UFAW Animal Welfare Series

Ninth Edition

The UFAW Handbook on The Care and Management of Laboratory and Other Research Animals

Edited by **Huw Golledge** and **Claire Richardson**

WILEY Blackwell

THE UFAW HANDBOOK ON

The Care and Management of Laboratory and Other Research Animals

The Universities Federation for Animal Welfare

UFAW, founded in 1926, is an international independent, scientific and educational animal welfare charity and membership society. UFAW's vision is a world where the welfare of all animals affected by humans is maximised through a scientific understanding of their needs and how to meet them. UFAW aims to discover what matters to animals, develop scientific solutions to animal welfare problems and disseminate evidence-based animal welfare information by:

- Funding scientific research
- Supporting the careers of animal welfare scientists
- Disseminating animal welfare science knowledge both to experts and the wider public
- Providing expert advice to government departments and other bodies, helping to draft and amend laws and guidelines

You can help make even bigger strides in animal welfare science by donating, leaving a legacy or becoming a member of UFAW. For further information about UFAW and how you can help promote and support our work, please contact us at the following address:

Universities Federation for Animal Welfare
The Old School, Brewhouse Hill,
Wheathampstead, Herts AL4 8AN, United Kingdom
Phone: +44 (0) 1582 831818
Website: www.ufaw.org.uk
Email: ufaw@ufaw.org.uk

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The Care and Management of Laboratory and Other Research Animals

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Edited by
Huw Golledge
Universities Federation Animal Welfare
UK

Claire Richardson
Universities Federation Animal Welfare
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Contents

List of contributors

†= have died before book was published

James R. Anderson
Department of Psychology, Graduate School of Letters,
Kyoto University
Kyoto, Japan

Kenneth A. Applebee
Applebee Advisory, Spectrum House
Hornchurch, Essex, UK

Jaco Bakker
Animal Science Department, Biomedical Primate
Research Centre
Rijswijk, The Netherlands

Simon T. Bate
CMC Statistics, GlaxoSmithKline Medicines Research Centre
Stevenage, UK

Melissa Bateson
Biosciences Institute, Newcastle University
Newcastle, UK

Kathryn Bayne
AAALAC International
Frederick MD, USA

Jackie Boxall
GSK, Stevenage, UK

Hannah M. Buchanan-Smith
Behaviour and Evolution Research Group, Psychology,
Faculty of Natural Sciences, University of Stirling
Scotland, UK

Kimberly M. Cheng
Avian Research Centre, University of British Columbia
Vancouver, Canada

Sonja T. Chou
Laboratory Animal Resources
NYU Shanghai, Shanghai, P. R. China

Nicola S. Clayton
Department of Psychology, University of Cambridge
Cambridge, UK

Donna Clemons
Comparative Medicine, AbbVie Inc.
North Chicago, IL, USA

John E. Cooper
Department of Veterinary Medicine, University of Cambridge
Cambridge, UK

Robyn J. Crook
Department of Biology, San Francisco State University
San Francisco, CA, USA

Steven J. Cubbitt
Cube Cleantech Limited, St John's Innovation Park
Cambridge, UK

Sietse F. de Boer
Behavioural Neuroscience group, GELIFES, University of
Groningen
Groningen, The Netherlands

Mike Dennis
Health Protection Agency
Porton Down, Salisbury, UK

Emma Desforges
Waltham Petcare Science Institute,
Waltham-on-the-Wolds, UK

Nicolas Dudoignon
Corporate Social Responsibility
Sanofi, Gentilly, France

Henrik Duelund Pedersen
Danish Medicines Agency, Regulatory & Clinical
Assessment
Copenhagen, Denmark

Ian J.H. Duncan
The Campbell Centre for the Study of Animal Welfare,
University of Guelph
Guelph, Canada

Cathy M. Dwyer
Animal Behaviour & Welfare, Animal & Veterinary Sciences,
Scottish Rural University College
Edinburgh, UK

Therese Edström
AstraZeneca R & D Mölndal
Mölndal, Sweden

Robert W. Elwood
Queen's University Belfast, School of Biological Sciences,
Belfast, UK

Heather Ewence
(formerly of) The Animal Health Trust
Suffolk, UK

Christopher G. Faulkes
School of Biological and Behavioural Sciences,
Queen Mary University of London
London, UK

J. Norman Flynn
Animals in Science Regulation Unit (ASRU),
Home Office Science, Security and Innovation, UK

Malcolm P. France
Consultant, Laboratory Animal Care and Management
NSW 2045, Australia

Eberhard Fuchs
Clinical Neurobiology Laboratory, German Primate Center,
Leibniz Institute for Primate Research
Göttingen, Germany

Carlos Garcia de Leaniz
Centre for Sustainable Aquatic Research, Swansea University
Wales, UK

Joseph Garner
Department of Comparative Medicine, Stanford University
Stanford, California, USA

Colin L. Gilbert
Babraham Institute
Babraham, Cambridge

Huw Golledge
UFAW
Wheathampstead, UK

Javier Guillen
AAALAC International
Pamplona, Spain

Marcel Gyger
Swiss Federal Institute of Technology, School of Life
Sciences, Center of PhenogenomicsLausanne, Switzerland

Mark Haselgrove
University of Nottingham, School of Psychology
Nottingham, UK

Penny Hawkins
RSPCA, Animals in Science Department
Horsham, UK

Meghan Holst
Department of Biology, San Francisco State University
San Francisco, CA, USA

Linda Horan
University of Strathclyde, Strathclyde Institute of Pharmacy
and Biomedical Sciences
Glasgow, UK

Ryan B. Howard
Department of Biology, San Francisco State University
San Francisco, CA, USA

Bryan Howard
(formerly of) University of Sheffield
Sheffield, UK

Shellene Hurley
Animal and Plant Health Agency, Pathology & Animal
Sciences
Addlestone, Surrey, UK

Ray W. Ingle
Natural History Museum
London, UK

Maggy Jennings
RSPCA, Animals in Science Department
Horsham, UK

Sylvia Kaiser
University of Münster, Department of Behavioural Biology
Münster, Germany

Petra Kirsch
University Hospital Hamburg-Eppendorf
Hamburg, Germany

Annette Klein
Institute of Zoology, University of Veterinary Medicine
Hannover
Hannover, Germany

Annika Kollikowski
Institute of Zoology, University of Veterinary Medicine
Hannover
Hannover, Germany

Jaap M. Koolhaas
Behavioural Neuroscience group, GELIFES, University of
Groningen
Groningen, The Netherlands

Christine Krüger
German Institute of Human Nutrition
Potsdam-Rehbruecke
Nuthetal, Germany

Julie M. Lane
The Food and Environment Research Agency
Sand Hutton, York, UK

Jan A.M. Langermans
Animal Science Department, Biomedical Primate
Research Centre
Rijswijk, The Netherlands
Population Health Sciences, Unit Animals in Science and
Society, Veterinary faculty
Utrecht University, The Netherlands

Rusty Lansford
Keck School of Medicine, University of Southern California
Los Angeles, USA

Gail Laule
Mandai Wildlife Group
Singapore

Graham Law†
University of Glasgow
Glasgow, UK

Stephen E. G. Lea
University of Exeter
Exeter, UK

Lena Lidfors
Department of Animal Environment and Health, Swedish University of Agricultural Sciences, Skara, Sweden

Maggie Lloyd
Red Kite Veterinary Consultants
Abingdon, UK

Jennifer Lofgren
Novartis Institutes for BioMedical Research Inc.
Cambridge, USA

Annet L. Louwerse
Animal Science Department, Biomedical Primate Research Centre
Rijswijk, The Netherlands

Arianna Manciocco
Istituto di Scienze e Tecnologie della Cognizione, Consiglio Nazionale delle Ricerche Rome, Italy

Robbie A. McDonald
The Food and Environment Research Agency
Sand Hutton, York, UK

Anthony McGregor
University of Durham, Department of Psychology
Durham, UK

Rachael Miller
Department of Psychology, University of Cambridge, Cambridge, UK
School of Life Sciences, Anglia Ruskin University, Cambridge, UK

Timothy H. Morris
The School of Veterinary Medicine and Science, The University of Nottingham
Sutton Bonington, UK

Guy Mulder
Research Models and Services, Charles River Laboratories, Inc.
Wilmington, MA, USA

Ruedi G. Nager
University of Glasgow, School of Biodiversity, One Health and Veterinary Medicine Glasgow, UK

Janja Novak
Division of Animal Welfare,Veterinary Public Health Institute, Vetsuisse Faculty, University of Bern
Bern, Switzerland

I. Anna S. Olsson
i3S - Instituto de Investigação e Inovação em Saúde and IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto
Porto, Portugal

