THE DESIGN AND STATISTICAL ANALYSIS OF ANIMAL EXPERIMENTS

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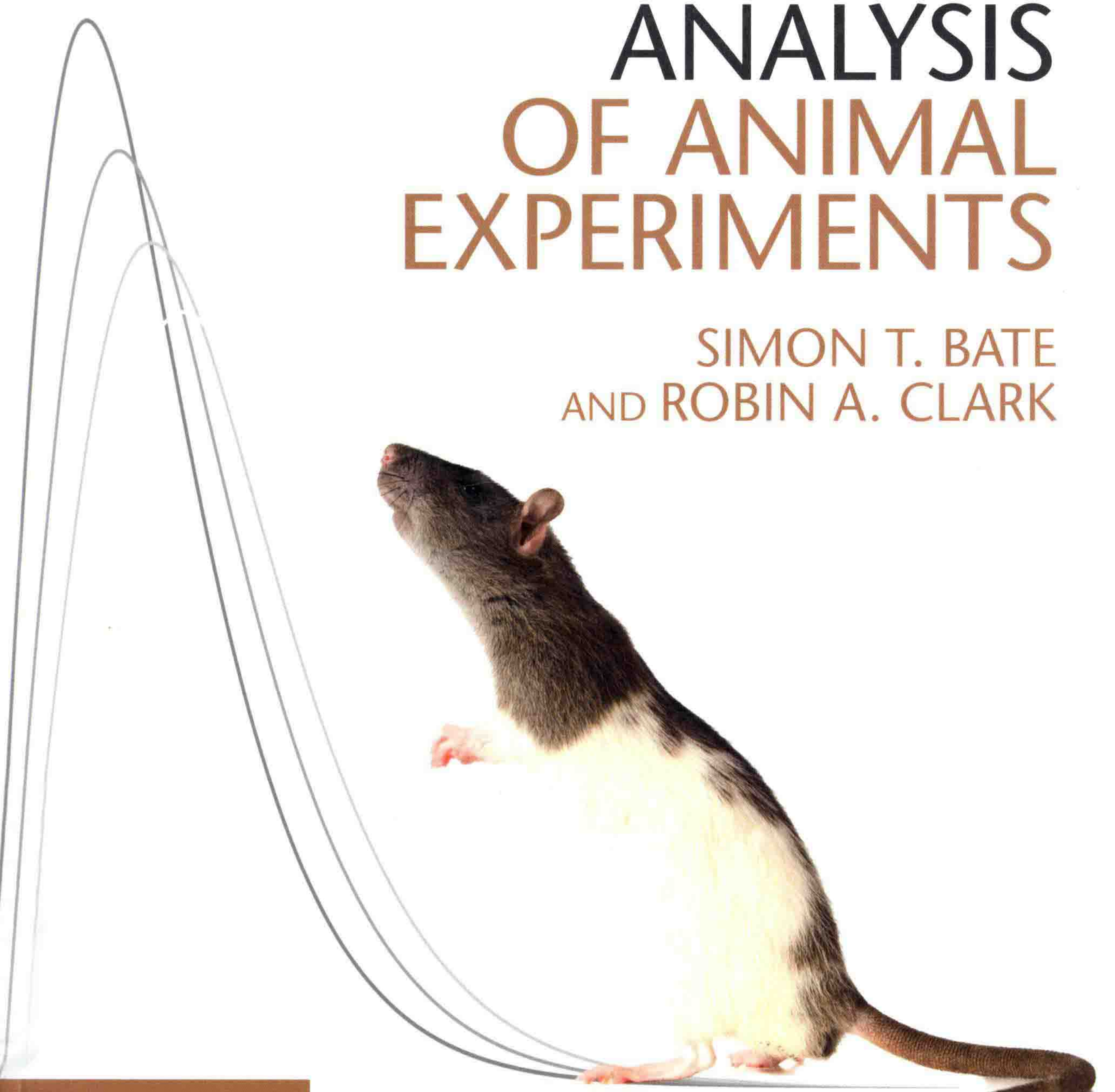
AND ROBIN A. CLARK



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The Design and Statistical Analysis of Animal Experiments

Written specifically for animal researchers, this is the first book to provide a comprehensive guide to the design and statistical analysis of animal experiments. It has long been recognised that the proper implementation of these techniques can help to optimise the number of animals used in an experiment. By using real-life examples to make them more accessible, this book explains the statistical tools that are routinely employed by practitioners.

