National Animal Ethics Advisory Committee

Occasional Paper No 6

Planning for refinement and reduction

ISBN 978-0-478-37534-3 (print)
ISBN 978-0-478-37535-0 (online)

ISSN 1173-6763 (print)
ISSN 1173-6828 (online)

January 2011

FOREWORD

This paper was presented at the 7th World Congress on Alternatives and Animal Use in the Life Sciences in Rome in 2009, and is reproduced by kind permission of Alternatives to Animal Experimentation (ALTEX), a quarterly journal for new paths in biomedical science.

The sixth in NAEAC's series about the use of animals in research, testing and teaching published from time to time by the Ministry of Agriculture and Forestry (MAF) under the auspices of the National Animal Ethics Advisory Committee (NAEAC), this paper was chosen by NAEAC for its obvious focus on refinement and reduction in the preparation of research programmes.

The provision of information and advice to animal ethics committees (AECs) is one of NAEAC's designated functions, and given that the Animal Welfare Act 1999 s80(2) specifically requires the promotion of reduction, refinement and replacement, this paper is recommended to AEC members as making a valuable contribution to efforts to minimise the impact of research projects on animals.

Virginia Williams

Chair, NAEAC

January 2011

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Planning for refinement and reduction

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Progress in Life Science Domains, ALTEX 27, Special Issue 2010

Summary

Experiments using laboratory animals do not normally occur as isolated "one-off" studies and there is often considerable scope for reducing overall severity and the number of animals used by careful planning of the complete programme. The paper illustrates this with examples and provides a commentary on the flowchart for planning a research programme developed by the FRAME Reduction Steering Committee. The flowchart reminds researchers that programme planning needs well-specified objectives and research into different ways of achieving them, and into the severity of the methods involved. The least severe sequence can then be chosen and the series planned to identify unexpected adverse effects and good endpoints early on, so later experiments can minimise severity. Starting with low-severity work can avoid unnecessary higher-severity studies and individual experiments can be designed to minimise numbers and severity. The flowchart should help experimenters plan minimal-severity programmes and be useful for ethical evaluation.

Keywords: planning, refinement, reduction, severity, experimental design

1. Introduction

A need for better training in the design of experiments and the strategy for experimental programmes became apparent from discussion at a number of meetings (see Howard et al., 2009). In response, the FRAME Reduction Steering Committee (FRSC) has been running training courses on this topic for postgraduates. In looking for material to bring together the various aspects of planning and carrying through an experimental programme, the Committee found the available flowcharts insufficient and has developed its own (Gaines Das et al., 2009). This covers not just the planning and design of individual experiments but also the strategy for the whole programme. The strategy is important, as experiments using laboratory animals do not normally occur as isolated "one-off" studies and there is often considerable scope for decreasing overall severity and reducing the number of animals used by careful planning and design in the context of the complete programme. Gaines Das et al. (2009) concentrated on planning to reduce overall numbers: this communication illustrates how this approach could be used to reduce the severity of an experimental programme.

Fifty years ago Russell and Burch (1959) recognised that "One general way in which great reduction may occur is by the right choice of strategies in the planning and performance of whole lines of research." They also recognised the ethical imperative to "reduce to an absolute minimum the amount of distress imposed". Reduction in animal usage reduces overall suffering by exposing fewer animals to adverse effects, but good programme planning can also minimise overall severity. Unfortunately, experimental design texts usually provide no guidance on how to design an individual experiment to minimise severity and are silent on how to organise a sequence of experiments. Ethical evaluation processes that judge only protocols may well miss possibilities for minimising overall severity through a suitable strategy for the whole programme. It is also a topic missing in FELASA's suggested syllabus for the training of researchers (see FELASA 1995). The UK has had an advantage in developing ideas in this area, as researchers have been obliged since 1987 to apply for projects covering an experimental programme of up to five years and a key section of the project licence application form has been the plan of work (see http://scienceandresearch.homeoffice.gov.uk/animal-research/publications-and-reference/publications/licences/project-licences/ for the latest example of this on the application form and notes with the form on planning and refinement). Many of the ideas developed over 50 years within the UK animal science community and by the UK Animals Scientific Procedures Inspectorate and its predecessor can be found in the fact sheet prepared by Morton (1998) and how they are encouraged in practice can be seen in the first report of the Animals Scientific Procedures Inspectorate (Home Office, 2004). Strategy for reduction is covered to some extent in Festing et al. (1998), the value of carefully-specified objectives in Fry (2004) and a step-wise approach to refining an experimental programme in Fry and Morton (2000).