Aleksandar Popovic
Research Models and Services, Charles River UK Ltd, Margate, UK

Kathleen R. Pritchett-Corning
Office of Animal Resources, Harvard University, Cambridge, MA, USA
Department of Comparative Medicine, University of Washington, Seattle, WA, USA

Jan-Bas Prins
The Francis Crick Institute, London, UK
Leiden University Medical Centre, The Netherlands

Ute Radespiel
Institute of Zoology, University of Veterinary Medicine Hannover
Hannover, Germany

Sonia Rey Planellas
Institute of Aquaculture, University of Stirling
Scotland, UK

Claire Richardson
UFAW
Wheathampstead, UK

Jon Richmond
Ethical Biomedical Research and Testing: Advice and Consultancy, UK

David Robb
Veterinary Services, Charles River Laboratories Edinburgh Ltd
Edinburgh, UK

Catriona M. E. Ryan
University of Exeter
Exeter, UK

Norbert Sachser
University of Münster, Department of Behavioural Biology
Münster, Germany

Peter Sandøe
Department of Food and Resource Economics, Science, Department of Veterinary and Animal Sciences, Health, University of Copenhagen
Denmark

Elke Scheibler
(formerly of) University of South Wales
UK

Marina Scheumann
Institute of Zoology
University of Veterinary Medicine Hannover
Hannover, Germany

Martina Schiestl
Faculty of Veterinary Medicine
University of Veterinary Sciences
Brno, Czech Republic

Daniel Schmidtke
Institute of Zoology
University of Veterinary Medicine Hannover
Hannover, Germany

Annette Schuhmacher
ssniff Spezialdiaeten
Soest, Germany

Laura Scullion Hall
(formerly of) University of Stirling
Stirling, UK

Christopher H.J. Sear
Cube Cleantech Limited
St John's Innovation Park
Cambridge, UK

Adrian Smith
Norecopa
Norwegian Veterinary Institute,
Norway

S. Clare Stanford
Department of Neuroscience, Physiology & Pharmacology
University College London
London, UK

Michelle Stewart
Mary Lyon Centre, MRC Harwell
Harwell Campus
Oxfordshire, UK

Richard Tinsley
School of Biological Sciences
University of Bristol
Bristol, UK

Graham Tobin
(formerly of) Harlan Teklad, Blackthorn
Bicester, UK

Carl S. Tucker
The QMRI (BVS) Aquatic Facilities, The Queen's Medical Research Institute
The University of Edinburgh
Edinburgh, UK

John L. VandeBerg
University of Texas Rio Grande Valley, Department of Human Genetics
South Texas Diabetes and Obesity Institute
Brownsville/Harlingen/Edinburg, Texas, USA

Marit K. Vernes
Animal Science Department
Biomedical Primate Research Centre
Rijswijk, The Netherlands

Elisabetta Visalberghi
Istituto di Scienze e Tecnologie della Cognizione
Consiglio Nazionale delle Ricerche Rome, Italy

Sara Wells
The Mary Lyon Centre at MRC Harwell
Harwell Science Campus
Oxfordshire, UK

Ute Weyer
Animal and Plant Health Agency
Pathology & Animal Sciences
Addlestone, Surrey, UK

Fleur Whitlock
(formerly of) The Animal Health Trust
Suffolk, UK

Michael J.A. Wilkinson
University of Glasgow, Biological Services, Veterinary Research Facility
Glasgow, UK

Sarah Williams-Blangero
University of Texas Rio Grande Valley, Department of Human Genetics
South Texas Diabetes and Obesity Institute
Brownsville/Harlingen/Edinburg, Texas, USA

Carole Wilson
University College London
London, UK

Christina Winnicker
Research Office of Animal Welfare Ethics Strategy and Risk
GlaxoSmithKline
Collegeville, Pennsylvania, USA

Jennifer Wittkowski
Institute of Zoology
University of Veterinary Medicine Hannover
Hannover, Germany

Hanno Würbel
Division of Animal Welfare, Veterinary Public Health Institute, Vetsuisse Faculty
University of Bern
Bern, Switzerland

Adrian Zeltner
Ellegaard Göttingen Minipigs A/S
Dalmose, Denmark

Elke Zimmermann†
Institute of Zoology
University of Veterinary Medicine Hannover
Hannover, Germany

Foreword

The use of animals in research is rightly a subject of a certain amount of public debate, but there is no doubt of the huge benefits to both human and animal health and well-being which has accrued as a result of highly regulated and scientifically based animal research. In this latest and impressive ninth edition, like its predecessors, this invaluable handbook from the Universities Federation for Animal Welfare (UFAW) continues to uphold the highest standards of animal welfare in research. It plays a crucial role in maintaining the social contract that allows for their ethical use.

The ninth edition comprehensively updates the eighth edition of 2010 and provides up-to-date, evidence-based information on the practical care and welfare of animals used in research to enable those who work with these animals to ensure the animals are cared for to the highest possible standard whilst also ensuring that they provide reliable research data.

Of 51 chapters, the first substantive one covers a priority issue, the Three Rs – *replacement*, *reduction* and *refinement* – which are a constant and continuing goal of all researchers. Very appropriately, the next chapter deals with the design experiments, which is critical to ensure statistically reliable results from as few animals as possible.

There follow a series of detailed chapters covering all the important general aspects of the care and use of laboratory animals, including welfare assessment, housing and the design of facilities, transport, nutrition and the legislation controlling the conduct of research. In addition to legislation, the use of animals now invariably requires ethical approval, the subject of another chapter. A further 33 authoritative chapters deal in depth with all types of animals used in research, including a major and brilliant chapter on laboratory mice. This makes the powerful point that providing enrichment and allowing animals control over their environments are critical to minimising their stress and maximising the biological relevance and predictive accuracy of any data derived from them. All of these chapters are written by experts in their fields from all over the world.

This superb handbook is of great value to researchers and to the welfare of the animals they use. Its authoritative and evidence-based exposition on such an important topic continues the exemplary contributions made by UFAW to the cause of animal welfare.

Emeritus Professor the Lord Trees, FRCVS,
FMedSci, HonFRSE
House of Lords,
London, SW1A 0PW,
United Kingdom
22 January 2024

1 Introduction

Claire Richardson and Huw Golledge

We are very pleased to introduce the 9th edition of *The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals*. The first edition of this book, published in 1947, was the first ever laboratory animal handbook published with the aim of improving the welfare of animals used in research, and it continues to be a key reference for those in the field. Putting the latest animal welfare science into practice to improve animal welfare is central to the mission of Universities Federation for Animal Welfare (UFAW), and this handbook exemplifies this practical approach to improving the lives of animals.

The previous (8th) edition of the handbook was published in 2010. Chapters have been updated to reflect rapidly growing advances in the field, and new chapters have been added on nutrition, feeding and animal welfare (Chapter 14); 3Rs considerations when using ageing animals in science (Chapter 16); ethical review (Chapter 18); the naked mole rat (Chapter 26); corvids (Chapter 45); and zebrafish (Chapter 49).

Our thanks go to Dr. Robert Hubrecht, co-editor of the 8th edition of the handbook, who instigated this new edition before retiring as UFAW's chief executive in 2019. Although he retired before the project was complete, we are very grateful for his knowledge, insight and organisational skills, which were instrumental in initiating this project. We are also grateful for Dr. Hubrecht's longstanding commitment to promoting animal welfare and science. Our thanks also go to Dr. Birte Nielsen, Research Director of UFAW, for her editorial guidance, knowledge and encouragement in completing the handbook.

We are grateful to the Wiley publishing team, particularly Adalfin Jayasingh and Rathi Aravind, for their patience and professionalism throughout the production stage.

Our greatest thanks go to the members of the laboratory animal science and welfare community that came together to both write and peer-review the chapters of this handbook. Colleagues from around the world volunteered their time to provide their expertise and share their passion for animals and science to write the individual chapters. In addition, numerous anonymous referees generously volunteered their time and expertise to provide invaluable comments on draft chapters, which have significantly improved the contents of each.

It has not always been an easy journey, and progress was particularly difficult during the COVID pandemic, so we are grateful for the patience of the contributing authors who submitted chapters early in the production phase and to the resilience of the authors who submitted later on and endured our nagging emails. It has been our pleasure to learn from all those involved.

As a charity, UFAW relies on its members and donors to carry out its work, so we thank them as well as the UFAW staff and trustees, without whom we would not have been able to dedicate time and resources to produce this updated handbook.

As with previous editions, we have, as editors, aimed to ensure that the chapters reflect UFAW's approach to the care and husbandry of animals used in research; however, the chapters are the individual authors' work, and the views they have expressed should not be taken as UFAW's official opinions. Similarly, it is, of course, the responsibility of those working with animals to ensure that the practices they adopt comply with national legislation.