A wide range of design types are considered in detail, including block, factorial, nested, crossover, dose-escalation and repeated measures. Alongside each design, techniques are introduced to analyse the experimental data generated. Each analysis approach is described in non-mathematical terms, helping readers without a statistical background to understand key techniques such as: *t*-tests, ANOVA, repeated measures, analysis of covariance, multiple comparison tests, non-parametric methods and survival analysis.

This is also the first text to describe technical aspects of InVivoStat, a powerful open-source software package developed by the authors to enable animal researchers to analyse their data and obtain informative results. InVivoStat can be downloaded at www.invivostat.co.uk.

Simon T. Bate is a Principal Statistician at GlaxoSmithKline, supporting pre-clinical research. He has spent over 12 years supporting the design and statistical analysis of animal experiments, including drug discovery research, toxicology studies and safety assessment. He presents many statistics courses around Europe, including the statistics module of the British Association for Psychopharmacology's Pre-Clinical Certificate.

Robin A. Clark is a Senior Analyst Programmer at Huntingdon Life Sciences, Alconbury. Originally qualified as a marine ecologist, he is now a software architect producing data collection and statistical applications for the pharmaceutical industry. Robin produced the user interface and designed the system architecture for InVivoStat.

To

RB, NRB, MTB, EC, EJC, ZMC

Preface

This book is aimed at practitioners who do not have a statistics degree and yet wish to apply statistics to help them arrive at valid and reliable conclusions while minimising the animal numbers required. Descriptions of the mathematical methods underpinning the topics covered in the book are purposefully kept to a minimum. If readers wish to gain a better understanding of the mathematics behind experimental design and statistical analysis then reading a more advanced textbook would help further their understanding.

The solutions to practical problems encountered when conducting animal experiments are explained using non-technical approaches. We believe that in many situations advanced statistical ideas can be employed successfully by researchers with no statistical qualification, using a combination of common sense and modern statistical analysis software packages. In our experience statistical ideas are often introduced to scientists using mathematical terminology. This can be off-putting to non-mathematicians and can leave researchers with, at best, only rudimentary statistical tools and at worst a fear of statistics.

To keep the descriptions of the statistical tools covered in this book as simple as possible, we shall occasionally give pragmatic explanations. While such explanations may not apply in all cases and in all scientific disciplines, this approach does allow us to introduce methods in a clear and concise way. By allowing ourselves the freedom to simplify the problems pragmatically, we aim to make statistical tools more accessible. The reader is invited, once they have familiarised themselves with (and hopefully found the benefit of using) the tools described in this book, to read more advanced texts on the subject.

This book is divided into seven chapters which loosely correspond to the procedure a researcher should take when planning the experimental design, running the experiment and evaluating the data generated. Following an introductory chapter and a second describing certain statistical concepts, the third chapter covers different types of designs. Designs are outlined, where possible, in simple non-technical language. This is followed by a chapter describing the randomisation of the experimental material. The fifth chapter discusses the statistical analysis of animal experiments and this is followed by a chapter describing how these methods can be applied within the statistical software package InVivoStat. The final chapter draws some conclusions about the ideas contained within the text.

A scientist can apply all of the methodology described in this book. Certain topics covered are more advanced than others and while we aim to make all subjects accessible, the reader should be aware that the help of a professional statistician may be advisable when first implementing some of the more advanced tools. However, once the readers have familiarised themselves with the ideas contained within this book, we hope they will have a fuller appreciation of the help statistics can offer to improve the conclusions that can be made when running animal experiments.

