The Directive requires identification of the likely adverse effects caused to animals used in scientific procedures, from birth (or capture) to death (or release or re-homing), and, wherever possible, implementation of refinements to reduce those adverse effects. This includes refinement of routine husbandry and care, as well as experimental interventions.

In this case, questions asked about refinements might include:

Source of animals

Where will the animals come from? If they are wild-caught, how will it be ensured that they are captured by "competent persons, using methods which do not cause the animals avoidable pain, suffering, distress or lasting harm" (Directive 2010/63/EU, Article 9)? Are records kept of any injured or stressed individuals, and mortality rates?

Techniques

Daily weighing. What are the likely cumulative adverse effects of catching, handling and confining the animals for daily weighing over a period of 12 weeks? For example, there is some evidence that repeated handling affects growth in *O. vulgaris* (Nixon, 1966). Since skin/gill lesions and reduced growth are expected effects of the parasite, might the adverse effects from repeated weighing reach a level at which they could interfere with scientific outcomes? Could the weighing technique be refined to reduce these harms? Is daily weighing necessary, or could the frequency be reduced?

Blood sampling. Similarly, what will be the immediate and cumulative effects of weekly blood sampling on the animals and what steps have been taken to minimise these adverse effects? What volume of blood will be taken on each occasion and could the volume be reduced? What is the scientific justification for taking weekly blood samples?

Behavioural testing. Will any aversive training techniques be used or will the animal be confined or constrained to conduct the tests? Consider possible adverse effects and refinements.

Humane killing. What method will be used to kill the animals, and is it the most humane and scientifically appropriate method available (based on current knowledge)?

Effects of the parasite

Is there anything that can be done to ameliorate the adverse effects caused to the octopuses by the parasite? E.g. Choice of substrate and material for tank enclosures to avoid additional damage to/pain from skin lesions, whilst at the same time providing a suitably enriched environment, e.g. with refuges? Assisted feeding if this becomes impaired?

Welfare monitoring and humane end-points

Systematic assessment and recording of all possible adverse effects is important in enabling implementation of refinements, including humane end-points—and is also likely to have particular scientific value in this study. Decisions will be needed about how frequently the welfare of the animals will be assessed during the different phases of the study (at least daily), which particular clinical signs will be recorded, and at what stage humane end-points will be implemented.

A 'scoring' system for adverse effects might be helpful in assessing the overall/cumulative severity of the clinical signs observed (see for example Hawkins et al., 2011), and in particular to determine when humane end-points have been reached.

On the question of humane end-points, it might be asked whether the study could be terminated earlier—i.e. before the arm/gill damage stage is reached, or once it is reached—in order to reduce the duration of adverse effects caused to the animals, without compromising the scientific value of the studies.

4.1.2. Prospective severity classification

Annex VIII of the Directive on *Severity Classification of Procedures* suggests that "chronic toxicity tests… with non-lethal end-points" should be classified as "moderate" severity, with the "severe" category being reserved for toxicity studies that have "death as an end-point", or where "fatalities are expected and severe pathophysiological states are induced". It is therefore likely that this study will fall within the moderate category. Nevertheless, the Directive requires that every effort is made to keep the adverse effects as mild as possible; and, at the other end of the scale, if the adverse effects unexpectedly approach a "severe" level, the animals should be humanely killed so as not to exceed moderate severity.

4.1.3. Justification

Answers to questions on replacement might suggest other ways of addressing the central aim (to understand the effect of the parasite on the octopuses), at least initially. However, if in vitro methods or studies of wild animals are not possible, the ethical evaluation process will need to:

(i) consider whether the harms have been minimised, and the benefits maximised, as far as possible,[11] and then
(ii) 'weigh' the harms and benefits of the study in order to decide whether it should be authorised, according to the terms of the Directive.

It is observed that affected octopuses are "unsaleable because of their appearance", so presumably the study is working towards ways of solving that difficulty. It might be asked whether and how far treatment of wild-caught animals is feasible and how describing the disease-course will help in dealing with the problem in the wild. If, on the other hand, the study is aimed at avoiding parasite damage in aquaculture of octopus, would it be better to direct efforts towards detecting and avoiding contamination of crabs?

