Nutrition Experiments in Pigs and Poultry

A Practical Guide

Edited by
Michael R. Bedford, Mingan Choct, and Helen Masey O'Neill

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Contributors

About the Editors

Mike Bedford completed his BSc in Animal Nutrition, Physiology and Production at the University of Nottingham in 1981. Subsequently he travelled to Canada to complete an MSc in 1984 with Dr J.D. Summers as supervisor at the University of Guelph on the topic of amino acid requirements of broilers and then went on to study for a PhD at the same university with Dr T.K. Smith as supervisor on the topic of polyamine metabolism in broilers. Two postdoctoral fellowships were then completed in Montreal (McGill University, 1988–1989) and then the University of Saskatchewan (1989–1991), the latter being on the topic of feed enzyme use in broiler diets. The second postdoc lead to his being employed by the project sponsor, Finnfeeds International Ltd, from 1991 to 2001, initially as a research manager and subsequently as research director. Mike then changed companies but maintained a similar job specification as the research director for Syngenta Animal Nutrition from 2001 to 2007, and then AB Vista from 2007 until the present day. In all cases the main subject area of his commercial placements related to the application of feed enzymes in monogastric nutrition, although other topics such as yeast and betaine have been part of his remit. Mike has published throughout his commercial life, producing numerous scientific articles and two editions of the book *Enzymes in Farm Animal Nutrition*, co-edited with Dr Gary Partridge.

Mingan Choct completed his Bachelor of Science at the Inner Mongolian Agricultural University in 1983 (China), Master of Science at the University of New South Wales in 1989 (Australia) and PhD at the University of Sydney in 1991 (Australia). He then worked at the Division of Human Nutrition of the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Adelaide from 1991 to 1995. In 1995, academia beckoned and Mingan

moved to the University of New England, Armidale, where he is currently a professor of animal nutrition. Mingan's academic interests include carbohydrate chemistry and nutrition, feed enzymes, energy evaluation and nutrition × disease interaction in poultry. He has supervised many postgraduate students and published numerous papers in journals and proceedings. He is an active member of the scientific community, serving as editor, assistant editor and on editorial boards of many journals. Mingan set up the Poultry Cooperative Research Centre (Poultry CRC) in 2003 with a total funding of AUS$174 million over 15 years to address scientific and educational challenges facing the Australian poultry industry. Mingan has been the CEO of the Poultry CRC since its inception. More details about Mingan's career and publications can be found at http://www.une.edu.au/staff-profiles/ers/mchoct.

Helen Masey O'Neill, known as Nell, completed her BSc in Nutritional Biochemistry at the University of Nottingham, which introduced her to the world of agricultural science. She then studied for a PhD with Professors Julian Wiseman and Sandra Hill, looking at the influence of storage and temperature treatment for the nutritional value of wheat for poultry. This led to an interest in feedstuff evaluation, especially in terms of energy. In 2010, after a period of levy board funded postdoctoral research and a teaching post, she joined the research team at AB Vista, working specifically on development and application of xylanases for use in pig and poultry diets. The vast amount of data generated within the team meant that it was prime for combined statistical analysis and the team developed 'Holo-analysis' techniques as such. During this programme of work, Nell has become interested in experimental design, particularly within the animal nutritional sector and with particular reference to how to best design experiments to test novel feed additives. This has informed and driven more data-oriented roles for Nell in recent years. Nell is also a member of the WPSA UK branch Council and actively involved in organising symposia and the annual Spring Meeting as well as working on the Inspire Symposium in 2014 at which this book was conceived. Nell also holds an Honorary Lectureship at the University of Nottingham where she contributes to several nutrition modules at Bachelors and Masters levels.

About the Authors

Rashed A. Alhotan is now a PhD candidate at the University of Georgia and a lecturer at King Saud University (Riyadh, Saudi Arabia). He received a BS degree in Animal Production from King Saud University in 2005 and an MS degree in Animal Science from the University of Nebraska at Lincoln in 2011. His current research is focused on improving poultry feed formulation by employing practical techniques to reduce nutrient variability, account for non-essential amino acids in finished feeds, and estimate the maximum safe level of feedstuffs using broken-line models.

Lynne Billard is University Professor and Professor of Statistics at the University of Georgia. She earned her BSc First Class Honors in 1966 and her PhD in 1969, both from the University of New South Wales. She is a former President of the American Statistical Association and International President of the International Biometric Society. She has over 250 publications, including six books, with applications in agriculture, biology, epidemiology, education and social sciences. She is a Fellow of the American Statistical Association, the Institute of Mathematical Statistics and the American Association for the Advancement of the Sciences, and an elected Member of the International Statistical Institute. She has received numerous awards, including the Wilks Award.