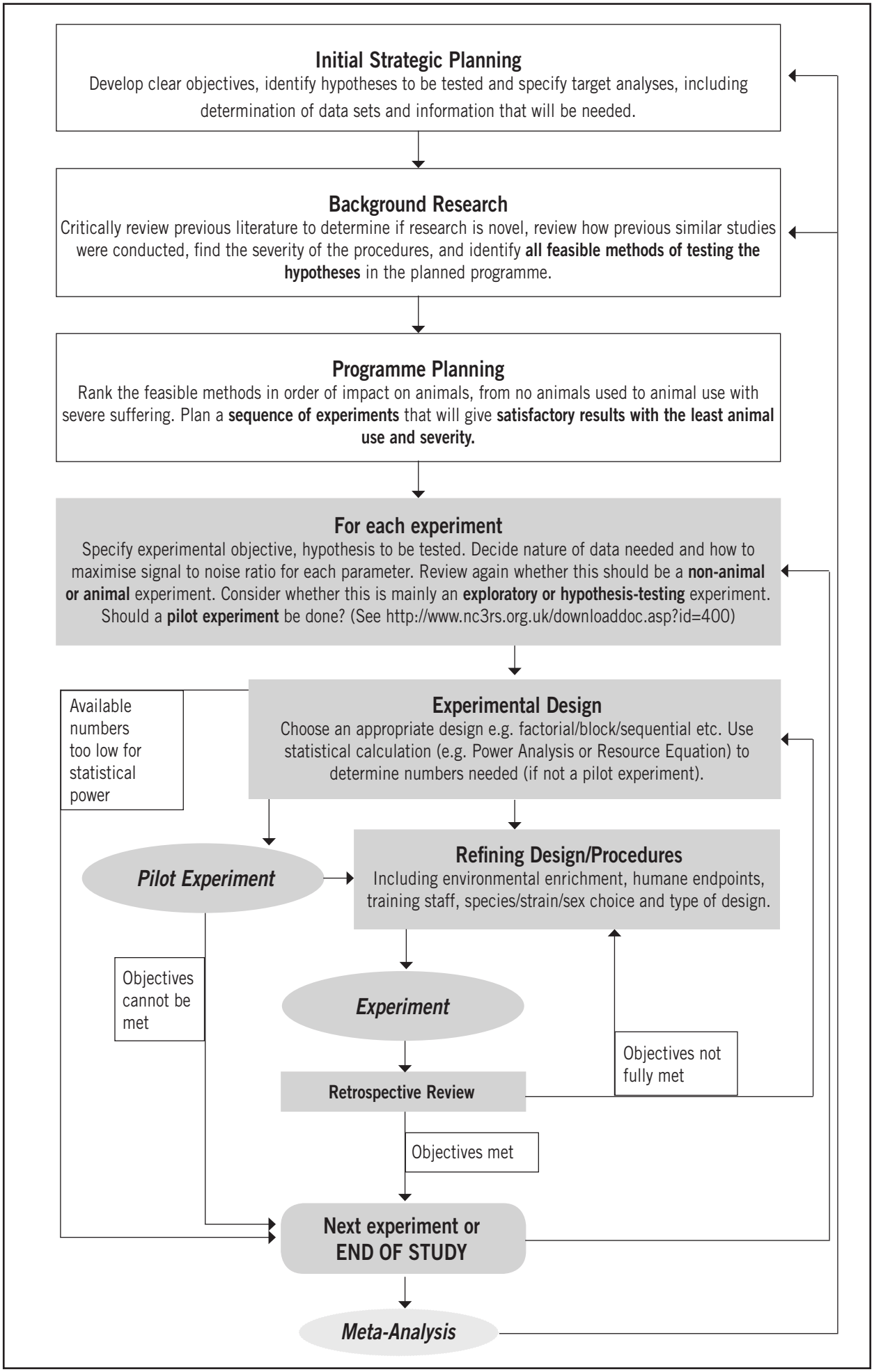

Figure 1: FRAME Reduction Steering Committee Strategic Planning Flowchart. Slightly modified and reproduced from Gaines Das et al. (2009) with permission from ATLA.