If, having considered these kinds of questions, the ethical review process is satisfied that the work is "justified from a scientific … point of view", in terms of the "validity, usefulness and relevance of the expected result" (Directive Article 38(2)(a) and Recital 39); that the use of animals cannot be replaced; and that the likely harms will be minimised as far as possible (see FELASA scheme in Table 2 for further thoughts on questions that might be asked on these points), the final step will be to 'weigh' the harms and benefits. That is, to consider whether the likely benefits are sufficient to justify a decision to authorise the work, given the harms likely to be caused to the animals.

Such 'weighing' is, of course, a matter of ethical (qualitative) judgement, not a quantitative procedure. The decision will need to take into account all relevant aspects of harm and benefit; and be informed by a range of expertise and perspectives.

4.1.4. Retrospective review

Should the study be authorised (perhaps as part of a bigger project), retrospective review will definitely be required if, as suggested above, some or all procedures are classified as moderate.

In this example, points for review could include:

- the actual severity of the procedures on the basis of day to day observations of the animals and their behaviour, and the effectiveness of the procedures for monitoring adverse effects and implementing humane end-points;
- whether any further refinements are possible and/or whether there could be scope to reduce numbers of animals used in future work of this nature;
- whether any humane alternatives have been developed, by the research team or others;
- the likely impacts of the scientific findings on management of the parasite, treatment of its effects, and improvements in the appearance/health and welfare, and associated saleability, of octopuses;
- a harm–benefit assessment based on these *actual* outcomes, to help inform future decisions;
- any developments in relation to the Three Rs that could be disseminated more widely.

[11] This could also include consideration of whether and how the Three Rs can be applied to the parts of the protocol involving the use of living crabs—especially as procedures on decapod crustaceans are regulated in some (non-EU) countries (see Box 1), and there is currently debate about these animals' ability to suffer.

4.2. Example 2: a study of molecular mechanisms of arm regeneration in *O. vulgaris*

A research team wishes to investigate the molecular mechanisms underlying the regeneration of the arm in adult *O. vulgaris*, following surgery to remove 90% of one arm. The aim of the research is to investigate whether the regenerative ability of the octopus arm could provide novel insights into tissue regeneration in humans. At specific time points (between one day and two months) after the surgery, animals will be killed and tissue removed for molecular, histochemical and histological study.

4.2.1. Opportunities for implementing the Three Rs

Replacement and reduction

Questions similar to those in Example 1 might be asked. For example, (how) have findings from previous in vitro or whole animal studies informed the objectives and design of the current study? Has, or could, in vitro work help to reduce the use of whole animals in the present proposal? On what basis has the number of time points been decided, and could the number be reduced? How many animals will be used at each time point, and how has the optimum number been decided? Has statistical advice been sought?

Refinement

Source of animals

Questions similar to those in Example 1 could also be asked here.

Surgery

By what surgical method will the amputation be performed? What methods of anaesthesia and analgesia will be used? Note that strong scientific justification will be necessary if there is any requirement for anaesthesia or analgesia to be withheld. Could the surgical, anaesthetic or analgesic methods be refined so as to further reduce pain and distress? (Remember, the Directive assumes that cephalopods *can* and *do* experience such adverse effects.)

Impact of arm amputation on the animals

What are the predicted impacts of loss of an arm on feeding, exploratory and other behaviour? Are there refinements—e.g. to methods of feeding, cage environment or other factors—that could help reduce these impacts?

Monitoring and humane end-points

How will the severity of adverse effects such as pain or distress be monitored, and what humane end-points will be set? What are the scientific reasons for keeping the animals for two months, and could that time be reduced? Is the method of killing the most humane possible and scientifically appropriate?

4.2.2. Prospective severity classification

Annex VIII of the Directive classifies "surgery [including organ transplantation] under general anaesthesia and appropriate analgesia, associated with post-surgical pain, suffering, or impairment of general condition" as a 'moderate' procedure. However, "surgical… interventions… that are expected to result in severe or persistent moderate postoperative pain, suffering or distress and persistent impairment of the general condition of animals" are classified as 'severe'. Which might apply in this case? Although the procedure would be 'severe' in vertebrates, could the surgery be moderate in cephalopods, with mild effects in the longer-term?