We hope that you find this handbook useful and that it helps to promote good welfare and good science.

The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, Ninth Edition.
Edited by Huw Golledge and Claire Richardson.

PART 1
IMPLEMENTING THE THREE RS IN RESEARCH USING ANIMALS

2 The Three Rs

Adrian Smith and Jon Richmond

Opening remarks

The Universities Federation for Animal Welfare (UFAW) actively promotes the welfare of animals bred, kept and used for experimental and other scientific purposes by:

- championing a scientific approach to animal care and welfare, providing evidence-based insights into '*what is meaningful to the animal*'; and
- advocating that '*best welfare is indeed best science*' and that we must '*. . . aim at well-being rather than at mere absence of distress*' (Russell & Burch 1959).

In 1954, UFAW commissioned work by William Russell and Rex Burch which led to the publication in 1959 of *The Principles of Humane Experimental Technique* (Russell & Burch 1959). Russell and Burch reasoned that high standards of animal welfare facilitate better animal-based science. They concluded that '*. . . humanity can be promoted without prejudice to scientific and medical aims*' and '*. . . the humanest possible treatment of experimental animals . . . is actually a prerequisite for successful animal experiments*'. . . '*If we are to use a criterion for choosing experiments to perform, the criterion of humanity is the best we could possibly invent . . . The greatest scientific experiments have always been the most humane and most aesthetically attractive, conveying that sense of beauty and elegance which is the essence of science at its most successful*'.

They championed the principles of Replacement, Reduction and Refinement, now universally known as 'the Three Rs'. These principles, and an understanding that they must be embedded in the planning, conduct and review of animal-based research and testing, are now an integral part of mainstream biomedical science and form the basis of legislation on animal research and testing in many countries (Guillén 2017)[1].

This chapter provides a contemporary overview of the principles, art and practice of humane experimental technique as it has evolved since Russell and Burch's landmark publication. This chapter focuses on general principles as a prelude to the more detailed and context-specific material in later chapters.

Introduction

It is now accepted that some classes of animal, such as vertebrates, can experience negative welfare states such as pain, suffering and distress, and all of those involved in the production, care and use of live animals for scientific or other experimental purposes have a moral, and in many cases a legal, obligation to minimise any justifiable suffering caused.

It also generally accepted that animal studies should only be undertaken when all of the following conditions are met:

- the scientific objectives are timely, of sufficient importance, attainable, and that the scientific and societal benefits will be maximised;
- there is no non-sentient replacement alternative;
- all relevant and practical Reduction and Refinement strategies have been implemented; and
- the design and conduct of the study minimise the animal welfare cost in terms of the total pain, suffering and distress that may be produced, rather than simply minimising the number of animals used.

Implementing humane experimental technique to minimise animal welfare costs requires knowledge and understanding of:

- behaviours and clinical findings in normal, healthy animals;
- the impact of animal care systems and scientific procedures;
- how animal welfare can be evaluated; and
- the development and application of informed, practical solutions to identify, manage and minimise the animal welfare costs.

[1]See also https://en.wikipedia.org/wiki/Animal_testing_regulations (accessed 10 Jan 2022)

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Simple words, complex meanings

The commonly used definitions of alternatives, Replacement, Reduction and Refinement, are deceptively simple. They conceal subtleties of meaning which must be fully understood in order to appreciate the power and relevance of the Three Rs.

The term 'alternatives', used by Smyth (1978) for the Three Rs, can mislead by creating the false impression that only Replacement is relevant, or that 'alternative' methods simply substitute for, but retain the scope and limitations of, the original animal models.

In reality, Replacement alternatives are not just substitutes for animal models: they are often better science, more powerful and versatile and the tools of choice. For example, the use of robotics and *in vitro* replacement systems for high-throughput screening of potential novel pharmaceuticals allows rates of progress not previously possible using animal models.

Reduction is better considered as optimisation of animal numbers. The intention is to minimise the number of animals required to provide suitably robust data. Using more is wasteful; using fewer at best requires that work is repeated and at worst results in misleading conclusions being drawn from the available data. There are occasions when the original estimates of the number of animals required prove on examination to be too few to meet the scientific objectives, and on these occasions properly applying the principles of Reduction will result in the justified use of more animals than originally estimated.

Reduction and Refinement are inseparable: the imperative is not to minimise the number of animals used, but to minimise the suffering that is caused.

The origin and evolution of the Three Rs

Early scientific use of animals was often curiosity-driven, and involved demonstrating biological phenomena, without necessarily having practical application (Barley 1999). A gradual shift then occurred towards understanding the underlying mechanisms and regulation of observed phenomena. This was, in turn, followed by a move to 'deductive science' – based on formulating and testing hypotheses, seeking results with practical applications, and publishing the results widely. These differing approaches to science are still reflected in the types of animal studies undertaken and animal models used (Festing 2011):

- exploratory models demonstrating biological phenomena or generating knowledge without necessarily being relevant to any immediate practical application;
- explanatory models elucidating the mechanisms;
- predictive models allowing problem solving and decision making.

The origins of the concepts of Replacement, Reduction and Refinement in relation to the use of animals in science date back to Victorian Britain (Richmond 2000). An editorial in the London Medical Gazette in 1839 advised that live animals should not be used

> *. . . till it is sufficiently clear that the fact pursued neither is, nor can be proved by any other evidence which is within reach, nor by any more mode of enquiry.* (Anon. 1839)

Principles of humane experimental technique were set out in more detail in Marshall Hall's publications from the same period. In an article in the Lancet in 1847 he wrote:

> *We should never have recourse to experiment in cases which observation can afford us the information required; No experiment should be performed without a distinct and definite object and without the persuasion that the object will be attained and produce a real and uncomplicated result; We should not needlessly repeat experiments and cause the least possible suffering, using the lowest order of animals and avoiding the infliction of pain; We should try to secure due observation so as to avoid the necessity of repetition.* (Hall 1847)

He composed the following seven principles (published posthumously by his widow in 1861)[2]

1. We should never have recourse to experiment in cases which observation can afford us the information required.
2. No experiment should be performed without a distinct and definite object, and without the persuasion, after the maturest consideration, that that object will be attained by that experiment, in the form of a real and uncomplicated result.
3. We should not needlessly repeat experiments which have already been performed by physiologists of reputation.
4. After due consideration that a given experiment is, at once, essential and adequate to the discovery of a truth, it should be instituted with the least possible infliction of suffering.
5. Every physiological experiment should be performed under such circumstances as will secure due observation and attestation of its results, and so obviate, as much as possible, the necessity for its repetition.
6. Facts should be laid before the public in the simplest, plainest terms. If there be a difference of opinion: '...add such views as may seem nearest the truth. These are neither wholly in accord with one opinion nor another, nor exceedingly at variance with both, ... a thing which may be observed in most controversies, when men seek impartially for truth'. (Celsus, translated from Latin)
7. In quoting the opinions of other authors, it should always be in their own words.

Russell and Burch originally defined:

- Replacement as '*any scientific method employing non-sentient material which may in the history of animal experimentation replace methods which use conscious living vertebrates*';
- Reduction as means of minimising, other than by Replacement, '*the number of animals used to obtain information of a given amount and precision*';

[2]https://www.ahajournals.org/doi/epdf/10.1161/01.CIR.48.3.651 (Accessed 12 Dec 2022)

- Refinement as measures leading to a '*decrease in the incidence or severity of inhumane procedures applied to those animals which have to be used*'.

The working definitions of the Three Rs commonly used today are often somewhat different to these originals (Buchanan-Smith *et al*. 2005; Tannenbaum and Bennett 2015; the NC3Rs web site[3]). More contemporary interpretations include:

- Replacement: methods which permit a given scientific objective to be achieved without conducting procedures on animals which impose any welfare cost;
- Reduction: methods for obtaining equivalent levels of information from the use of fewer animals in scientific procedures, or for obtaining more information from the same number of animals;
- Refinement: methods which alleviate or minimise potential pain, suffering or distress, and which enhance animal well-being.

The acceptance and application of the principles of humane experimental technique after Russell and Burch's book in 1959 was followed by a period of reduction in animal use and significant welfare gains, at a time of increasing investment and rapid advances in the biomedical sciences. The production and use of animals for experimental and other scientific purposes are, however, now increasing again, primarily due to the production and use of genetically altered animals[4,5]. Nevertheless, the principles of humane experimental technique, albeit with revised definitions, are proving to be sufficiently relevant and flexible to be applicable to areas of animal use not foreseen at the time of Russell and Burch's publication.