Manuel Da Costa is a PhD Candidate in the Poultry Science Department at the University of Georgia. He earned his DVM and MSc, ICBAS from University of Porto in 2010 and his MS from North Carolina State University in 2013. His work has been mainly focused on poultry health and management, with a major emphasis in nutrition, and mathematical modelling. He has had five papers published in peer-reviewed journals, and more than 20 abstracts presented at international meetings. He has also won several awards, including the 2015/2016 Phibro Animal Health Fellowship for work done in animal gut health.

David Lindsay was an Animal Scientist and a member of the Faculty of Agriculture at the University of Western Australia for 33 years. He was its Dean for 11 years and Director of the Institute of Agriculture for 9. He retired in 1999 to concentrate on developing and teaching courses in Communication of Science in Australia and internationally. He has written several books on scientific writing, the latest, *Scientific Writing = Thinking in Words*, published by CSIRO Publishing and a French edition, *Guide de rédaction scientifique* published by Éditions Quai, both of which appeared in 2011. A Spanish version, *Guía de redaccíon científica – de la investigation a las palabras*, was published by Editorial Trillas in 2012. He is a fellow of the Australian Academy of Technical Sciences and Engineering and was made an Officer of the Order of Australia (AO) for his research into reproductive physiology of farm animals.

John Patience is a Professor of Applied Swine Nutrition in the Department of Animal Science at Iowa State University, a position he has held since 2008. Patience, a native of Ontario, earned his BSc (Agr) and MSc Degrees from the University of Guelph, and PhD from Cornell University. At Iowa State University, his research focuses on the supply and utilization of energy by the pig with special emphasis on fibre and fat and the application of exogenous enzymes; on the relationship between diet composition and the function and physiology of the gastrointestinal tract; on ingredient evaluation; and on general feeding and management of the wean-to-finish pig. He has published more than 100 refereed journal articles and presented more than 350 invited lectures across the US and Canada and internationally. He is a former President of the Canadian Society of Animal Science and of the

Midwest Section of the American Society of Animal Science and is currently an Associate Editor of the *Journal of Animal Science*. He was elected a Fellow of the CSAS in 2010 and won the FASS-AFIA New Frontiers in Animal Nutrition Award in 2015.

Gene Pesti is Professor of Poultry Science and Animal Nutrition, University of Georgia. He earned his BSA from the Ohio State University in 1972, his MS from Auburn University in 1976 and his PhD from University of Wisconsin-Madison in 1980. He has over 200 publications including three books on poultry nutrition and feed formulation, and poultry husbandry. He is a Teacher Fellow of the National Association of Colleges and Teachers of Agriculture and has won numerous awards, including the Broiler Research Award from the National Chicken Council, the Nutrition Research Award from the American Feed Industry Association, the Research Award from the American Egg Board, and the Evonik Degussa Award for Achievement in Poultry Science.

Acknowledgements

A large volume of potentially useful research work is often rendered unpublishable by a simple design fault, the use of an incorrect feeding standard, a statistical analysis issue, or the lack of proper diet characterization. These mistakes seem to be repeated over and over again. We know this from our experience in reviewing papers and from failed experiments of our own. Thus, we decided to write this book, hoping that it might help pig and poultry nutrition researchers avoid costly mistakes in experiments and analyses.

We have many people to thank for input that helped shape this book. Thank you to Dr David Cadogan, Mr Geoff Clatworthy and Dr Tim Walker, three animal nutritionists in Australia with a combined commercial experience exceeding 100 years, for their invaluable inputs into Chapter 2. Dr Shubiao Wu was instrumental in spotting the problems associated with the calculations for the Practical Diet Replacement Assay presented in Chapter 3. Professor Hank Classen, Dr Jean Noblet, Dr Roger Campbell, Professor Bob Swick and Professor Frank Dunshea provided insightful comments on Chapter 3. Ms Hylas Choct helped illustrate Fig. 6.1.

Mrs Liz Roan proof-read and copy-edited all the chapters. Her timely and expert editorial skills made our jobs of editing this book so much easier.

We are proud of our collaboration and the way we worked on this book. We completed the book without having a single face-to-face meeting after the development of the idea – a testament to team work and efficient application of communication tools. The whole project ran almost as scheduled despite our busy workloads, and in Nell's case having her first child, Esther, who kept her company from start to finish.

We are indebted to our families for their love, support and tolerance during the many hours we were anti-social and off-limits after work, on weekends, or while travelling.

Mingan, **Mike** and **Nell**

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Foreword

So you want to do poultry or swine nutrition research?

What do you need to know and how will you go about doing it with the maximum potential for scientific and industrial acceptance and application? This text (*Nutrition Experiments in Pigs and Poultry: A Practical Approach*) provides a great framework for answering these questions. The book is comprehensive and includes factors to consider when planning an experiment, such as the appropriate design, the nature and characterization of diets, how to assess nutritional value and how best to report the results of the research. In other words, planning from hypothesis to data collection to reporting. It also examines the use of holo-analysis to maximize the value derived from the scientific literature. Although scientists will undoubtedly have portions of this knowledge, it is unlikely that even the more experienced among us have it all. Having this important information logically presented in one document fills a publication gap, as no single source of information covers this material in the same way.