Could this procedure actually be mild throughout for cephalopods, given that they engage in autophagy (Budelmann, 1998)? How could this be assessed objectively?

4.2.3. Justification

In considering whether the study meets the requirements for authorisation, the review process will need to be satisfied (as per Example 1) that:

- the study is "justified from a scientific … point of view", in terms of the "validity, usefulness and relevance of the expected result";
- there is a need to use animals, because alternative approaches cannot achieve the aims of the work;
- the likely harms to the animals have been identified and will be minimised as far as possible—by refinement of the procedures and care of the animals, and by optimising experimental and statistical design; and
- there is likely to be sufficient benefit (novel insights into tissue regeneration in humans) over harm to justify authorisation of the work.

See Directive, Articles 38(1) and 38(2)(a)–(d).

In this study, there might also be 'reduction' benefits for other studies, if, after the octopuses are killed, there is an opportunity to "bank" surplus tissue for use in other projects. This would be in accordance with Article 18 of the Directive, which implies that "organs and tissues of animals killed" should be shared wherever appropriate.

4.2.4. Retrospective review

Again, the general questions outlined in Example 1 could also apply here.

In this case, retrospective review might, in particular, help to resolve any uncertainties about the actual severity of effects caused to the octopus by amputation of an arm, on the basis of day to day observations. This could then inform the approach and design of any similar studies in future—so that the Three Rs can be implemented as fully as possible.

4.3. Example 3: care of cephalopods undergoing senescence

A female *O. vulgaris* captured from the wild is being maintained in a laboratory aquarium, and will be used in a particular project once sufficient other females have been obtained to yield statistically significant results. She is in good health, showing normal exploratory behaviour and skin patterning, and has been attacking and eating crabs. After two weeks, she produces an egg mass and begins 'nursing' it. Another team in the research institution hears of this, and asks to use 12 of the fertilised eggs immediately and, when they become available, the same number of week-old hatchlings in a study of developmental gene expression. The PI agrees to this and arranges supply of the eggs and hatchlings (when available), as requested.

After producing the eggs, the mother begins to lose interest in crabs and a few days later stops eating. The PhD student who routinely monitors the animals notices that when the tank is opened for inspection the animal shows no interest, has some unusual white spots on the skin, and appears to be losing weight. The student is concerned that the animal may be suffering, records these observations and reports them to the PI who says this is normal for senescent animals and in any case it is only another few weeks until hatchlings will be available for the other research group.

The PhD student is unhappy with the PI's decision and talks to the designated veterinarian, who inspects the animal and suggests that it is killed immediately by anaesthetic overdose. The PI does not agree, and discusses the issue with the Director of the research institute. The Director then asks the institution's animal welfare body (AWB) to consider the matter and recommend a course of action.

As Box 2 shows, two of the AWB's tasks are to advise on the welfare of laboratory animals and the application of the Three Rs in on-going projects and all the whilst the animals are being cared for in the laboratory. A well-constituted and effective AWB will bring a range of relevant expertise and perspectives together to consider just the kind of issue illustrated in Example 3, in a constructive, collegiate manner.

The immediate question is whether or not the female octopus concerned should be humanely killed. But the particular case also has wider implications for the treatment of senescent cephalopods in general.

As Anderson et al. (2002) make clear, senescence is a normal stage in an octopus's life. Both males and females show signs of senescence before death. Females guard, clean and oxygenate their eggs, becoming so dedicated to these tasks that they dramatically reduce, and often stop, feeding. In captivity *O. vulgaris* females may lose around 50% of their body weight whilst brooding their eggs. Male octopuses near the end of their lives also stop feeding, tend to develop small white lesions, and become more active—though often this activity is relatively uncoordinated (see Anderson et al., 2002 for further discussion).

These signs of senescence are also possible signs of pain or other suffering as suggested in this volume (Andrews et al., 2013). However, it is unclear whether or to what extent senescence is an unpleasant, distressing or painful experience for the animals concerned.

The AWB will want to decide a course of action acceptable to the PI, PhD student and designated veterinarian (or suitably qualified expert), and also develop clear guidance to help resolve similar questions in future.

Andrews et al. (2013) propose that, in general, the "precautionary principle" should be invoked, in that octopuses showing signs of senescence should be humanely killed, unless there is a clear scientific or animal welfare justification for keeping them alive.