Acknowledgments

We would like to thank all our colleagues, both past and present, for providing so much inspiration for this book. We hope this text reflects the discussions, consultations and the range of examples that we have worked on together. There are simply too many scientists to mention, but you know who you are! We hope the wide variety of interesting practical problems we have faced together is reflected throughout this book.

Special thanks also to the statisticians who have advised us over the years and shaped our views on this subject matter. Simon would especially like to thank Philip Overend and Andrew Lloyd for their input and encouragement and Janet and Ed Godolphin for their help and support over the years.

We would like to give an extra special thanks to Gillian Amphlett, Gill Fleetwood, David Willé, Clare Stanford and Nathalie Percie du Sert for giving up their time to review sections of the book, and providing ideas, advice and feedback. Their input has been most helpful and improved the text considerably.

Finally we would like to thank our friends and family for putting up with the long unsociable hours we spent writing this book.

Contents

1

Introduction

Many researchers, either directly or indirectly, rely on statistical ideas when carrying out animal experiments. While some statistical tools are well known and are applied routinely, other tools are less well understood and so are less well used. The overall aim of this book is to discuss statistical methodologies that can be applied throughout the many stages of the experimental process. Researchers should be able to carry out most of the techniques described, although the advice of a professional statistician is advisable for some of the more advanced topics. Making use of these techniques will ensure that experiments are conducted in a logical and efficient way, which should result in reliable and reproducible decisions.

The particular types of study addressed in this book, as the title suggests, are studies involving animals. We attempt to cover all of the statistical tools that the animal researcher should use to run successful studies. Of course many of the problems faced by the animal researcher are common to other disciplines, and hence the ideas contained within this book can be applied to other areas. It should be noted that certain topics described in the text have been simplified to allow non-statisticians to apply the ideas without professional statistical support. Such pragmatic descriptions, while simplifying the technical details, are not universal and will not be applicable in all scientific disciplines.

There has been much interest in the use of statistics in animal research, in particular in the application of the 3Rs, replacement, reduction and refinement, as described by Russell and Burch (1959):

Every time any particle of statistical method is properly used, fewer animals are employed than would otherwise have been necessary.

Many authors have since highlighted how important the use of good experimental design is when conducting animal experiments; see Festing (1994, 2003a, 2003b) and the references contained within. Some of the more practical, as well as statistical, aspects of experimental design and statistics when applying the 3Rs are described in the book by Festing *et al.* (2002). There have also been surveys into the use of statistics in refereed journals; see McCance (1995) and more recently Kilkenny *et al.* (2009). The latter draws attention to some of the mistakes that can be made by researchers when designing and analysing animal experiments. The reliability of the reporting of animal experiments has been considered in, for example, Macleod *et al.* (2009) and Rooke *et al.* (2011). These articles highlight that papers describing experiments that do not employ suitable randomisation techniques and/or blinding may contain biased results.

The main goal of this text is to demonstrate how statistics can aid the reduction and refinement of animal studies. The efficient use of statistics, both in terms of complex experimental design and powerful statistical analysis, can reduce the number of animals required. Statistics can also help the researcher understand the processes that underpin the animal model and help identify factors that are influencing the experimental results. Such an understanding will inevitably lead to a refinement in the experimental process and a reduction in the total number of animals used.

Statistics, as a discipline, provides researchers with tools to help them arrive at valid conclusions. However, statistics, along with the application of some common sense, can also increase the understanding of the animal model through the application of graphical and mathematical techniques. For example, graphical tools play an important role in helping the researcher understand the effect of the features of the experimental design and also uncover overall patterns present in the data. The application of a formal statistical test, without first investigating the data graphically, can lead to the researcher drawing incorrect conclusions from the data. Consider the following real-life case study, which used graphical, as well as statistical, tools. If a conventional statistical analysis had been carried out, without first investigating all of the information gathered within the experiment, then the conclusions would have been misleading.