So who will benefit from this information? Clearly less experienced scientists will benefit the most, and I would suggest that this book is a perfect opening day gift for graduate students (required reading) and postdoctoral fellows. I know that I will be doing that in my lab. However, it also holds value for more experienced scientists, as a reminder of best practice or for providing a perspective they may not have considered. This book is a good addition to resources available for mentoring the next generation of scientists.

It is not possible to adequately describe all of the contents of the book, but many aspects of the book ring particularly true for me. First and foremost is the importance of planning and critical literature review (including learning from previous mistakes) before an experiment is undertaken. When developing a protocol, each decision regarding experimental design must be made with complete recognition of its impact on the results and

interpretation of the data. We are reminded to not just do what has been done in the lab before or what was found in the literature, but to decide on research details after careful consideration. Decisions must ensure that the experimental design can accurately test the research hypothesis and reach the research objectives. Clarity of hypothesis and objectives is paramount. Response criteria should be selected that match the experimental design and permit logical interpretation. Just because an assay is up and running in the lab does not make it appropriate. The research of animal nutritionists has potential for commercial application, so the experimental design and data collection should, as closely as possible, match the conditions where the research will be applied. This includes designs that result in performance standards representative of the genetic capacity of the animals being tested. Finally, communication of results must ensure clarity of understanding to maximize knowledge transfer to readers.

I congratulate the editors, Mingan, Michael and Nell, for coming up with the concept of the book and putting together an excellent group of contributing authors. I have known the book editors for some time, and in particular Michael and Mingan. As a senior scientist, I have had the opportunity to watch both become internationally recognized science leaders, one employed in industry and one employed in academia, but both with the same passion for research and the research process. This book demonstrates the unselfish commitment of the editors to get research right and have it also be relevant to the industry that will use it.

In conclusion, this book is a good reminder of the time and effort required for the completion of high-quality research. Its contents should decrease the chance of mistakes, the wasted effort of poor research and failure to publish, and the failure of research to achieve industrial application. Animal nutrition is an applied discipline that requires good science, but with an eye to application by the animal industry.

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1 General Principles of Designing a Nutrition Experiment

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1.1 Introduction

The clear goal of animal nutrition is to facilitate the optimal use of resources for production of a desired trait. Animals are produced for meat, eggs, milk, wool, leather and many other outputs that have significant economic value. The cost of producing these outputs largely depends on the cost of the feed employed and the concomitant efficiency of that feed to produce the output of interest. Commercial least-cost formulation programmes are routinely employed to establish the lowest cost route for meeting these needs. The success of such programmes is dependent upon both the accuracy of the requirement and ingredient nutrient content data employed. Nutrition experiments are central to this process as they provide the very information that drives this optimization. As a result, it is important to ensure that when an experiment is conducted, the data generated are both accurate and relevant to the intended application. There should also be a minimum requirement for reporting of methods and data, so that the context in which the data are reported is known. This is important not only for the data at hand, but also for retrospective analysis where data from multiple publications can be combined to determine if a holistic model can more accurately predict the optimum nutrient content for a given output of interest. Clearly, the success of such reviews in deriving a satisfactory model is dependent upon the consistency of reporting of the relevant independent variable in the publications considered. Sadly, in many works, that reporting is far from consistent and, as a result, considerable opportunity for discovery is lost (Rosen, 2001). The focus of this chapter is to highlight the multiple considerations that need to be taken into account if the data generated are to be of value to academia and industry at large. It is split into the two areas of interest to the commercial

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feed manufacturer: nutrient requirements research and ingredient nutrient contents research.

1.2 Nutrient Requirements Research

The hypothesis of any nutrition trial must be that the animal will respond in some manner to the nutrient in question and nothing else. Setting such a hypothesis at the outset then drives the design of the trial. The aim is usually to determine the relationship between a given nutrient (with or without additional factors such as environmental or husbandry related factors, breed, age, sex, etc.) and a variable of interest. Such a variable may be weight gain or feed conversion ratio (FCR) or an index of interest (e.g. a digestibility, physiological or metabolic indicator). In its simplest form an experiment may, for example, examine the effects of a single nutrient on growth rate. In this case, the goal is to isolate and control all other sources of variation so that any change in performance is clearly attributable to the dose of the nutrient investigated. Provided growth rate is limited at all times by the nutrient investigated, then the experiment can be considered a success and the data can be used to estimate the requirement for that nutrient for any desired rate of growth up to the point where growth rate is no longer limited by the nutrient under test. It is at this point that the 'requirement' for that nutrient for maximum growth rate is established. There are, however, multiple caveats that need to be considered even in such simple experiments when 'requirements' are being determined. These are:

1. Environment.
2. Cage versus pen.
3. Feed form.
4. Energy – amino acids, carbohydrates and fat.
5. Fibre.
6. Other nutrients.
7. Age.
8. Breed and sex.
9. Disease status.