Progress with the Three Rs is not solely driven by a desire to improve animal welfare. Methodological improvements are required to overcome the limitations of existing animal models and open up new lines of scientific enquiry. In practice, 'alternative' methods based on the Three Rs are generally more technically advanced, cost effective, reliable, easily scalable and may be more scientifically valid than those traditionally used. The development and use of Adverse Outcome Pathways (AOP) to improve regulatory testing regimens is an example of these principles being applied in practice (Vinken 2013) to better protect the public and the environment.

Thus, the case for the Three Rs can be made simultaneously on three grounds:

- better animal welfare;
- better science in terms of quality and rate of progress; and
- logistics and economics.

Where implementation of the Three Rs is at times hampered by the lack of scientific evidence about, or a consensus on, what constitutes 'best practice', a useful approach to promoting the Three Rs and high-quality, humane animal-based research can be the Three Ss of good Sense, Sensibilities and Science (Rowsell 1977; Smith & Hawkins 2016).

[3]https://www.nc3rs.org.uk/the-3rs (accessed 10 Jan 2022)

[4]http://ec.europa.eu/environment/chemicals/lab_animals/reports_en.htm (accessed 10 Jan 2022)

[5]https://www.gov.uk/government/collections/animals-in-science-statistics (accessed 10 Jan 2022)

A holistic approach to the Three Rs

Russell and Burch recognised the need for a holistic, rather than sequential, approach to the Three Rs, particularly with respect to Reduction and Refinement. Tensions can exist, balances may have to be struck, and synergies exploited. Decisions must be made on a case-by-case basis in the context of the specific scientific objectives being pursued.

To minimise animal suffering, Reduction and Refinement must be considered concurrently. For example, there are technologies that involve initial surgical preparation but which then reduce the total number of animals required, minimise the stress animals subsequently experience and improve the quality of the findings. Implantable telemetry devices (Kramer & Kinter 2003) can allow the remote capture of intermittent or continuous streams of 'physiological data' while animals undertake normal activities unstressed by disturbances to the social group, sedation, handling or restraint. These technologies may permit the numbers of animals per study to be reduced by the capture of serial data and the reuse of telemetered animals. In such cases there are trade-offs to be made between the welfare costs of the initial surgical preparation, the reduction in the number of animals required to give meaningful results, the procedural stresses that can be avoided after recovery from surgery and the improved nature and quality of the data that can be gathered (Brockway *et al*. 1993; Schnell & Gerber 1997).

Serial diagnostic imaging can also reduce numbers, at the cost of the serial general anaesthetics that are normally required to restrain the animal during imaging.

These, and other, examples illustrate the need to take a holistic rather than a sequential approach when putting humane experimental technique into practice.

Replacement

Although we do not currently have the means to replace all forms of animal use without slowing scientific progress, Replacement is especially relevant to fundamental and applied biomedical research, regulatory testing and the use of animals in education and training. Replacement alternatives typically offer a range of benefits over the animal models they supersede, often allowing more rapid progress and in some cases providing scientific insights that were not possible using animal models.

Replacement alternatives must be based on sound science and produce responses that correlate with those of biological systems which they model. Their development requires an understanding of the underlying biological mechanisms and their responses, and is often dependent on the availability (or *de novo* generation) of reliable animal or human reference data, and new technologies.

Russell and Burch distinguished between 'absolute' Replacement, with no sentient animal use (for example, computer models), and 'relative' Replacement, using animals in procedures not causing pain, suffering or distress (for example, humane killing to obtain tissue, experiments under full terminal anaesthesia and the use of immature forms believed to be incapable of experiencing pain, suffering or distress).

Progress with Replacement has, to date, been largely with single-stage processes involving biological effects mediated by clearly understood single-event mechanisms, and for which there is high-quality human or relevant animal reference data. Devising non-animal models of dynamic and complex biological interactions is more difficult, but progress is being made, for example by producing so-called organs-on-chips (Marx *et al*. 2016; Trapecar 2021; Vulto & Joore 2021).

The range of Replacement options can be wide and varied, and may include:

- strategies avoiding the need to generate new animal-based data;
- systems allowing elements of evidence gathering, analysis or decision making to be undertaken without live animal use;
- animal-based methods and models providing the required insights without causing procedure-related pain, suffering or distress to sentient animals.

Replacement strategies

In some instances, new scientific objectives can be achieved without the need for animal use. Examples include:

- Rationalising and harmonising regulatory requirements and provisions to dispense with inessential tests. For example, the Abnormal Toxicity Test (Schwanig *et al*. 1997) is no longer required for the evaluation of a wide range of biologicals used in clinical practice.
- Harmonising international validation processes, regulatory testing requirements and decision making, to eliminate the need to use animals in different protocols to inform multiple regulators in different geographical regions about a single toxicological endpoint.
- Reformulating scientific objectives to allow relevant insights to be gained using existing data or new non-animal data.
- Reviewing published work to ensure that relevant existing data are not overlooked and animal experiments inadvertently replicated. There is, however, an important distinction to make between inadvertent, unnecessary duplication (the unintended repetition of studies that have already been completed and reported) and justified, intentional replication. The latter may be necessary to confirm findings, introduce new model systems, evaluate procedural changes, restart programmes of work after periods of inactivity, or when changing laboratories.
- Data sharing where previous relevant findings have not been published: for example, accessing data generated for in-house decision making or contained in regulatory submissions.
- Sharing tissues and samples from animals killed or used for scientific purposes.

Replacement methods

Where new data are required, a wide range of Replacement methods can be considered:

- The use of physico-chemical properties to predict biological effects to screen or fully evaluate test materials. Examples include the use of pH and buffer capacity to predict potential severe ocular irritation or corrosion (OECD, 2017); peptide reactivity assays to screen chemicals for skin sensitisation potential (Lalko *et al*. 2012); and the use of computer and mathematical models allowing molecular structure to be correlated with specific biological activities[6].
- The use of non-sentient organisms. Examples include the use of bacteria (Ames *et al*. 1973), roundworms (Leung *et al*. 2008) and fruit flies (Perrimon *et al*. 2016).
- The use of immature forms of sentient species incapable of experiencing pain, suffering or distress; for example, fish larvae to evaluate aquatic toxicity (Lilicrap *et al*. 2016).
- The use of *ex vivo* and *in vitro* systems, of animal or human origin, at the level of the organ, tissue slice, cell culture/suspension or sub-cellular component (Marx *et al*. 2016; Vulto & Joore 2021). These may be absolute replacements (for example, non-primary cell cultures that do not require to be maintained using foetal calf serum), or relative replacements (for example, animal primary cell cultures, or other cell culture systems requiring the use of serum).
- The collection of material shed by animals (e.g. their fæces, hair, saliva and urine) from which DNA can be retrieved, as an alternative to invasive capture and sampling methods (e.g. Bischof *et al*. 2020). The collection of environmental DNA is especially relevant in field research. Genotyping can be used to identify and track both individuals and populations.
- Human studies, subject to appropriate ethical safeguards. Data may be gathered in the course of volunteer, clinical-trial, post-marketing surveillance or epidemiological studies. New technologies (for example, improved methods of diagnostic imaging, and preclinical markers of biological effects) can offer new opportunities to work with ethically human subjects.

The pros and cons of animal use in education and training have been hotly debated for many years, both for ethical reasons and in the light of the Three Rs (Zemanova *et al*. 2021). A wide range of Replacement alternatives can be used in education and training, once the educational objectives have been clearly defined. These can be used to demonstrate biological phenomena, processes and interactions; train participants in manual skills and develop proficiency in problem solving. These 'alternatives' include models, films and videotapes of procedures, interactive software simulations and virtual reality systems. The NORINA database contains information on 3,000 alternatives and supplements to animal use in education and training, at all levels of academia and industry[7].

The high-fidelity fallacy

All model systems, whether they are animal or non-animal models, mimic only limited aspects of the human condition or other target system (Sams-Dodd 2006). This must be kept in

[6]https://echa.europa.eu/support/registration/how-to-avoid-unnecessary-testing-on-animals/qsar-models (accessed 10 Jan 2022)

[7]https://norecopa.no/NORINA (accessed 10 Jan 2022)

mind when the most appropriate model is selected, and findings analysed, interpreted, generalised and extrapolated. Scientists must be fully aware of the scope, and the limitations, of the models that they use (Pound & Ritskes-Hoitinga 2018).

Russell and Burch (1959) warned of the 'high-fidelity fallacy': the false assumption that high-fidelity (the closeness in biological terms of a model system to the actual system of interest) dictates the preferred model system. Non-human primates can be considered to be high-fidelity models of man as '*in their general physiological and pharmacological properties*' they are '*more consistently like us than are other organisms*'. However, any instinctive preference for high-fidelity models '*ignores all the advantages of correlation*', whereby '*the responses of two utterly different systems may be correlated with perfect regularity*' (Russell and Burch (1959)).

