Planning for *Reduction*

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Summary — *Reduction* is one of the Three Rs which can be readily achieved in practice. This can be done by careful consideration of the experimental strategy and the implementation of good experimental design. Moreover, strategic planning leads to 'best' scientific practice and can have a positive impact on both *refinement* and *replacement*. The FRAME Reduction Steering Committee has developed a flow chart for an overall strategy for planning and conducting biomedical research. This, and important planning considerations for each of the steps proposed, are discussed. The strategy involves taking an initial overview and undertaking related background research, then planning a sequence of experiments expected to give satisfactory results with the least animal use and minimal severity, choosing an efficient design for each experiment in the sequence, reviewing the results of one experiment before progressing to the next, and conducting an overall analysis at the end of the programme. This approach should minimise animal use and maximise the quality of the resultant scientific output.

Key words: *experiment, experimental design, reduction, strategic planning.*

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Introduction

Reduction, a key element of the Three Rs, can be defined as the use of fewer animals to obtain a particular level of information, or obtaining more information with a given number of animals so that fewer animals are used overall (1). It has been described by Russell and Burch (2) as the mode of progress "most obviously, immediately, and universally advantageous in terms of efficiency". *Reduction* can most effectively be achieved by the right choice of strategy and the correct sizing of individual experiments. A complete strategy must incorporate the aims of the work to be carried out, the choice of experimental animals and techniques over the whole programme, and the ultimate method for the reporting of the work with its impact on the area. For each experimental stage, the strategy should consider the parameters to be measured, the experimental design to be employed, and the methods to be used for analysis of the results. To ensure full and effective use of any animals, it should also include the steps to be taken to allow all data to be available for consideration by other professionals. This is particularly relevant with the current emphasis on 'meta-analysis'.

A first, and an essential, step in the planning of any experimental programme is a clear statement of the objectives of the programme. However, these must be defined within the context of the overall strategy of which the single experiment is typically only a small element. The narrower objectives of an individual experiment necessarily impact on the experimental design. However, the overall, broader objectives of the work should not be ignored, and the wider aims of any scientific work as a possible basis for further research and *refinement* should also be borne in mind.

Unfortunately, while the broad objectives of the work are normally evident in publications which describe research and testing involving laboratory animals, the objective(s) of each experiment reported may not be made clear, and the experimental design and statistical methods used for analysis often cannot be adequately assessed. A number of surveys have found that many papers lack key details and give insufficient information for the reader to judge whether the experimental design was efficient, or to assess critically the statistical analysis (see, for example, 3–6). Moreover, there is often a tendency to approach *reduction* and experimental design in the short term, and outside the context of the overall strategy. Thus, the number of animals required may be considered only for a single limited experimental procedure with narrowly-defined parameters.

Many of the ideas behind achieving *reduction*, in both an experimental programme and the experiments contributing to it, have already been explored (see, for example, 1). In this paper, we deal with a planning approach that puts those ideas into a practical format. To this end, we present a strategic planning scheme and then consider steps in the experimental design and the ways in which these steps can impact upon *reduction*.

Study Planning and Experimental Design

Experiments which involve the use of laboratory animals do not normally occur as isolated 'one-off' studies. They are typically intended to address specific questions which form part of larger studies or projects. In such cases, the role of the experiment within the larger study is an essential part of the background information to be taken into account when designing the experiment. Even when a single experiment may seem to be a proper approach, a careful consideration of the objectives may indicate that two or more experiments may be more efficient in terms of animal use (7). In a regulatory context, experiments may be one of a sequence of on-going tests of particular products or product types. While the individual test to show that regulatory requirements are met may appear isolated, it should nevertheless be considered in the overall context of such testing. Similarly, the logistics of housing or procedures, may favour smaller experiments and 'one factor at a time' testing and shift the focus from the entire research programme to the individual experiment.

The overall strategy is sometimes implicit and not fully recognised by the experimenter involved in planning a particular experiment within the scheme. This view is reinforced by the concentration on the individual experiment typically found in experimental design and statistical texts. Nevertheless, all animal experiments should be considered in the context of an explicitly-recognised overall strategy. We have therefore indicated an outline format for an overall strategy (Figure 1).

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 individual animal experiments can be considered. The steps indicated in Figure 1 are discussed in more detail below.