In all cases, the reader should consider whether the conditions of the experiment reflect the conditions under which the data are to be applied. If the experimental conditions and those under which the data are applied differ significantly, then the relevance of the information, whether it is requirements or nutrient contents of ingredients, needs to be considered. Clearly, no single set of experimental conditions will replicate all potential commercial applications and, as a result, commercial nutritionists have to consider the data available along with knowledge of the conditions under which their animals are raised. As a result, almost all nutritionists will formulate diets with significant 'safety margins' employed for critical nutrients to prevent significant losses in performance. Such 'safe' nutrient specifications are based on multiple data sets and types of experiments, tempered by personal experience.

Thus, a great opportunity exists to improve feed efficiency through more accurate and relevant determination of nutrient requirements and ingredient nutrient contents. This chapter will alert both research scientists and commercial nutritionists to the factors that must be considered when estimating nutrient contents of ingredients and requirements of the animal.

1.2.1 Environment

Temperature

Commercial animals are grown under a variety of conditions that influence the requirement for many nutrients. It is well known, for example, that in hot climates most animals will restrict intake and, as a result, requirements for some nutrients on a g/100 g basis will increase (Dale and Fuller, 1979). High temperatures also alter the metabolism of the animal so that processes that were not apparent in an animal at thermoneutral temperatures will now need resourcing. Synthesis of heat shock proteins is one such example. Heat shock proteins have been shown to have significant and far-reaching benefits on intestinal integrity and oxidative status as well as secretion of digestive enzymes, and as a result modify digestive efficiency (Gu *et al*., 2012; Hao *et al*., 2012). The synthesis of such reactive proteins can be moderated by many other nutrients, e.g. ascorbic acid (Mahmoud *et al*., 2004; Gu *et al*., 2012; Hao *et al*., 2012), resulting in the performance of the animal being moderated by nutrients other than that under test. Thus the determined nutrient requirement for optimum growth rate may be dependent not only on the temperature under which the animal was raised, but also on the concentration of heat shock mitigating and exacerbating nutrients/conditions that subsequently modify the severity of the thermal stress endured. The entire diet should thus be reviewed carefully when considering data from heat-stressed animals. Conversely, if animals are to be grown commercially under high temperature stress then such nutrients/conditions should be considered.

Similarly, as temperatures fall below the thermoneutral zone, the animal has to commit resources in order to maintain body heat. Such activities clearly consume additional nutrients, which will increase the requirement for these same nutrients if maximum gain is to be achieved (Ahmad *et al*., 1974). As the temperature at which an experiment is conducted can have profound effects on the determined requirements, it needs to be accurately documented if the reader is to be aware of the implications of the conditions of the test for their application.

While most thermal stresses modelled are chronic rather than acute, and are more often than not routinely reported, there are significant effects noted when birds are exposed to an acute stress in what would otherwise be considered a normal, thermoneutral environment. This is especially true for young animals exposed to acute cold stress as it can severely influence the health status of the animal (Lubritz, 1994) in a manner that compromises the value of the data derived. Cycling heat or cold stress is also different from

chronic effects, as animals adapt and alter intake patterns accordingly. Birds exposed to cycling heat stress, for example, learn to eat less during the cooler periods in anticipation of the impending temperature rise (Teeter *et al*., 1992). Clear reporting of not only the average daily temperatures to which the animal is exposed, but also the daily minimum and maximums and the age at which such events took place, is essential if value is to be extracted from the work. Moreover, the application of such data needs to consider if the birds grown commercially have been or will be exposed to acute, chronic or cycling thermal stresses, as this will influence the success of the application of a nutritional strategy.

Lighting

Light intensity and day length and/or length of dark periods influence several aspects of metabolism and hence nutrient requirements. Higher light intensity (particularly red light) encourages activity and feed intake but also aggression in poultry (Prayitno *et al*., 1997) and, as a result, energy and nutrient recovery and expenditure are altered, and thus requirements will be adjusted accordingly.