High-fidelity models are generally not required in practice. What is essential is not that a model system 'looks like' the system of interest, but that it behaves like it. The essential quality of a good model system is high *discrimination*: its ability, in the context of a defined biological process or outcome, to produce responses which correlate with the response of the system which they model. Replacement alternatives (for example, isolated tissues, cell cultures and computer models) generally possess high discrimination but are, inevitably, low-fidelity. In the words of Russell and Burch, they '*reproduce one particular property of the original, in which we happen to be interested*'. This concept is partially fuelling a transition away from animal experimentation to studies of human cells, tissue or organs, particularly within the realm of toxicology where it is, at present, most feasible (Kimura *et al*. 2018).

Reduction

Reduction and Refinement must always be considered simultaneously. Focusing purely on decreasing numbers can lead to solutions that produce a disproportionate increase in the pain and distress caused to the animals that are used (Richmond 1999).

Reduction can be considered to comprise any strategy or method which:

- other than by Replacement reduces the need for animal studies; or
- minimises the number of animals required to achieve a defined scientific objective; or
- permits more data or product to be obtained from the animals that must still be used.

Experimental design

Elements of experimental design such as sample size, statistical power, variation, precision and the proper application of appropriate statistical methods are important means of determining the number of animals required and interpreting the data that is generated (see Festing & Altman 2002, and the Norecopa web site[8]). However, there are equally important non-statistical considerations. These include selection of suitable experimental subjects, husbandry and care systems, procedural details and other means of controlling and minimising unwanted stressors and unnecessary variables. See, among others, Chapter 15 (The use of positive reinforcement training techniques to enhance the care and welfare of laboratory and research animals).

Having exercised due diligence to ensure a new animal study is not unknowingly duplicating the work of others and that there are no suitable Replacement options, it is important to be aware of, and consider, the full range of Reduction options and opportunities.

The sequence in which a series of objectives are pursued and experiments are carried out is an important consideration. One of the most effective means of minimising the numbers of animals required for a programme of work is to apply tiered and hierarchical approaches to enable the early identification and discarding of models, materials and hypotheses not destined for further evaluation or development, thus avoiding the need for unnecessary animal studies. Using the assessment of the ocular safety of materials as an example (Gallegos Saliner & Worth 2007):

- consider a test material's structural and physicochemical properties;
- evaluate *in vitro* test results;
- conduct dermal safety tests; and
- identify strong skin irritants and corrosive materials.

These can combine to enable a reliable evaluation to be made of likely ocular safety without undertaking tests on live animals. When ocular safety tests on live animals are still required, testing first on a single animal can reduce the number of animals used, as materials giving strong positives in a single animal do not require confirmatory testing in more animals. This example also links to Refinement, by dispensing with the need to use animals to test the materials most likely to cause the greatest degrees of pain, suffering, distress and lasting harm.

Preliminary *in vitro* data can reduce the number of animals required for definitive studies. For example, cytotoxicity data is now used to reduce the number of animals used in acute toxicity studies by determining the appropriate doses of test materials to be used in the animal studies (ICCVAM 2001).

Small proof-of-concept studies, if they fail to demonstrate the expected outcomes, obviate the need for failed large-scale definitive studies.

Definitive studies can often only be planned in detail once preliminary animal data are available. Pilot experiments are useful: these are small-scale preliminary studies to examine and fully develop the working hypotheses and logistics of proposed definitive studies[9]. Even though in many cases the results will not be published, they will be used to design improved definitive studies by providing insights into:

- likely inter-individual variation and the number of animals required to obtain robust scientific results;
- the most appropriate dosing, and sampling routines;
- the nature, incidence, severity and timing of possible physiological, behavioural changes and adverse effects, and the required observation schedules;

[8]https://norecopa.no/prepare/4-experimental-design-and-statistical-analysis (accessed 10 Jan 2022)

[9]https://nc3rs.org.uk/conducting-pilot-study (accessed 10 Jan 2022)

- how adverse effects can best be avoided, elucidated or managed;
- importantly, they may identify and provide an opportunity to tackle unexpected, technical problems and extraneous experimental variables before larger-scale studies are undertaken.

The number of animals required to meet the scientific objectives reflects the required degree of precision and certainty. The number of animals required should be no more than is necessary to meet the scientific objective.

There may be opportunities for reducing the number of animals required by taking account of the prevalence of the outcome of interest (Hoffmann & Hartung 2006). It may require less data to identify candidate test materials with a common property, than with an uncommon property.

Where test materials are only to be assigned to general categories, requiring only an estimate of their biological properties, smaller numbers of animals may be sufficient, rather than the larger numbers required to calculate more precise or absolute values.

Control groups are in all other respects exposed to identical conditions, observations and investigations. They are used as standards for comparison, making conclusions about the relevance and significance of the results more robust by demonstrating that the test system is appropriately responsive and capable of correctly identifying biologically active and inactive test materials. They assist also in eliminating alternative explanations of experimental results: the possibility that the experimental subjects were prone to, or incapable of, giving appropriate positive or negative results. They may also be valuable in demonstrating other potential confounding variables within the test system, for example, the chance that some unrecognised, intercurrent problem influenced the responses observed.

When there is a need for control data, the number of animals can, in some circumstances, be minimised by the use of a single concurrent control to evaluate simultaneously a range of test materials for the same biological property, or by the use of historical controls, or when a number of test materials are tested in the same laboratory on the same day. The routine use of concurrent positive controls, to demonstrate that the test method as applied in a laboratory can produce an appropriate positive response, is generally unnecessary if the routine testing programme itself regularly produces both valid positive and negative results.

In some circumstances, a relatively large amount of extra information can be gained from the use of small additional satellite groups to pursue more than one scientific objective within a single experiment. For example, toxicokinetic data can be gathered in the course of single-dose toxicity studies (EMEA 1995).

The degree of uniformity (lack of variability) within and between experimental subjects is an important determinant of the number of experimental subjects required, and all reasonable efforts should be made to control relevant genetic and epigenetic factors. The use of purpose-bred animals permits varying degrees of control of genetic variability and microbiological status, and for many of the commonly used species the availability of inbred and isogenic strains allows the use of smaller group sizes than is possible with outbred or random-bred animals (see Chapter 4: An introduction to laboratory animal genetics). In some instances, it has been argued that the use of genetically identical animals allows scientific progress to be made that would otherwise be impossible (Festing & Fisher 2000). However, the relative merits of using inbred and outbred animals are still being debated (Tuttle *et al*. 2018).

Variability may be further reduced by providing a controlled and standardised environment, with the most uniform populations and results being produced when the environment is optimal for the animals' well-being (Chance 1957; Chance & Russell 1997). Whether or not research animals should be kept under controlled conditions is, however, currently the subject of debate (Würbel & Garner 2007; Karp 2018).

Stressed animals will inevitably have different baseline behaviours, physiological findings and range of responses to experimental interventions, from unstressed animals. Therefore, all reasonable efforts should be made to identify and remove or minimise unnecessary stressors (Poole 1997; Garner 2005).

Retrospective analyses of results may show that the number of animals needed could in future be reduced without loss of precision. This has been found to be the case with some vaccine potency assays (Hendriksen & Steen 2000).

Re-use of animals

Re-use may be defined as the second or subsequent scientific use of an animal that has already completed a series of procedures for a defined scientific purpose when the use of a naïve, unused animal would have also been scientifically satisfactory. While re-use may reduce the total of number of animals required for programmes of work, it has to be balanced against the increased, cumulative suffering experienced by the individual animal. Common examples include the re-use of animals as blood donors; and, subject to suitable recovery periods, the re-use of dogs or non-human primates in pharmacokinetic studies.

Re-use should only be considered when the following conditions are met:

- The first use has not compromised the suitability of the animal for the second or subsequent use (for example, animals which have been exposed to a pathogen or immunogen will not give a naïve response if subsequently re-exposed);
- Animals experienced only minimal pain, suffering and distress, and no lasting harm, from their earlier use;
- The animals have been shown on a case-by-case basis by a competent person, after completion of the first use, to have been restored to a normal state of well-being.

The re-use of animals is frequently regulated by legislation.

Optimising animal production

Matching the production of animals and the availability of animal tissues to known or likely demand avoids waste. Common examples include cryo preservation of genetically

altered animal lines (Glenister & Rall 2000) rather than maintaining 'tick-over' colonies, preservation and archiving and sharing of other tissues and samples, and through tissue-sharing schemes such as AniMatch[10].