In this case, the female's nursing activity is essential for survival of the developing embryos, and allowing these to continue to develop to hatching could, in turn, be justified by the request for week-old hatchlings for use in a study of developmental gene expression (which, depending on the procedures involved, would require authorisation from the relevant competent authority). Supplying the hatchlings would presumably also make it unnecessary to capture and remove another female from the wild, which could be preferable on ethical grounds. However, once the hatchlings have been supplied, adoption of the precautionary principle would require humanely killing the female octopus and any remaining embryos, unless there is scientific justification for keeping her alive.

Whilst other scientific justifications can be envisaged for keeping senescing animals alive, the most likely is the study of senescence itself—because, as Anderson et al. (2002) point out, other areas of research on octopus physiology and behaviour are likely to be confounded if senescent animals are used. Depending on their potential adverse effects, scientific studies involving senescing animals are likely to require authorisation from the relevant competent authority.

In general, decisions such as those outlined above are for researchers, the designated veterinarian and animal care staff to decide, in dialogue with the AWB (as appropriate). Where there is doubt about the legal position, the relevant competent authority should also be consulted.

4.4. Example 4: studies of squid communication

A PhD student wishes to investigate social recognition and communication between individual squid, comparing chromatophore patterning of different *Loligo* and *Sepioteuthis* species, when placed in a variety of "social situations". Squid will be reared from hatching in the laboratory, in two ways: (i) in small shoals of around 20 animals, in enriched tanks; and (ii) in individual enriched tanks, out of view of each other, until they are used. Studies will involve comparing behaviour in "social encounters" within pairs or small groups of individuals placed in observation tanks for video recording of behaviour. Individual behaviours will be compared across a range of different social encounters, both within and between the groups reared in isolation and in shoals and, e.g., with varying age of animals (from juvenile to adult) gender, size, and familiarity.

4.4.1. Does the study involve regulated procedures (i.e. is project authorisation required)?

A procedure becomes regulated under the Directive when it may cause an animal "pain, suffering, distress or lasting harm equivalent to or higher than that caused by the introduction of a needle in accordance with good veterinary practice". This includes psychological effects such as anxiety, above the threshold level. Clearly, however, it is difficult to compare levels of stress caused in behavioural experiments with adverse effects caused by injections.

A little more assistance is offered by Annex VIII of the Directive together with the first of a planned series of consensus documents intended to "facilitate harmonised implementation" of the new regulation (European Commission, 2011). These documents list examples of some below-threshold procedures, which include "open field testing" and "application of external telemetry devices that are expected to cause no impairment to socially adapted animals and do not interfere with normal activity and behaviour". It is also noted that repeated application of such below-threshold techniques could cause cumulative welfare effects that might reach or exceed the level of a 'mild' procedure. If this were to happen, the techniques would become regulated.

Since none of the examples of sub-threshold procedures in the above documents exactly compares with proposed procedures, it is a matter of judgement whether they will be regulated or not. Clearly, the national competent authority responsible for authorisation of regulated procedures will have the final say on such a matter, but cephalopod researchers' views, along with local AWB input, may well be important in such decisions.

Whether or not the study is regulated, it is important that the normal principles of humane and ethical research are followed as far as possible. Some relevant questions are noted below—and answers to them might also help in determining whether the work falls within the remit of the EU Directive.

4.4.2. Replacement and reduction

It is difficult to see how this behavioural study could be replaced by a non-animal method that could answer the research questions (although it might be asked whether field observations could be used as a non-regulated alternative). However, questions similar to those raised in examples 1 and 2 might be asked about "reduction": how will group sizes and number of replicates be determined and optimised for this particular study, and will statistical advice be taken?

4.4.3. Adverse effects and refinement

4.4.3.1. Effects of rearing squid in isolation. Where vertebrates are concerned, "studies involving short-term deprivation of social partners, short-term solitary caging of adult rats or mice of sociable strains" are regarded as "mild" regulated procedures; whereas "complete isolation for prolonged periods of social species, e.g. dogs and non-human primates" would be classed as "severe". However, it is unclear how these categories relate to individual rearing of squid.