Day length influences not only locomotion and skeletal integrity, but also intake. Longer dark periods tend to reduce intake, gain, yield and leg problems (Brickett *et al*., 2007; Lien *et al*., 2007, 2009). Day length can also influence the efficiency of the intestinal tract. In birds, it is suggested that long dark periods encourage more of a meal feeding pattern which results in greater use of the crop. This use extends the time available for wetting of the feed, allowing for more efficient subsequent digestion and thus may contribute to reduced nutrient needs to achieve optimum performance. Longer dark periods have also been associated with increased rates of retro-peristalsis from the caecum (Godwin and Russell, 1997). This can increase mineral and nutrient recovery from the diet as a significant amount of fibre digestion takes place in the caecum. Not only does the reflux of caecal volatile fatty acids (VFAs) and enzymes (bacterial phytases, NSPases) provide energy and minerals for the host, but it is also proposed that the refluxed VFAs stimulate entero-hormonal pathways, which result in digesta being held in the stomach for longer, potentially improving gastric and hence overall digestive efficiency as a result (Masey O'Neill *et al*., 2012; Singh *et al*., 2012).

Fluorescent lighting can contribute to synthesis of vitamin D, which will clearly influence the dietary requirement for this vitamin, and also impinge on the calcium (Ca) and phosphorus (P) metabolism of the animal, which may alter the determined requirement (Willgeroth and Fritz, 1944). Thus the lighting source, as well as intensity and day length, should be reported as a minimum for nutrient requirement studies.

Humidity

While very often overlooked, and hardly ever reported, high humidity when combined with temperature can result in heat stress and the concomitant problems noted above.

Air quality

The concentration of carbon dioxide (CO_2) and ammonia has a remarkable influence on the wellbeing and performance of the animal. Unfortunately, reporting of air quality is commonly overlooked in many trials. CO_2 in excess of 4000–6000 ppm can lead to lethargy, poor performance and perhaps increased mortality in young animals (Reece and Lott, 1980; Donaldson *et al*., 1995). Environmental ammonia concentrations above 30 ppm can lead to poor feed conversions, lower weight gains and increased susceptibility to disease (Johnson *et al*., 1991; Beker *et al*., 2004). Particulates can provoke considerable respiratory health problems in animals. All of these factors have a significant impact on the partition of nutrients to growth and hence the nutrient requirements of the animal. Thus, measurements of air quality should be reported, especially when larger-scale floor pen trials are conducted and such quality issues are most likely to arise.

Feeder type and space

Research trials often provide significantly more feeder space per animal compared with space allocations used in commercial practice. Evidence exists to suggest that restricting the space per animal at the feed and water trough can reduce subsequent performance, particularly if the diet has more fines than pellets (Lemons and Moritz, 2015). When space is more than adequate, the intake of both water and feed is limited only by the animal's appetite, and the results obtained could be considered relevant for all unstressed conditions. If the trial presents results where the feeder space is not adequate for all animals to achieve *ad libitum* intake, not only will this create greater variation in individual intakes (as the more dominant animals will secure a greater proportion of intake compared with subservient animals), but also the nutrient densities determined necessary for optimum performance will relate to what is essentially a partial restriction on intake. Water availability sits in the same arena as feed, since a restriction on water availability will limit intake as the animal strives to balance one with the other. Removal of availability of water rapidly precipitates a very rapid drop in intake. Importantly, it is not only chronic but also acute shortages that need to be avoided in the design of a trial.

A key consideration with both water and feed availability is not just the number of drinkers or feeder space provided per animal, but also whether these nominal spaces are physically accessible to the experimental animals. Incorrect positioning, whether it be placing the feeder in a corner or raising a nipple drinker too high for smaller animals to reach, effectively restricts intake. Furthermore, behavioural studies have shown that some individuals in group housing situations develop a preference for specific feeders/drinkers, and if access to their favoured route is blocked or restricted, then these individuals will be feed restricted even though there may be more than adequate feed available elsewhere in the pen from alternative feeders/drinkers (Marini, 2003).

The relevance of the above points relates to the fact that most papers state 'feed and water were available *ad libitum*'. Clearly, there are many

factors that need to be considered to ensure that this is actually the case and that both were indeed available to all animals on an *ad libitum* basis. Unfortunately, very few papers report feeder space per animal along with water drinker space/number, which prevents the critical reviewer determining whether this may or may not have influenced the responses observed.

1.2.2 Cage versus pen and stocking density

Animals group-housed in pens, depending upon stocking density, clearly have greater opportunity for locomotion, social interaction and copraphagy compared with their caged counterparts. Thus the energy needs, and the potential for recycling of nutrients and utilization of bacterial metabolites present in the faeces, will differ and so influence the results obtained. Furthermore, the size of the group in which each individual is housed will influence social interaction and hierarchical effects on the ability of the individual to reach feed and water, which is compounded by available feeder and drinker space. The nuances of social hierarchy can lead to stress for those at the bottom of the pecking order. Such stresses, often observed both behaviourally and hormonally, alter the metabolism of the sufferer and, as a result, their nutrient needs. Indeed, high stocking densities have been shown to radically increase the optimum dietary density of some nutrients (e.g. tryptophan) that play a role in the alleviation of stress. The National Research Council (NRC) requirements for tryptophan for 3–7-week-old ducks is estimated to be 0.17%, but when they were stocked at 11 birds/m^2 (much higher than the optimal 5–7 birds/m^2), optimum growth rates and efficiency, liver levels of antioxidants and muscle quality parameters were achieved at 0.78%, four times the nominal requirement level (Liu *et al*., 2015).