Refinement

Refinement improves the quality of life of every animal bred, kept and used for experimental and other scientific purposes, and potentially benefits every programme of work using live animals.

Animal welfare is a complex issue: it comprises not only the health of an animal, but also its state of well-being. It has both physical and psychological dimensions which can be compromised not only by unpleasant stimuli, but also by the denial of that which is pleasurable. It is important to be aware that there are many causes of suffering and distress other than pain. Refinement is not just a matter of minimising the incidence of adverse effects, or the number of animals used; it is about minimising the total, cumulative pain, suffering, distress and lasting harm that may be caused to animals bred, kept and used for scientific purposes. Thus, a higher incidence of findings not indicative of a high welfare cost, such as reduced weight gain, may be preferable in welfare terms to a lower incidence of endpoints clearly indicative of higher levels of suffering.

Consideration of Refinement starts the moment there is an intention to breed or keep an animal for experimental or other scientific purposes. It continues throughout the production and scientific use of the animal until it is humanely killed or otherwise disposed of; and, as in the case of Reduction, it does not end until the lessons learned are incorporated into future practice.

All reasonable efforts should be made to ensure that animals used for biomedical research and testing have normal baseline physiological parameters and behaviours, by refining systems for their care and use (Poole 1997; Bayne 2005).

Assessing animal well-being

To make proper provision for animal welfare it is essential to understand and recognise what is '*meaningful to the animal*' and to do '*what is right for the animals*' (Russell & Burch 1959). Recognition of an abnormal state depends on an awareness of, and familiarity with, normality in the species, strain and individual under observation.

The behavioural and physiological responses of animals to adverse effects are not uniform between species, strains, individuals of the same species and strain, or even in the same individual at different times (Scharmann 1999). Assessment of welfare must therefore take place at the level of the individual animal.

Welfare is assessed by taking into account behavioural, physiological, clinical and laboratory findings (see Chapter 6: Brief introduction to welfare assessment: A Toolbox of techniques). Of these, behavioural findings and changes are often the earliest, most sensitive and most meaningful indicators.

In the absence of evidence to the contrary, it should be assumed that any stimulus, experience or pathology that produces pain and discomfort in man, also does so in sentient animals (Home Office 1965; Smith & Hawkins 2016). Confidence in indices of welfare is best placed in findings which:

- occur in an appropriate context;
- progress with the nature and severity of the insult or pathology;
- are predictive of the ultimate welfare, clinical or pathological outcomes;
- can be controlled with appropriate specific, supportive or symptomatic treatment.

For example, signs considered to be indicative of pain should occur in contexts where there is reason to suspect or believe pain may be present, and should decline with prompt, effective analgesic administration.

However, it is important to recognise that:

- animals may be distressed, though not in pain, and therefore display signs which analgesics will not alleviate – this may be seen for example in animals with locomotor impairments due to neurological damage;
- analgesics can have direct pharmacological effects unrelated to pain relief producing behavioural and physiological changes, and altering clinical findings (Roughan & Flecknell 2000);
- identifying and managing chronic pain and distress, where the signs can be harder to detect, poses particular difficulties (Flecknell & Roughan 2004).

As the judgement of animal well-being ultimately rests with humans, a degree of critical anthropomorphism is perhaps inevitable. 'Critical' in this context implies empathy tempered with objective knowledge of the animal, its needs and normal behaviours, preceding events and the significance of any signs which may be seen.

Expert judgement can be required to understand the scope, limitations, possible interpretations and significance of even seemingly objective findings. Pitfalls to be borne in mind include the following:

- demonstrating behavioural or physiological differences may be contingent upon the animal's environment;
- preference testing (Kirkden & Pajor 2006) may only identify the least objectionable rather than the best option, and short-term preferences may not be indicative of long-term preferences, needs and benefits;
- although technology is improving, measuring even basic physiological phenomena and behaviours sometimes requires additional interventions that add welfare costs or influence the parameters being measured.

Severity scoring systems

A number of disturbance indices, pain, and severity scoring systems have been developed to assist with the assessment of the welfare of animals used for experimental purposes (for

[10]https://www.animatch.eu (accessed 10 Jan 2022)

example, Hendriksen & Morton 1999; Hawkins *et al*. 2011; Smith *et al*. 2018b; Zintzsch *et al*. 2017 and the Norecopa web site[11]). These can be used to identify protocols with high welfare costs where work on Replacement or Refinement might most usefully be commissioned, and to evaluate the impact of treatments and potential Refinement measures (see also Chapter 6: Brief introduction to welfare assessment: A 'toolbox' of techniques). They encourage the use of appropriate observation schedules, standard documentation and plain non-technical language with a limited range of keywords to identify, describe and record findings. These simplify staff training, provide a systematic approach to evaluating and documenting welfare and clinical findings, and facilitate communication within and between research groups.

Such systems are based upon indices of welfare, often with continuous variables categorised to reflect what we believe to be meaningful differences in levels of significance and suffering. Combinations of signs tend to be more significant than the occurrence of any sign in isolation. Although they must be adapted to reflect the research objectives, models and protocols, they should be valid whether impaired welfare is due to the immediate or delayed, local or systemic, or primary, secondary or tertiary effects of the procedures.

Observation schedules

Arrangements must be made to check animals under study at appropriate times to gather data and safeguard their welfare. All animals should be checked at least once a day by a competent person capable of recognising and arranging for welfare problems to be promptly remedied. The frequency of checks should be increased when problems are likely, or have already occurred. A policy for the availability of competent personnel seven days a week must be in place before the start of the experiment.

Findings of relevance to the animals' welfare, including normal findings, must be recorded, along with the action taken and the animal's response.

Contingent and direct harms

The welfare costs to animals bred, kept and used for experimental and other scientific purposes have two distinct components (Russell & Burch 1959):

- 'contingent' welfare costs (harms), comprising the welfare-negative aspects of animal production and care, whether caused deliberately or by omission;
- 'direct' costs (harms) resulting from the experimental procedures.

Animal facilities and care practices must facilitate high standards of animal welfare and high-quality research by eliminating, or identifying, controlling and minimising, unwanted variables. At the same time, the best possible, appropriate provision should be made for the physiological, social and behavioural needs of the animals (see later chapters such as Chapter 9: Planning, design and construction of efficient animal facilities and Chapter 10: Enrichment: animal welfare and scientific validity).

Contingent harms

Many elements of animal accommodation and care affect the welfare of animals and their response to experimental interventions (Poole 1997; Bayne 2005). These can affect the validity and reproducibility of findings to the extent that experimental results may only be valid for, and reproducible within, the specific conditions under which they were obtained. Key considerations are the animals' physical and behavioural needs, and how provision for these can best be made within the context of their production, care and use.

Ideally, the standards of animal care and accommodation provided would be based on objective evidence of what is required to make best provision for animal welfare. At present, much of the evidence required to derive and support such standards does not exist, with guidelines and regulations being based on a combination of empirical findings and what is believed to be existing good practice. These set only the minimum expected or acceptable standards of care and accommodation (see, for example, the EU legislation web site[12]).

Whenever possible, in order not to delay innovation and the introduction of better evidence-based care systems, standards should be written as performance standards (what outcomes they are intended to achieve) rather than as engineering standards (prescribing only the required inputs).

Pair- and group-housing

Animals, other than those that are naturally solitary, should be socially housed in stable groups of compatible individuals. It has been shown in many species that housing with one or more socially compatible conspecific significantly reduces stress, and that being kept singly in isolation compromises both an animal's welfare and its suitability as an experimental subject (Kappel *et al*. 2017). Care is required to ensure that pair- and group-housed animals are socially compatible, mindful that population density and group size influence the physiological and psychological state of the animal and affect experimental responses.

There will be some circumstances, for example the use of a single instrumented animal, when the companion animals will not be experimental subjects, yet will be exposed to any contingent harms.

Animals should only be singly housed on veterinary or other welfare grounds, or justified scientific need; in which case animal care and veterinary staff should be involved in the decision making, and additional measures taken to optimise animal welfare.

Space requirements and structure

Animals should be provided with a sufficiently spacious and complex environment to facilitate a wide range of normal activities and behaviours, taking account of their physiological and ethological needs. The preferred systems will vary

[11]https://norecopa.no/more-resources/severity-classification (accessed 10 Jan 2022)

[12]https://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm (accessed 10 Jan 2022).

according to species, strain, age, physiological condition, stocking density and group size, and whether animals are kept as stock, for breeding or for experiments.

Basic physiological and ethological needs (such as freedom of movement, appropriate social contact and the ability to withdraw from social conflict; the performance of meaningful activities and access to food and water) should never be restricted without good cause, and then only to the justifiable minima.