Many cephalopods are solitary or semi-solitary, but squid forms shoals—e.g. for feeding and reproduction—and, in this sense at least, may be regarded as a "social" species. Squid behaviour may be affected by size of tank and may become erratic when individuals are separated from their shoals (Hanlon, 1990). However, rather little is known about the social behaviour of individual squids (Boal and Gonzalez, 1998; Hanlon and Messenger, 1996); and, beyond recognition of species and gender, there is as yet little evidence available for or against social recognition in squid (Boal, 2006).

In this light, it might be asked, for example, whether the individual rearing of squid could be reduced or avoided in the proposed project; whether some or all of the 'encounter' experiments could be carried out using squid that are group-housed in an enriched tank; and how keeping individual squid isolated from others might impact on the later social interactions being investigated.

Careful observation and recording of the behaviour and appearance of the individually housed animals might also help to inform future

judgements about the potential adverse effects and severity of rearing squid in isolation.

4.4.3.2. Effects of behavioural testing. What will be the size of the chamber in which behaviour is studied, the duration of exposure and frequency of behavioural testing—and how might these factors influence the adverse effects experienced by the squid? Are any refinements possible?

If the behavioural challenge is to be repeated over the longer-term, what are the likely cumulative effects on the animals—e.g. on behaviour, body condition, feeding, growth, behaviour (etc.)? See list of potential adverse effects and discussion in Andrews et al. (2013). Will the smaller, juvenile squid experience stress when they encounter a larger, more mature animal in a confined space? Is there any danger of attack? Will the tank have a refuge for the smaller animals?

Where aggression is possible in such staged encounters, Guidelines from the Association for the Study of Animal Behaviour (2012) recommend that continuous observation, provision of escape routes, and (depending on context) use of protective barriers are considered as possible refinements. In this study the encounters between animals will be video recorded. Will a researcher or member of animal care staff also observe the squid, to monitor for aggression and any other unexpected or anticipated difficulties? Is there a facility to catch and remove animals quickly if they are injured or obvious targets of aggression?

4.4.3.3. Fate of the animals at the end of the study. The squid will not have been subjected to any invasive or toxic studies, so (following health checks) could they be released into the wild or re-homed (e.g. to an aquarium), or re-used in another study? Would any of these options be an ethically more acceptable fate than humane killing?

4.4.4. Severity classification

As discussed above, it is uncertain whether any of the techniques involved in the study would count as regulated procedures and, if so, what severity of adverse effects they might cause.

Careful observation and reporting of the actual severity of the procedures within this study might provide information to inform further prospective judgements on the severity of these behavioural methods.

4.4.5. Justification and retrospective review

It is uncertain whether this work will require prior authorisation by the competent authority—but whether or not it does, the researchers, funding body and institution will want to be satisfied that any potential harms to animals have been minimised, by application of the Three Rs (see above); and that the likely benefits have been maximised. In practice, the study might yield new information relevant to sociality in squid, that could help in coming to a more informed view about the likely impacts of this, and similar studies, on the animals (as discussed above).

If the study is not regulated, retrospective review will not be required. Nevertheless, it might be beneficial for the AWB, in dialogue with the researchers, to 'look back' at this kind of work in order to:

- consider the actual adverse effects (if any) on the animals;
- identify any refinements that could be shared with others doing similar work; and
- consider further the question of whether or not these husbandry and behavioural methods can cause adverse effects that meet or exceed the threshold for regulation under the new Directive.

4.5. Example 5: "fixing" cuttlefish developmental stages

A project involves 'fixing' cuttlefish hatchlings in 80% alcohol, three days after hatching, for anatomical studies of development.

4.5.1. Is this 'fixing' a regulated procedure (i.e. does it require project authorisation)?

The Directive covers "live cephalopods", but the stage of development at which procedures on cephalopods are regulated is not specified. However, larval forms of fish and amphibians are covered from the stage at which they begin "independently feeding" and it is reasonable to assume that the same provisions apply to cephalopods. Cuttlefish are capable of independent feeding from about 3 days after hatching, and so the animals used in this study will, in principle, be covered by the Directive.