In Europe the replacement of Directive 86/609, with its introduction of "projects", could place more emphasis on ethical evaluation of the overall severity of the programme. In the proposal put to the European Parliament in November 2008 Article 37 requires the assessment of whether "the project is designed so as to enable procedures to be carried out in the most humane ... manner." This could translate into closer scrutiny of the combined severity of a series of individual experiments. There is also an emphasis on retrospective review – another feature in the FRSC flowchart. Generally, wider appreciation that good planning avoids wasteful use of animals and can reduce the extent of animal suffering caused by an experimental programme is likely to raise ethical awareness of the value of looking beyond the individual protocol. The FRSC flowchart, reproduced in Figure 1 with an addition on researching the severity of procedures, is designed as a framework to help both researchers and those assessing programmes achieve good scientific outputs with minimal animal use and suffering.

2. Planning a programme for minimal severity

2.1 Initial planning

The first and most important parts of any strategic plan are a) setting the aims or general objectives and b) doing the relevant background research pertaining to these objectives. When the broad objectives are clearly specified, an appropriate programme can be planned within which possibilities for studies not involving animals can be identified and individual animal experiments can be considered. It is important to distinguish between the general aims of the programme and the hypotheses the individual experiments would test. Without clarity at both of these levels it is difficult to consider all feasible methods of testing the hypotheses of interest without using animals, or to see non-animal methods that could be developed for the purpose. In the severity context specified objectives are needed for setting objective-dependent endpoints for the individual experiments (see below).

In the initial strategic planning stage, decision points in the programme should also be considered. At a decision point the progress so far is reviewed and decisions taken on whether in the interests of minimal animal use and severity the plans should be changed, additional monitoring or revised procedures incorporated or the programme abandoned. If the first part of the programme involves development of a new animal model, for example, it may be necessary to accept that a satisfactory model is illusory and save further suffering by taking a different approach.

2.2 Background research

The flowchart suggests that background research should not be limited to checking to avoid duplication and seeking one practicable route to pursuing the aim of the programme, but should include:

- identifying the range of possible routes and both their relative need for use of animals and their relative severities;
- obtaining information on the conduct of experiments similar to those envisaged and any adverse effects encountered;
- gathering information on the likely constraints, such as time taken for procedures and availability of people and of animal accommodation; and
- researching the techniques and sampling methods proposed, the severity and possible adverse effects involved and their limitations.

Articles on specific techniques or procedures may identify adverse effects and there may also be clues in experimental reports. Unexplained differences in group sizes, for example, may indicate that some animals unexpectedly died or had to be withdrawn from a study on humane grounds.

2.3 Programme planning

Using the information obtained from experience and background research, a series of studies likely to use minimal numbers and involve least severity can be structured around the planned decision points. As pointed out in Gaines Das et al. (2009), this programme planning should also consider and take into account constraints that may frustrate achieving the objectives or involve unnecessary animal use or suffering. These include limitations on the

resources that can be drawn upon, availability of accommodation, restrictions on sample collection and handling and how much data can be obtained in a given time period.

For minimising severity, a critical element is planning the sequence of experiments so that at an early stage adverse effects, and how to avoid or control them, are identified and humane endpoints are set and reviewed in practice. Any programme is likely to have unpredictable unknowns, such as technical difficulties with a published procedure new to the laboratory, unanticipated adverse effects of a substance or procedure or combination of experimental treatments. An objective of the first experiments, or the first experiment in each subordinate series, should be to identify such unknowns and suitable humane endpoints.

When developing a new animal model the background research should enable the planner to pre-set criteria by which the model will be judged as successful, and a decision point at which the results with the model are assessed against these criteria should be planned into the programme.

3. Examples of planning for minimal severity

In vaccine studies the aim of a programme may be to test new vaccines, with different studies on efficacy and safety. Animals are likely to be subjected to unnecessary suffering if the safety studies, which typically involve giving double or more the expected dose of the vaccine, are optimistically planned to start before the results of efficacy studies are known, since a proportion of the vaccine tested will show insufficient efficacy.