Environmental enrichment

The laboratory environment can never reproduce the complexity of an animal's natural environment, nor adequately model human societal interactions. The intention is generally to mimic critical natural environmental factors so that normal, strongly motivated behaviours can be expressed, reinforced and maintained (Blanchard & Blanchard 2003). Not all natural behaviours are appropriate in the laboratory setting (Fraser 1993). They may represent what the animal needs or wants to, would not normally choose to do (for example, in response to environmental stressors), or will only choose to do when the need arises.

Environmental enrichment options can be categorised as:

- Social enrichment, generally characterised by housing with compatible conspecifics complemented by space of sufficient volume and complexity to permit an appropriate range of species-specific interactions and interaction with man. In many circumstances, social enrichment is both more effective than inanimate physical enrichment, and a prerequisite for the effectiveness of physical enrichment. Appropriate early social experience can be essential for the development of a normal behavioural repertoire. Conditions at breeding and rearing facilities play therefore a large part in determining the subsequent suitability of animals as experimental subjects or future breeding stock.
- Physical enrichment, including the provision of an adequate amount of suitably structured space, materials to manipulate, sensory stimuli and a varied diet. To prevent or reduce stress-induced behaviours, animals should be given a degree of control over their environment by encouraging species-appropriate physical exercise, foraging, manipulative and cognitive activities.

A creative and critical approach is required. Not all potential changes are beneficial; and if one form of enrichment is chosen others, which may be more effective, have to be rejected.

It is important that appropriate options are identified and critically evaluated in terms of their immediate and long-term impact on the animals' well-being and the research objectives (Bayne 2005; Benefiel *et al*. 2005). Assessing the impact of potential environmental enrichments depends on the ability of staff to evaluate the animal's state of mind and welfare state. It cannot be overemphasised that the most important resources required to devise and evaluate environmental enrichment opportunities are competent and caring staff.

Environmental enrichment is discussed in more detail in Chapter 10: Enrichment: animal welfare and scientific validity.

Restraint

During many husbandry and scientific procedures, animals may be restrained to minimise the risk of injury to the subject and handler, and facilitate the performance of the procedures. Restraint can be stressful, producing changes in physiological parameters and behaviours depending on the nature, duration and degree of restraint, particularly when the restrained animal is also removed from its enclosure or social group. Appropriate restraint will depend on the species and the nature and duration of the procedure for which the animal is being restrained, with the most refined method of restraint being that which causes the least stress to the animal and its social group.

Training of animals to accept reasonable restraint procedures is possible in a range of species, and has been shown to reduce the resulting physiological and behavioural changes (Wolfensohn & Honess 2005). Procedural training can in some circumstances encourage animals to allow the safe performance of routine procedures without the need for restraint (see Chapter 15: The use of positive reinforcement training techniques to enhance the care and welfare of laboratory and research animals).

Marking and identification of animals

Individual animals bred, kept and used for experimental and other scientific purposes need to be identified. This is generally achieved by marking individual animals, although biometric methods (identification based on an individual animal's natural physical characteristics) would be preferable. Faced with a choice of effective identification and marking methods, the preferred means is that which causes the least pain, suffering or distress to the animal. Many guidelines are available for marking[13] and identification[14] of research animals.

Transport of animals

The transport of animals, between or within establishments, can be stressful. All reasonable efforts must be made to avoid or minimise any stress that may be caused, and to ensure that animals are acclimatised to a new environment before being used for scientific purposes (see Chapter 12: Transportation of laboratory animals). Journey times should be minimised, the least stressful modes of transport used, and appropriate contingency plans should be in place.

The acclimatisation period will vary with the stresses imposed by transportation; the differences in the housing and care systems; and the species, strain and the condition of individual animals. It may be necessary to take expert advice to determine the appropriate minimum period for acclimatisation, and to confirm that animals have recovered before being used for scientific purposes.

In some cases, welfare costs can be minimised by transporting ova or embryos rather than live animals. This, and the resulting rederivation, is also a means of disease control when acquiring animals from facilities with different or unknown microbiological status.

[13]https://norecopa.no/search?q=guidelines%20marking&fq=db:%223r%22 (accessed 10 Jan 2022)

[14]https://norecopa.no/search?q=guidelines%20identification&fq=db:%223r%22 (accessed 10 Jan 2022)

Humane killing

The majority of animals produced and used for scientific purpose are humanely killed as part of, or at the end of, their scientific use; as are surplus stock animals.

Humane methods of killing, when properly applied, ensure rapid loss of consciousness without producing signs of pain or distress, result in the death of an animal with a minimum of physical and mental suffering, and should not interfere with any scientific data which is to be collected postmortem. They should also be aesthetically acceptable, and must incorporate careful and compassionate animal handling routines that avoid or minimise the stress due to any necessary restraint or the need to remove the animal from its enclosure or social group (see Chapter 17: Euthanasia and other fates for laboratory animals, and the Norecopa web site[15]). All require expertise which can only be developed by appropriate staff training, and the provision and maintenance of appropriate facilities and equipment.

After a humane killing method is applied, death must be confirmed in all cases before removing tissues or storing or disposing of cadavers.

Direct harms

A number of procedures applied to animals for experimental purposes impose welfare costs. The welfare costs tend to vary in proportion to:

- the degree of sentience and needs of the individual experimental subject;
- the nature, duration, intensity and frequency of the challenge;
- the biological systems and mechanisms involved;
- other factors which aggravate or ameliorate the suffering experienced by an individual experimental subject.

Choice of experimental subjects

Selection of appropriate experimental subjects requires understanding and control of factors including the animal's genotype; environmental conditions; other elements of animal husbandry, accommodation and care; and microbiological status.

The choice of species is relevant to refinement. Some species:

- are afforded specific legal protection;
- are believed to have a greater capacity to experience pain and distress (sometimes referred to as 'neurophysiological sensitivity'). Where there is flexibility in the interpretation and implementation of regulatory testing requirements, selection of the 'lowest' appropriate species should be on scientific considerations, not custom and practice or availability;
- have specific, complex husbandry requirements difficult to provide in the research context. Choosing the species whose needs can best be catered for in the laboratory setting may constitute Refinement.

Animal models of disease, animals expressing harmful natural genetic mutations and some lines of genetically altered animals (Wells *et al*. 2006) have specific problems and needs in addition to, or different from, those of normal animals of the same species. These special needs must be considered, identified and met when such animals are bred, kept or used for scientific purposes.

Wild-caught animals

The environmental, ethical, welfare and scientific benefits of using purpose-bred animals are so great that the use of non-purpose-bred, and in particular wild-caught, animals requires special justification. Where it can be justified, capture should be performed by competent persons using humane methods, minimising the impact of capture both on the captured animals and the remaining wildlife and habitat (see Chapter 7: Welfare and 'best practice' in field studies of wildlife). Arrangements should be in place for animals in poor health to be examined promptly by a competent person, and appropriate action taken.

Proper provision must be made for the transportation, acclimatisation, quarantine, housing, husbandry and care of wild-caught animals, mindful that their health status, behaviours and needs are likely to be different to those of animals bred in captivity. The eventual fate of wild-caught animals should be given due consideration before work begins.

Dosing

Research protocols commonly require that animals are dosed with test materials, and detailed advice on limit volumes and practical issues is available elsewhere (see Diehl *et al*. 2001 and the recommendations in the species-specific chapters in Part 2 of this book). In many cases, the most refined options to meet the scientific objective can only be determined by pilot studies. If the intention is to mimic natural exposure, to maintain a particular level at a target site, or to produce a specific effect (and not produce unwanted effects) pilot studies may be required to identify the appropriate dose or exposure.