Aims

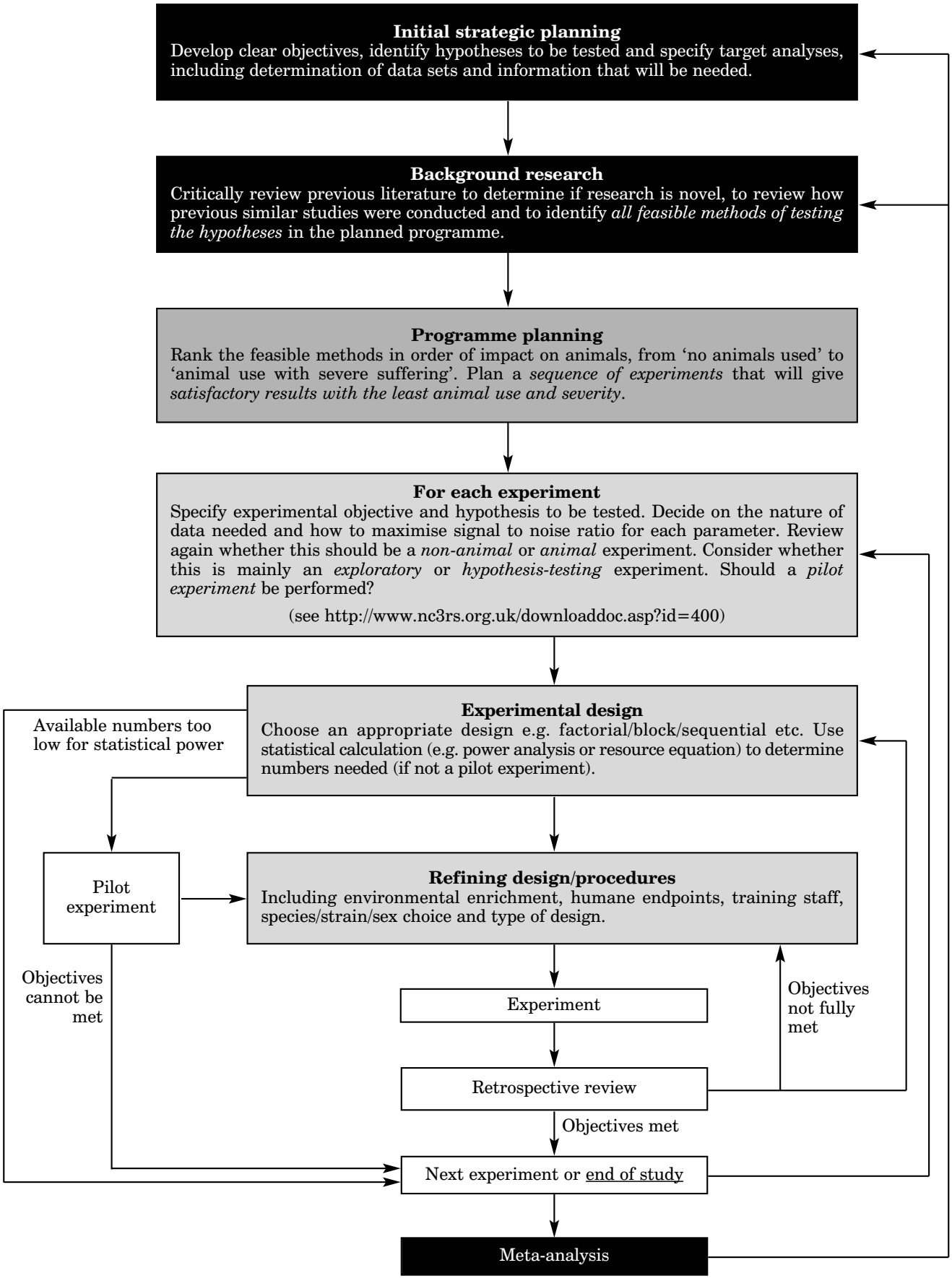
If the objectives are not clearly stated, both for overall strategy and for the particular narrow experiment, then none of the other points can be properly considered. Typically, in the initial stages, the objectives or the research hypotheses will be broad and general, while the objective(s) which can realistically be addressed by any individual experiment will be much more restricted, commonly the testing of a particular statistical hypothesis. Thus, in an example from a regulatory context, the general objective is to test that a vaccine is safe and efficacious, but an individual experiment may only test the hypothesis that the potency of the vaccine is within specified limits with a given probability. This does not exclude the possibility that some characteristics of the responses in the individual experiment may also address other issues. For example, a gross and unexpected response in the potency test could indicate the vaccine was contaminated, so the test could also serve as a screen for this rare event.

The distinction between the broader general objectives and the more restricted ones which can be achieved in individual experiments must be recognised when the programme is planned. Failure to make this distinction may lead to inappropriate designs which attempt the impossible, and produce the opposite of *reduction*.