A programme may be staged to achieve all the objectives that can be met at low severity first, only proceeding to higher severity studies when the low severity approach is exhausted. The sequence of questions could be –

Question	Example of response
What is the overall objective?	To determine the effects of hypoxia on sympathetic nerve activity.
What can be done without animals?	No relevant studies – an intact vascular nerve network is needed.
What can be done under terminal anaesthesia?	All the studies on the effects of hypoxia up to 24 h.
What can be done with only mild severity?	Studies on the effects of prolonged mild hypoxia in which the experiments involve >24 h exposure, then terminal anaesthesia.
What can only be done at more than mild severity?	Investigation of effects of prolonged, more severe hypoxia. Confirmation studies with implanted electrodes in conscious animals.

False assumptions are then exposed and basic information obtained without subjecting animals to the more distressing procedures. In some cases the results from the milder studies may well indicate that the more severe ones are not needed.

The development of a mouse model of acute pancreatitis illustrates how planning to minimise severity could operate to save much animal suffering. Acute pancreatitis is a very painful condition in humans. It carries substantial morbidity and can be fatal. It is worse in those who are obese and the reasons for this are unknown. Deficiencies in current treatment and management of patients with pancreatitis, the lack of understanding of the

mechanism for the higher severity in obesity and the need for the interactions of multiple body systems to mimic the condition justify use of animals to study how the condition can be ameliorated. The objective of the programme is to investigate why acute pancreatitis is worse in obesity by first developing an obese animal model, then using pharmacological dissection to detect mechanisms and compare these with those found in non-obese animals. Background research indicates that intra-peritoneal injection of a combination of two interleukins should induce pancreatitis and that a genetically obese mouse should be a good prospective model, but also that acute pancreatitis in rodents produces substantial suffering. In acute pancreatitis in man, and non-obese rodents, there are early changes in serum amylase and other enzymes and marked histological alterations to pancreatic cells. Certain serum components are good early predictors of the severity of the pancreatitis. Reasonable criteria for the obese mouse model could be the demonstration of comparable serum and histological changes.

The programme sequence could be a pilot experiment with a dose and combination of the agents expected to produce pancreatitis, with serial blood sampling and post mortem histology as measures, and signs of abdominal pain or a set time after the injection to induce pancreatitis as an endpoint. This should give a good indication of whether the model has prospects and whether the severity controls and endpoint can be refined. It could be followed by a factorial experiment using different interleukin doses and combinations to determine the optimal dose combination for producing raised serum levels and the characteristic histological changes. A further experiment could determine the time course of the enzyme and other serum changes more precisely, and then one with the animals killed at set time points to follow the histo-pathological changes in the pancreas. Finally, it might be necessary to allow progression to full blown acute pancreatitis in a few animals to confirm that the demonstrated signs are genuinely those of early development of the condition. This would establish the model and the pharmacological studies could then use the optimal induction and sampling arrangements determined from these experiments. The pharmacological experiments could routinely end at the time when definitive early changes were reliably detectable, sparing animals the further suffering of a progression to later stages of the disease.

The approach taken by Sennello et al. (2008) to developing an obese mouse model of acute pancreatitis seems to have been very different. The first experiment reported in the paper is a survival study, with the number of mice that died after the interleukin injection as the key parameter. When all mice in the obese group of 10 mice died but none in the non-obese group, and this was confirmed in a repeat experiment, the dose was increased for groups of 10 of the latter until 30 percent died. A further experiment confirmed that multiple organ failure occurred. Subsequent experiments then followed the time course of the serum changes and the pancreatic histology. Clear changes were detectable two to six hours after injection, whereas mice took 24–48 hours to die. This sequence is quite different in severity from that outlined above.

4. Achieving minimal severity at the experiment level

The lower half of the flowchart mainly concerns the individual experiments. The flowchart assumes researchers will be familiar with the important points on refining experimental procedures and designing experiments for efficient use of animals well covered elsewhere (For the former see the UK National Centre for the 3Rs Information Portal at http://www.nc3rs.org.uk/landing.asp?id=38, Morton 1998, or the section on *Avoiding or Minimizing Distress in Laboratory Animal Use* in the report of the US National Academy of Sciences 2008, and for the latter see Festing et al., 2002). There are some additional considerations for designing for minimal severity, however.

4.1 Setting clear objective(s) for each experiment

As pointed out elsewhere (Gaines Das et al., 2009; Fry, 2004), this is important for formulating a design that uses the right number of animals. In the context of refinement it is crucial to setting objective-related endpoints, i.e. the points when individual experiments have met the objective or clearly cannot achieve it. Continuing beyond this endpoint involves risk of animal suffering or distress, for which there is no justification (see Fry, 1998).