Refinement is relevant to consideration of:

- The route of administration.
  - With oral administration, admixing the test material with food or water (providing stability and palatability are not problems) or administration in liquid, tablet or capsule form, may be more refined than gavage-dosing. The timing of the doses, and volumes administered, must neither compromise the animals' normal food and fluid intake, nor cause discomfort or other volume-related effects.
  - Test materials may be administered parenterally by injection or cannula. Other than administration directly into the circulation, this can lead to varying rates of uptake depending primarily on the injection site, the general condition of the animal and the volume and formulation used.
    - Administration by intraperitoneal injection is a special case: it results in the test material being partitioned and taken up simultaneously into the systemic circulation and hepatic portal circulation (where it may be metabolised by the liver before it enters the systemic circulation). How test materials partition between the portal and systemic circulations depends on the

[15]https://norecopa.no/more-resources/humane-killing (accessed 10 Jan 2022)

nature and volume of the test material, varies from subject to subject, and in the same subject from day to day.
    - Topical application of test materials to skin or mucous membranes may require some form of restraint, or other measures, to ensure the test material remains in place and is not ingested by the animal or its cage mates.
- The frequency and duration of dosing.
    - These are generally determined by the properties of the test material (for example, its bioavailability and biological effects), its interaction with the experimental subject (for example, its half-life, how it is metabolised, where it accumulates and how it is excreted) and the study objective.
- The equipment used.
    - For injection procedures, the smallest bore needle capable of delivering the volume required in an acceptable time should be used.
    - The need for multiple injections, and the associated restraint procedures, may be dispensed with by the placement and use of cannulae to permit repeated (or continuous) administration. To constitute Refinement, their use must be balanced against the welfare costs of the procedures to insert the cannulae, the restraint and other cannula-care procedures that may be required, and the possible cannula-related problems.
- The volumes to be administered.
    - For intravascular administration, the volume and the time over which materials are administered should avoid unwanted volume-related effects, and should not produce any biological changes due to the nature and volume of the vehicle used.
    - For injection into closed spaces (for example, intramuscular or intradermal injection), the volumes and rates of administration should avoid adverse effects due to pressure effects or over-stretching of tissues.
- The formulations to be administered.
    - The formulation and volume of test materials used are generally determined by the frequency of administration, the required accuracy of dosing, the nature and solubility of the test material, the required dose and preferred concentration.
    - In general, for parenteral administration the closer the osmolarity, pH, buffer capacity, viscosity and temperature of the test material are to normal body fluids, the greater the biocompatibility and the less discomfort and stress will be caused.

Non-invasive sampling

A range of biochemical parameters can be estimated or measured without the need to obtain blood samples. A number of hormones and metabolites can be measured in urine and faeces, allowing estimates to be made of recent circulating levels in unrestrained animals, mindful that there is a time lag between their production, release and excretion. In some cases, animals can be trained to deposit excreta in suitable receptacles without being restrained or removed from their social groups.

Although physiological responses to instantaneous stressors cannot be measured in urine or faeces, determination of salivary levels can provide a minimally invasive means for measurement of short-term responses for some materials, and for detecting and quantifying other metabolites and biomarkers (Chiappin *et al*. 2007).

Blood sampling

Blood sampling is one of the most common procedures used in animal research, and advice on volume limits (which should always be considered the justifiable maxima rather than the norm) and other practical issues is available elsewhere (e.g. Diehl *et al*. 2001 and the NC3Rs web site[16]), and in the species-specific chapters in Part 2 of this book.

Refinement is relevant to:

- The nature of the sample.
    - In many species, venous blood can be obtained from superficial veins by venepuncture or venesection.
    - Arterial blood is generally obtained by direct arterial puncture or closed cardiac puncture (the insertion of a needle directly through the chest wall into the left ventricle of the heart under general anaesthesia). Cardiac puncture is only appropriate for sampling under general anaesthesia from which the animals are not allowed to recover.
    - Blood obtained by retro-orbital puncture is not a physiological fluid: such samples comprise admixed capillary and venous blood, contaminated with other tissue fluids, in which a variety of clotting factors have been activated. Its haematological and biochemical parameters are neither physiological nor representative of blood anywhere in the systemic circulation. The technique is also likely to cause tissue damage and discomfort.
- The frequency of sampling and the volumes required.
    - The volumes, rates of withdrawal and frequency of sampling must be designed to prevent hypovolaemia and anaemia. Average blood volumes and limit sampling volumes are generally calculated on the basis of body weight (Joint Working Group on Refinement (JWGR) 1993; Wolfensohn & Lloyd 2003), but must be interpreted in the knowledge that the safe sampling limits are typically lower in animals whose welfare is already challenged. Microsampling is being used increasingly, particularly in the pharmaceutical and chemical industries[17].
    - If frequent samples are required, cannulation should be considered as a means of minimising the stress of sampling.

Reward or punishment?

Behavioural testing often requires that experimental subjects remain interested in performing prescribed tasks, and various means have been devised to motivate experimental subjects to undertake such tasks on demand or for longer periods.

[16]https://www.nc3rs.org.uk/3rs-resources/blood-sampling (accessed 10 Jan 2022)

[17]https://www.nc3rs.org.uk/microsampling (accessed 10 Jan 2022)

Methods of motivating test subjects may be based upon rewards/positive reinforcement (for example, access to a preferred food or drink as a reward for displaying the desired behaviours) or punishment/negative reinforcement (for example, exposure to an air-puff or mild electric shock to discourage other behaviours). In some cases, the reward may be made more desirable by a period of food or water deprivation (e.g. Prescott *et al*. 2010).

The most refined and ethically justifiable paradigms are those that rely solely on reward/positive reinforcement systems without prior deprivations. There are considerable opportunities to refine food deprivation in rodents[18]. Punishment/negative reinforcement regimens require specific justification.

Anaesthesia and analgesia

The informed and responsible use of anaesthetics and analgesics to prevent and manage pain is an essential component of contemporary animal research. A detailed review of current best practice in the use of anaesthetics and analgesics is beyond the scope of this chapter, and authoritative information can be found elsewhere (see, for example, Flecknell 2015, the Research Animal Training (RAT) web site[19], and the species-specific chapters in Part 2), but there are general principles particularly relevant to Refinement.

General anaesthetic agents affect many physiological mechanisms and parameters, and care must be taken to ensure this does not compromise experimental data or animal welfare. Appropriate steps should be taken to monitor and maintain the circulation, respiratory function, fluid balance, and the body temperature of the anaesthetised subject within normal physiological limits throughout surgery and until the effects of anaesthesia have worn off.

Recovery from general anaesthesia can be hazardous, and animals should not be left unattended until the effects have worn off, any necessary specific, symptomatic or supportive treatments have been given and their effectiveness determined. Consideration should be given to administering the first dose of analgesia, sometimes referred to as pre-emptive analgesia, before recovery from anaesthesia, since total post-operative analgesic requirements are reduced when the initial dose of analgesic precedes the animal's ability to feel pain.

Post-surgical analgesia must be the norm, and it should be administered as required to control pain and speed the restoration of normal behaviours, such as food and water intake, thus shortening the post-surgery catabolic phase and improving animal welfare. This requires appropriate observation schedules, with treatments based on the findings in, and needs of, individual animals.

Surgery

Surgical procedures must only be carried out by competent persons; using the best available surgical and animal care techniques; and the anaesthetic and analgesic regimens best suited to the species, the nature and duration of the procedure and the scientific objective. Surgery should be performed using aseptic technique in areas designed for, and dedicated to, this purpose.

The availability of trained, competent staff to take responsibility for the care of animals during the post-operative period must be confirmed before surgery is scheduled. To make best provision for post-operative care, it is recommended that complex surgical procedures are carried out as early in the working week, and working day, as possible.

Humane endpoints

Humane endpoints, minimising the direct welfare costs of justifiable animal-based research, are essential components of humane experimental technique, and a cornerstone of refinement (Richmond 1999)[20]. Humane endpoints incorporate all reasonable and practical steps that can be taken to minimise justifiable suffering by avoiding, or promptly recognising and remedying, unnecessary adverse effects arising during scientific procedures. Humane endpoints must be described in meaningful terms and be promptly recognised and acted on by those entrusted with the welfare of the animals.

To some, the term 'humane endpoint' mistakenly represents '*the earliest indicator in an animal experiment of severe pain, severe distress, suffering, or impending death*' (OECD 2000). That is a dangerous misconception.

Contrary to the narrow OECD definition, humane endpoints are often particularly appropriate when levels of pain and distress being experienced are not high and death is not imminent.

Humane endpoints in practice

Humane endpoints must be objective and evidence-based in order to:

- avoid the needless culling of animals whose welfare is less compromised than believed, or before the scientific objective has been achieved;
- prevent evidence indicative of significant suffering being missed;
- inform judgements about the severity of different procedures and models;
- evaluate potential refinements.

Although they must be designed within the context of the project, experiment and experimental group, they are best thought of as being applied to the individual animal: with early indicators often being the most meaningful, both with respect to welfare problems and to prevent scientific outcomes from being compromised by later undesirable changes, due to unnecessary and unwanted secondary or tertiary effects (Hendriksen *et al*. 2011).

Humane endpoints take account of legal, ethical, welfare and scientific considerations, and must cater for a number of eventualities, including the following:

- having achieved the experimental objective (or when it is recognised it cannot be achieved), even if there is no

[18]https://norecopa.no/3r-guide/fasting-in-rodents (accessed 10 Jan 2022)

[19]https://researchanimaltraining.com/elearning (accessed 10 Jan 2022)

[20]https://www.humane-endpoints.info/en (accessed 10 Jan 2022)