1.2.3 Feed and water form and quality

Commercial animals are fed specific diets for specific growth periods. Usually, a crumble is fed as the starter with smaller then perhaps larger pellets as the bird ages. It is well known that feed form influences intake, feed wastage and feed efficiency (Abdollahi *et al*., 2013) and in some cases the effects observed with mash diets are not replicated in pelleted diets (Rosen, 2002a; Pirgozliev *et al*., 2016). The grist size of the ingredients used and the pelleting conditions employed will all influence the hardness of the pellet – which can directly influence performance and the subsequent digestibility of the diet (Amerah *et al*., 2007; Abdollahi *et al*., 2009, 2013). Pelleted diets containing wheat are more viscous than mash (see soluble fibre, below), which can reduce fat digestibility and subsequently reduce availability of the fat-soluble vitamins. If diets are fed in mash form, the grist size has a significant impact on the length of time the feed spends in the gizzard, which markedly influences the digestibility of the whole diet (Amerah *et al*., 2007; Svihus *et al*., 2008). As a result, the form of the feed employed and its grist following

grinding should be relevant to the commercial application it is representing. A caveat with regards to commercial practice relates to where pellet quality is measured. Often, the feed can leave the feed mill as high-quality pellets with few fines, but the pellet quality is substantially reduced on arrival in front of the animal following transport and delivery down the feeder via an augur. Thus, the commercial operator needs to take the pellet quality confronting the animals in the system into account when considering whether pellet quality should modify nutrient specifications.

Water is often not considered, but clearly is of great consequence to the performance obtained in a trial. If the water supply is limited or removed, even for just a couple of hours, the growth response obtained will not be relevant to animals that have not suffered such a restriction. Water quality is of particular note if it is rich in minerals, e.g. calcium. In some parts of the world where hard water is prevalent, the water supply can contribute the equivalent of 0.1% Ca in the diet. This is critical if the goal is to determine the Ca or P requirement of the animal, and influences the results of all other trials run under such circumstances. Microbial quality of the water also needs to be taken into account as it can influence health status and growth rates significantly (King, 1996). Bell drinkers are notoriously more likely to carry high bacterial loads than nipple drinkers, for example, and as a result the nutrient needs for optimum performance may be significantly modified by such simple choices.

1.2.4 Energy – amino acids, carbohydrates and fat

Many studies are conducted to determine the energy requirements of an animal or the energy content of an ingredient. The tenet, as with all nutrients, is that the animal responds to increased dietary energy to a point at which no further response is achieved. The energy needs of an animal are for maintenance, anabolic and catabolic activities that are met through the aerobic and/or anaerobic oxidation of the energy source. The difficulties with this approach are that energy is not a specific nutrient but is supplied by virtually all carbon-based feedstuffs. As a result, energy can be supplied by nutrients such as amino acids, starch, fibre, fat and sugars. Since many of these potential energy contributors also have a functional role independent of the energy contribution, their oxidation removes them from the pool for use in their function. For example, oxidation of an amino acid will define its fate as an energy source and not a component in a protein. Indeed, its oxidation may incur an energy cost in the disposal of the nitrogen component of the amino acid. The use of the amino acid as an energy source will depend upon the balance and supply of other, more desirable energy sources such as glucose or fatty acids. Indeed, the energy needs of an animal will also depend on the nutrient supply for growing tissues – specifically amino acids. If the diet is deficient in specific amino acids, or protein in total, then maximum growth rate will be limited and consequently the energy needs of the animal will not be the same as those for an animal with all the amino acids needed for its

potential. Thus, experiments designed to estimate the energy needs of an animal or the energy content of an ingredient need to ensure that the animal is not constrained by any other nutrients at any point in the energy titration. In practice, this necessitates an amino acid-dense diet. However, it is important that the diet used is not so oversupplied with some nutrients that there is a need to use energy to dispose of those nutrients that are supplied to excess. An example of the latter case is an amino acid-imbalanced diet whereby all amino acids are supplied at or above requirement, but some are significantly oversupplied, more so than would ever be deemed commercially relevant. This will drive the animal to deaminate the excess; and since synthesis of uric acid and disposal in the urine of poultry is a very energy-expensive process, this may interfere with the interpretation of such studies.