4.2 Pilot experiments or dose-setting procedures

The flowchart highlights the value of incorporating pilot experiments at suitable points in the programme and provides the link to a National Centre for the 3Rs online document on the subject. These preliminary experiments are likely to be worthwhile use of animals to establish proof-of-concept or to provide useful information on technical problems, the time course of an experimental outcome, or how much resource is needed for a full-size experiment. In regulatory toxicology studies and some pharmaceutical work the dose-setting procedure is essentially a type of pilot study.

Research pilots and dose-setting runs should be planned to provide information to help minimise severity. This means having observation schedules that will detect adverse effects and indicate their severity and duration, so that in the definitive studies suitable monitoring arrangements can be made, opportunities for refinements considered and incorporated, and severity-related humane endpoints identified. Noting the time course of the experimental effect can help with setting objective-related humane endpoints.

4.3 Designs to minimise severity

Factorial designs

These are recommended for their efficient use of animals (Shaw et al 2002), but can also be used to minimise severity by pointing to optimal conditions for a series of studies where the conditions carry significant severity. For example, a group needs to set up an hypoxia model to study alterations in sympathetic nerve activity produced by exposure to low oxygen tensions. A mouse strain is known to show the effect but is sensitive to low oxygen tensions and can go into respiratory distress. Good data on the minimal extent and duration of hypoxia are not available. A factorial design for the exploratory experiment, in which several levels of oxygen deprivation and different durations are used in various combinations in the same experiment, will be the most efficient way of seeing the minimal level and duration of hypoxia needed to produce an effect large enough to study. This combination can then be used for performing the subsequent series of experiments with minimal adverse effects from the hypoxia.

Sequential designs

Sequential experimental designs, which allow the cumulative analysis of data, are efficient in that the study only continues until the objective is reached. Compared to other designs, where estimates of the number of animals needed have to be made in advance and risk either overestimating or using too few (and thus wasting them), severity is reduced as only the number of animals actually needed experience distress. Definitive experiments of this nature (e.g. Waterton et al., 2000) need careful planning and analysis as assumptions have to be made about the comparability of the conditions for successive groups of animals and the risk of time bias. The help of a statistician is highly advisable.

However, a modified sequential design, the “up and down” approach, is one of the standard methods for determining acute oral toxicity in regulatory studies (Bruce 1985; Rispin et al. 2002), and a similar approach (but without lethality or predicted lethality as the endpoint) could be considered when planning research studies for which it is suitable. For example, a dose of compound expected to show some effect if the compound is efficacious is given to two animals and they are observed for a set period. (Using a pair of animals gives some control for variability, but if the likely effect is severe, as in acute oral toxicity tests, then only one animal at a time should be used). If no or little effect is seen, the dose is increased for the next pair and so on until a useful efficacy of the substance can be determined or a cut-off point at which it can be deemed ineffective is reached. If the first pair shows higher effects than needed, the dose for the second pair is decreased. This continues until a minimal useful effect level is found. Using such an approach for research work where a substance of unknown effect is being given, or an agent is being tried on a new genetically modified mouse line, should help minimise severity by minimising the numbers exposed to adverse effects of unknown or unpredictable severity.

Use of unequal group sizes

Where the experimental procedure involves considerable severity, distributing the animals into a large control group and small experimental group or groups can provide as powerful a design as one using equal group sizes, with only a modest increase in total number required (Ruxton, 1998). Again severity is reduced because many fewer animals are exposed to the suffering of the experimental procedure. Planning for this means the extra animals required are ordered in time, the arrangement of any extra cages for the large control group is properly set out and the analysis and interpretation of the results are considered.

5. Conclusion

Good overall planning allows early identification of factors that may affect severity, such as unanticipated adverse effects and, where possible, stages the experiments in a programme to achieve objectives at the lowest level of severity before proceeding to higher severity. Careful consideration of the steps and experiments needed to meet well-specified objectives and adjusting the sequence of experiments to involve minimal severity is necessary to "reduce to an absolute minimum the amount of distress imposed ..." (Russell and Burch, 1959). The FRAME flowchart brings together the ideas on planning for reduction and refinement into a single sheet and includes points not made in previous overviews. It should help experimenters plan programmes of minimal severity and may also be a useful checklist for those doing ethical evaluation.

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