Background research

Consideration of all feasible methods of testing the hypotheses of interest and the reasons for the specific use of animals, is an ethical requirement for all animal experiments and is essential if laboratory animal use is to be minimised. If it is established that animal experiments are necessary and appropriate for a stage in the programme, then existing information about the animals and techniques to be used must be available to those responsible for the design of the experiments involved. Information about parameters which have been/could be measured and the way in which these relate to overall strategy is important, as is the narrowing of experimental aims. In particular, the collection of additional ancillary information may offer opportunities for further *reduction* and/or *refinement* in the longer term. Such information may also provide additional support for the particular experiment (8). The techniques used and the way in which they are performed in practice must also be explicitly detailed. Implications resulting from the use of particular techniques are not always obvious (9). Frequently, parameters are measured for blood or other samples by using a variety of *in vitro*, immunoassays and other assays. The limitations of the sampling and the characteristics and designs of these assays form a critical part of the overall design, and this information should be included in the background research. It is easy to forget, for example, just how little blood can be taken from a mouse without evoking blood loss compensation mechanisms that may affect the experiment, and the limitations of an assay may mean that larger numbers of animals have to be coped with or that scope for repeat sampling must planned in. This could affect whether the overall programme can be achieved within a particular time frame or with a certain number of animals. The reliability or reproducibility of a biochemical test appropriate for a particu-

Figure 1: Strategic planning scheme for conducting biomedical experiments



lar stage in the programme which does not use live animals, may be such that the overall planning should include a preliminary validation stage or take a different path.

Experimental programme

Planning a sequence of experiments needs to take into account not only practicalities such as resource availability, but should also consider whether preliminary pilot experiments would be advisable at any stage. Time should also be allowed for reviewing the results, ideally after each experiment. The results obtained up to that point, should inform the objectives of the next experiment, and may indicate a different design or a change in the programme. Sometimes, an alternative to using live animals may be seen to be feasible, when it was not before. Often, experiments provide data in addition to that which was sought, which may suggest other research hypotheses, and in some cases (e.g. toxicological screens) the prime purpose may be to explore whether unexpected responses occur (see discussion in 1). Therefore, the sequence should have scope for modification.

Performing pilot studies is often worthwhile. As an NC3Rs document points out (see http://www.nc3rs.org.uk/downloaddoc.asp?id=400), they can reveal deficiencies in the design of a proposed experiment or procedure, allowing these to be addressed before time and resources are expended on large-scale studies. They may provide information about variability under the existing experimental conditions, and indicate whether or how this might be controlled. They may also provide vital information on the severity of proposed procedures or treatments; severe effects should be avoided for ethical reasons, and because they may evoke responses from the animals that invalidate the experimental results.

Two considerations for the individual experiments need careful thought at the programme planning stage. One is how to minimise the variation in each parameter ("noise") and maximise any effect on the parameter from a treatment ("signal"). For example, having a single person taking all the readings needed may minimise inter-operator variation in reading, but the programme may have to be planned around the availability of that person. Or an effect may be best seen in one part of the diurnal cycle, so the experiments may need to be planned to permit all the measurements to be taken at that time of day. Another consideration is how experimental units are defined and how their proper randomisation can be achieved. Randomisation is the process by which experimental units are randomly assigned to experimental treatments. The goal is that experimental units are distributed randomly across all factors that influence the outcome measures except for the factors being experimentally manipulated. It may be difficult to avoid some bias — for example, when picking animals from a group, the least nervous may be the ones that first get picked up. This can be avoided by numbering the animals, generating a sequence of random numbers, and picking the animals with those numbers in that sequence. However, this takes additional time, and the organisation of when and how to number the animals may need to be part of the programme plan. If the nature of the experiment requires that all animals in a cage are treated identically, then the experimental unit becomes the cage, and suitable plans for randomisation of cages may be required.

Experimental design and numbers of animals

Many statistical texts focus on the design and numbers of animals required for individual experiments with clearly-defined, narrow aims. One factor which often receives limited attention in these texts is a consideration of the available facilities and the feasibility of the experimental procedures in the context of the design. Designs in the 'real world' are frequently constrained by practical considerations. If, for example, the maximum number of mice per cage in a particular facility is eight, then a design based on ten mice per cage, which may be statistically 'ideal' in some sense, would not be possible and alternatives would have to be considered. The length of time required to carry out procedures cannot be overlooked, and failure to allow for this in design and analysis can lead to misinterpretation of results. Thus, time required for procedures and order of treatment should form part of the experimental design. If laboratory staff can only carry out ten procedures per morning, then any experimental design must make allowance for this. For example, multiple mornings may be required, with 'day' being a blocking factor in the experimental design. A checklist which considers essential steps for experimental design has been developed and provides a helpful guide (10). Care must be taken at this stage that practical considerations have not altered the contribution of the individual experiment to the achievement of the overall objective.