Example 1.1: Reducing blood cholesterol levels in mice

A scientist wanted to test the hypothesis that a novel compound had a beneficial effect on reducing high-density lipoprotein (HDL) cholesterol levels in a transgenic C57Bl/6J strain of mice. A blood sample was taken pre-treatment and the baseline cholesterol level for each animal measured. The mice were then randomised to either the drug treatment group or the control group and dosed with either the drug treatment or vehicle twice daily for two weeks. At the end of this period, a terminal blood sample was taken and the HDL cholesterol level measured.

As the scientist wanted to make use of the baseline information in the statistical analysis, it was decided that the percentage change from baseline would be a suitable response to investigate. This would, the scientist hoped, effectively remove the animal-to-animal differences by normalising to the baseline level. While there was evidence of a decrease in HDL cholesterol level in the group of animals administered the drug treatment (a 20% decrease from baseline in the drug treatment group compared to a 10% decrease in the control group) this was not deemed statistically significant using an unpaired *t*-test ($p = 0.191$). A means with standard errors of the mean (SEMs) plot of the data (see Section 5.3.5) is presented in Figure 1.1.

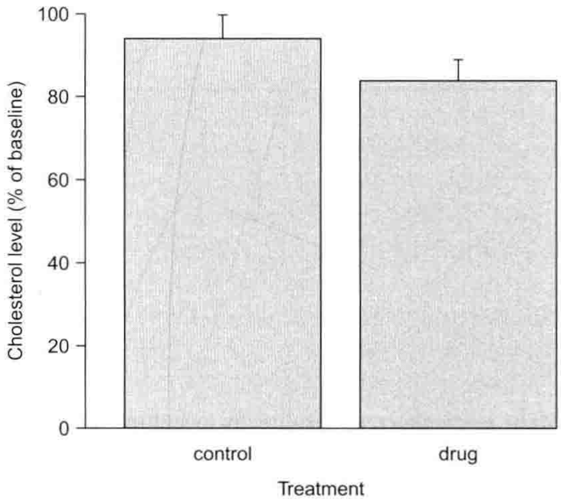

Figure 1.1. Plot of treatment means with standard errors for the percentage of baseline cholesterol response for Example 1.1.

As a follow-up the scientist also analysed the terminal HDL cholesterol level. From this analysis it appeared that there was a statistically significant increase in cholesterol level in the drug-treated group compared to the control. A plot of the means with SEMs of the terminal HDL cholesterol level is presented in Figure 1.2.

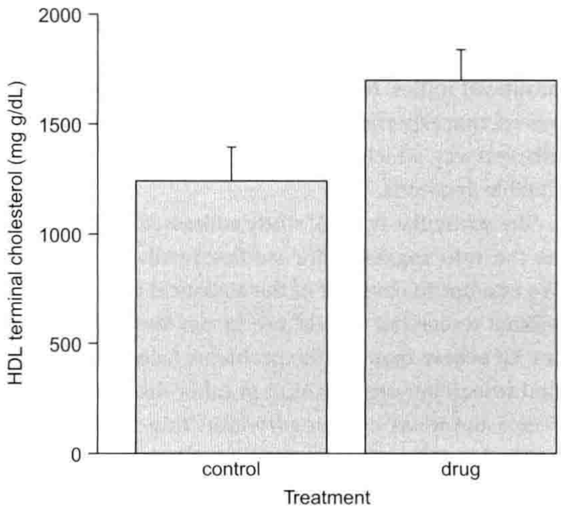

Figure 1.2. Plot of treatment means with standard errors for the terminal HDL cholesterol for Example 1.1.

Based on the results of this experiment, should we conclude the drug increases cholesterol levels? And why did the two analyses give such different conclusions? These questions can be answered by a simple scatterplot of the measured HDL cholesterol levels. If we plot terminal vs. baseline HDL cholesterol levels, an underlying problem with the experiment becomes clear. The scatterplot is presented in Figure 1.3.

From Figure 1.3 it can be seen that there are two distinct groupings along the *X*-axis. The plot reveals that, in terms of the HDL baseline cholesterol level, the animals belong to one of two