A further consideration is whether the energy source plays a physiological role as well as an energy substrate role. Fat, fibre and carbohydrates can all interact with the intestinal tract in a manner that can alter rates of pancreatic enzyme secretion, peristalsis, transport rates of nutrients from the intestinal lumen to the blood, and the growth and maintenance of the intestinal tract. Such effects are mediated through the detection of these components or their fermentation products throughout the length of the digestive tract and the secretion of hormones (such as IGF, PYY and insulin) in response (Croom *et al*., 1999). If any of these signals are at a threshold of response, the apparent response to the energy titration trial may be misinterpreted. For example, dietary fat is known to interact with the intestinal tract and influence the secretion of several hormones, the consequence of which is known as the ileal brake (Gee *et al*., 1996; Hand *et al*., 2013). Such a phenomenon holds back digesta in the gastric phase and seems to improve digestibility of protein and consequently amino acids. Problems can occur if an experiment is set up to determine the energy content of an added fat and the doses employed start below but titrate to levels above this threshold. Clearly if there was any benefit in performance due to improved amino acid digestibility, which was not the focus of the study, the results of the study could easily be misinterpreted.

Energy is therefore a very difficult value to address as it is supplied by so many nutrients and feed components and the efficiency of use of a particular energy source may well be influenced by the contribution of other energy-bearing components as well as the specific fat and amino acid considerations noted above.

Further considerations in this regard are covered in Chapter 5.

1.2.5 Fibre

Chapters 4 and 5 deal with fibre in more detail. However, fibre can have such an influence on the digestibility of so many nutrients that it simply cannot be ignored when designing a nutritional experiment. There are two principal considerations: (i) insoluble fibre and passage rate; and (ii) soluble fibre and nutrient digestibility rate.

Insoluble fibre and feed passage rate

Insoluble fibre that is 'functional' has significant effects on the passage rate of feed throughout the intestinal tract. 'Functional fibre', as it has been termed, encourages not only development of, and retention of feed in, the gizzard, but also more rapid movement of digesta through the small intestine. Provided this is not fed to excess, the effects are often beneficial, as the intestine functions more efficiently and total nutrient extraction from the diet is enhanced. Fibre source and particle size influence the 'functionality' of the fibre and hence the effects noted (Hetland *et al*., 2004). This effect of insoluble fibre overlays and interacts with the feed form (pellet or mash) as discussed earlier.

Soluble fibre and nutrient digestibility rate

If the test diet contains significant quantities of viscous cereal grains (rye, barley, oats, triticale and wheat, in descending order of viscosity), this will significantly compromise the digestibility of fats, proteins and carbohydrates. Viscosity slows diffusion rates of both enzymes and nutrients proportionately with their molecular weight. As a result, digestibility of the very large fat micelles formed in the process of their digestion in the intestine is more significantly compromised than that of simple sugars or minerals. Any studies investigating the digestibility of fats, proteins, etc. should be done using cereals that are relevant for the commercial nutritionist (Dänicke *et al*., 1999). Use of a maize-based diet will radically overestimate the energy content of a fat source if the same were to be fed in a rye-based diet commercially. If the commercial application of fats in viscous diets is accompanied by the use of a relevant NSPase (which reduces viscosity) then clearly the test diets should also include an NSPase.

1.2.6 Other nutrients

When an 'optimum' in performance is observed, it is assumed that this is the point at which performance can no longer be improved with the nutrient of interest. If, however, the performance reaches a plateau due to the emergence of a limitation in the concentration of another nutrient, then the true optimum performance may actually be considerably greater than that achieved in the study. It may really be that far more of the nutrient under test would have actually been needed to optimize performance, had this nutrient remained the only limitation on performance. This is a critical condition of the test, i.e. that the nutrient under test is at all times the constraint on growth rate. Interesting problems can develop when there are antagonisms between nutrients, and the continued addition of the nutrient under test may actually reduce the availability of the antagonized nutrient to the extent that the latter now becomes limiting. An example is the lysine/arginine antagonism where excessive lysine reduces the efficiency of utilization of arginine by stimulating the catabolic enzyme arginase in the liver and kidney of the chick (Allen

and Baker, 1972). It is essential for any nutritional paper to list all ingredients and their inclusion levels so that the reader can calculate the expected nutrient contents of the diet and thus put the results into context from their own perspective. Provision of a table of calculated contents of all nutrients of interest or relevance is also considered as a minimum in such work as it provides the context from the author's viewpoint.

Much of the nutrient content data that could be reported in a paper is omitted for no particular reason other than brevity and, as a result, future insights into the trial may be limited. Retrospective analysis, or holo-analysis, of literature data is often attempted in order to tease out associations between input variables and output variables of interest from multiple papers addressing the subject area of interest. It is often noted that, in some analyses, the ingredient and calculated nutrient composition of the diet influences the response to the nutrient(s) of interest. Rosen (2002a) illustrated one example of this where the association of fat and the presence of an ionophore coccidiostat affected the response observed to the inclusion of a phytase and thus presumably P deficiency. Such an association was not foreseen at the outset of the analysis and highlights the lost opportunity for discovery of such items of information if reporting is incomplete.