Parameters measured

For each experiment, decisions must be taken about the nature of the data needed and the ways in which these data can be precisely collected. The *refinement* and *reduction* components of the Three Rs are linked. *Refinement* can lead to *reduction*, especially in cases where *refinement* leads to improved precision of measurements. This can be particularly

important where it is possible to replace more-severe methods with less-severe methods, or qualitative measures with quantitative measurements, as for example where a temperature measurement has been used to replace a binary reaction (11). In every case, the severity of the procedure(s), the precision of the parameter(s) measured, and the feasibility of the procedure(s), all need to be considered in conjunction with one another.

Methods of analysis

The results of an experiment are not complete until the data produced by the experiment have been analysed. The methods to be used for the analysis will depend on the design of the experiment, and should be considered at the time the design is developed. Both design and analysis should conform to good statistical practice, and there are numerous texts devoted to the details of analysis. However, correct analysis requires substantial information about the 'animal' interactions and experimental conditions. An issue which frequently arises is the appropriate definition of replicates when animals are housed together in cages, or treated in groups. The definition of replicates (experimental units) has a major impact on the interpretation of the statistical analysis. If all of the animals in a cage are given the same treatment in an experiment, they will all be affected by any factor which applies to that cage, so it is the cages and not the individual animals that are the independent replicates of the treatment for the purposes of statistical tests (see, for example, 12). The risk of 'pseudoreplication' has been long been understood for ecological work (13) but, in our experience, it is not well recognised among biomedical researchers, whose publications rarely indicate how the animals given a particular treatment were distributed among cages.

Factors which may be critical to correct analysis, but which may not be explicitly recorded with the data supplied for analysis, include characteristics of the individual animals (frequently recorded as part of good laboratory practice, such as weight, sex, general condition), caging or other grouping, order of treatments, order of measurement of responses and time required for measurements, and member(s) of staff carrying out the treatment. The complete records of any experiment should include these factors as part of the data. Although all the factors may not be considered in particular analyses, this should be a decision explicitly made and reported by the analyst. Care should also be taken that baseline measures do not differ between treatment groups at the start of the experiment, and consideration should be given to the use of individual characteristics (e.g. weight) as covariates in the statistical analysis.

Retrospective review

An experiment is not complete until it, and its analysis, has been reviewed retrospectively. This review should be carried out as soon as possible after the experiment, so that all the members of staff involved are aware of what has happened and of any lessons which can be learned. Communication among all the interested parties, including the biological scientist(s) responsible for the project, the statistician(s) responsible for design and analysis, and the laboratory staff responsible for animal care and procedures, makes a review valuable and constructive. The role of the experiment and its impact on the broader programme objectives should also be considered. The review should also consider whether any changes should be made to the programme (see above).

A final step for any experimental programme should be publication of the results. Such publications should contain sufficient detail that individual experiments could be repeated. Description of all the relevant factors in publications is important for *reduction*, since any transfer of experimental method can be more readily achieved, and unnecessary repetitions can be avoided. However, not infrequently, publications omit important information (see, for example, 5 and 14).

The long-term availability of all data should be encouraged. Focus on the factors required to make the data available may enhance the experimental design and approaches to it. Moreover, the availability of the data allows for re-analysis by using different statistical methods or different assumptions. This can clearly lead to *reduction* if the available data mean that experiments do not have to be repeated. In addition to its likely uses in future *refinement* and *reduction*, the requirement that data are made available may also encourage better planning and record keeping. Clearly, such a requirement should be known from the outset, at the time when any experiment is planned.

Conclusion

Reduction should be considered from a long-term and medium-term, as well as from a short-term, narrowly focused aspect. In the medium term, the experimental programme should be planned to obtain the most value from the least animal use and minimal severity at each stage and, in the short term, the scientist should use the minimum number of animals consistent with the aims of each particular experiment. However, in the longer term, data sharing and full collection of ancillary variables may contribute substantially to future *reduction* and *refinement*. It is possible that there may be some conflict between the short-term,

medium-term and long-term approaches, and these will require consideration on a case-by-case basis.

The implementation of sound strategic planning, with clear recognition of broad general hypotheses and the way in which the necessarily more-narrow hypotheses of individual experiments contribute to them, form a basis for scientifically-valid *reduction*.

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Glossary

Factorial design — An experimental design where more than one experimental factor is examined and experimental units take on all possible combinations of the factors.

Experimental unit — The biological material that is measured AND can be independently assigned to any of the experimental treatments. In animal experiments, the experimental unit is often the animal, but if all animals in the same cage receive the same treatment, they are not independent, as they will share any cage effect that there may be. Therefore, the cage is the correct experimental unit.

Blocked design — An experimental design that controls for a nuisance factor known to influence the outcome measures of interest. Blocks represent categorical factors (for example, room, cage or time period) to which experimental units can be assigned randomly.

Power (or statistical power) — The probability that a false negative result will NOT occur in a statistical test. Alternatively, it can be seen as the probability that you will not fail to find a difference that really exists.