However, the "killing of animals solely for the use of their organs and tissues" is not a "procedure" that requires authorisation from the competent authority (Article 3(1)) *provided that* the animals are killed "with the minimum pain, suffering and distress" and the method is an "appropriate method of killing" as set out in Annex IV of the Directive (Article 6). Cephalopods are not included in Annex IV, but it can be argued that immersion in 80% ethanol (a tissue fixative) is unlikely to qualify as an appropriately humane method, given that ethanol "anaesthesia" is aversive at much lower concentrations of around 1–4% (Andrews and Tansey, 1981; Estefanell et al., 2011; Mooney et al., 2010).

This suggests that the fixing procedure is likely to be regulated, and, if so, could only be carried out if specifically authorised by the competent authority on the basis of scientific justification (Article 6(4)).

4.5.2. Prospective classification of severity

As noted, if the ethanol fixing method is used without first killing the hatchlings by a humane method (e.g. magnesium chloride), it is likely to be regarded as a regulated procedure, because ethanol is aversive. In line with Annex VIII of the Directive, the procedure might therefore be classified as "severe", because it causes death of the animals.

4.5.3. Is there scientific justification for the fixing procedure and could it be refined?

Are there scientific reasons why it is not possible to anaesthetise or kill the animals by another more humane anaesthetic technique (e.g. magnesium chloride) and then fix in 80% ethanol (or another suitable fixative)? If so, the scientific case will need to be put to the ethical review process for discussion and evaluation. If not, a refined (i.e. more humane) method should be used.

4.5.4. Retrospective review

This case explores an area where humane techniques are under development, and is something that the AWB and national competent authority might consider further, as more information becomes available about the efficacy of methods of anaesthesia for cephalopods and their use prior to fixing. Both bodies could play a role in disseminating the new information and developing policy for humane practice.

5. Conclusion

As this paper emphasises, implementation of the new EU Directive is imminent, and researchers, including cephalopod 'experts', will need to begin to prepare now. Action points suggested by the above discussion include the following:

i. make contact with the National Contact Point (government body) responsible for implementing the new Directive in your country, in order to be kept informed of progress in transposing the law and impacts on your research. As noted above, National Contact Points for all EU countries are listed at http://ec.europa.eu/environment/chemicals/lab_animals/ms_en.htm;
ii. where relevant, make your views known and/or offer expertise to the National Contact Point, to help make sure that the new provisions are implemented in a way that is in keeping with the spirit of the legislation but is also practically workable for cephalopod research;

iii. if you work in an establishment where vertebrates are used in scientific procedures, ask colleagues who currently carry out regulated animal studies for information and advice to help gauge the you ensure that you are in compliance in particular:
iv. ensure that you meet the requirements for authorisation of the place(s) at which you carry out research (even if the research solely involves killing cephalopods by recognised methods for use in in vitro studies). This includes specific requirements for:
   - care and accommodation of animals;
   - education, training and competence of staff;
   - nomination of specific personnel with functions related to the welfare of animals;
   - a local animal welfare body;
v. consider whether your work involves (or will involve) procedures that could cause 'pain, suffering, distress or lasting harm' to cephalopods (at any point from hatching onwards). If it does, contact the National Contact Point and ensure that you can meet the Directive's criteria for authorisation of projects.
   Note: The Directive works on the assumption that procedures likely to cause pain, suffering, distress or lasting harm in vertebrates will cause similar effects in cephalopods. Regulation will include, e.g., stressful behavioural or captive breeding studies, fixing developmental stages for in vitro work; and killing animals by methods not recognised in the Directive;
vi. consider the supply of animals for your work, and comply with Article 9 of the Directive:
   - if wild-caught animals are used, could they be replaced by purpose-bred animals—and would this be in the animals' best interests?
   - if it is possible to use purpose-bred animals, can the work be carried out at the breeding centre? If not and the purpose-bred animals will have to be transported over long distances, would it be less stressful, overall, to continue to use wild-caught animals?
   - if a scientific or animal welfare justification can be made for using wild-caught animals, how will you ensure that the animals are captured by competent persons, with minimal adverse impacts on animal welfare? For example, could you set up a benchmarking scheme to record sick, damaged or traumatised animals and avoid that supplier if appropriate?
vii. consider the record keeping and reporting required under the Directive and, when appropriate, ensure that systems are in place to meet national and EU statistical requirements, including effective assessment of actual severity.

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