1.2.7 Age

The requirement for many nutrients falls with age, though it may increase for some others. In the case of some ingredients, the rate at which the requirement falls may be relatively rapid and is probably unknown. An example is phosphorus. A diet may start off as being very deficient in P but by the end of the test period it may actually be surplus to requirement, in which case the 'negative control' may not restrict growth rate as much as expected and sometimes not at all (Bedford *et al*., 2016).

Amino acid requirements, as a percentage of the diet, also fall with age, indeed some more than others (Dozier *et al*., 2008). Energy requirements, on the other hand, tend to increase. As a result, the test needs to be conducted over a time period that correlates with a standard industry practice if the data are to be relevant and valuable.

One obvious problem noted many times is that digestibility experiments using mature animals are not relevant for younger animals. Not only are the absolute values for the digestibility of energy, amino acids, fat and Ca and P usually lower in the young animal, but also in some cases ranking of samples can be significantly different as well. For example, not only was the apparent metabolizable energy (AME) of 18 samples of maize in 10-day and 42-day broilers shown to be lower in the younger birds, but also the correlation between 10-day and 42-day AME was particularly poor, suggesting that use of an older animal to screen out poor samples for younger animals could be fatally flawed (Collins *et al*., 1998).

Some ingredients or additives need to be fed for a period of time to enable the animal to adapt fully and thus express the phenotype that correctly values

the product tested. In some cases this means that the product needs to be fed from day-old, particularly if this is how it would be used commercially. An example is phytase and NSPase where the review by Rosen (2002a) noted that failure to feed from day-old resulted in the loss of almost all the value of the enzyme. Indeed, the practice in many phytase studies to feed a phosphorus-adequate diet for the first five days before putting the animals on a test diet results in considerable buffers of Ca and P being laid down in the bones. This reduces the challenge mounted by feeding the subsequent 'low P' negative control and as a result the phytase dose needed to restore performance to the level of the positive control is markedly underestimated. Moreover, the fact that the P requirement of the bird is falling rapidly with age means that the practice of feeding a P-adequate diet for the first five days removes the most sensitive phase of the chick's life from the test. This is a significant error perpetuated in the literature, particularly since the industry practice is to feed phytase from day-old, a practice that is not modelled frequently even today. Recent challenges to the suggestion that NSPases should be fed from day-old also need re-evaluation. Although the authors concluded that the statistics suggested that the enzyme only needs feeding for the last 14 days of life (Santos *et al*., 2013; Cardoso *et al*., 2014), plotting the data suggests otherwise. In the case of this paper, use of a highly protected means separation technique resulted in poor resolution in the study, and hence large numerical differences in performance went unnoticed. Selection of the correct statistical techniques for determination of the response to a nutrient, additive or ingredient is an enormous topic and needs to be considered carefully. The model employed should have relevance in biology and replication should be adequate. Such considerations are dealt with in much more detail in Chapter 2.

1.2.8 Breed and sex

Different breeds and strains within breeds have different requirements for optimum growth rates as a result of the pressures under which they have been selected. High-yielding strains, for example, will require more lysine for optimum breast meat yield than slower-growing strains, particularly during the period of growth when breast meat deposition is at a maximum. However, it is not necessarily differences in requirements for optimum performance between strains per se, rather the end goal for the different strains (breast yield vs body weight, for example) that drives requirements towards different economic optima (Waldroup, 1997; Corzo, 2005; Kim, 2012). Clarity is therefore required in the description of the strain being used and the outcome desired. Although it is clear that genetics underlie the majority of the differences between random bred lines and modern strains, it is still quite clear that the nutrient requirements identified for each strain to achieve their potential are quite different.

It is well established that males grow more rapidly and efficiently than females and concomitantly their requirements for maximum gain and efficiency are also higher. Unfortunately, the majority of research focuses on

males only and, as a result, the end user is left with far greater uncertainty with regards to how to feed the females (Corzo, 2005). Indeed, it is only in separate-sex-fed flocks that advantage can be taken of the differences between sexes. In many situations, however, the production of chickens is based on as-hatched flocks, which results in compromised nutritional offerings to both sexes. One final consideration is that the sexes may differ in their response to a particular stressor. As an example, the lysine requirement of the female, but not the male, was increased when the birds were heat (37°C) stressed (Han and Baker, 1993; Corzo, 2005).

1.2.9 Disease status

Requirements for nutrients are altered markedly with disease and immune system status. One example is that the requirement for threonine and serine is elevated during coccidiosis, presumably as the need for cellular repair and mucus synthesis is particularly reliant on these amino acids (Kidd, 2000). Animals that are in the midst of an inflammatory challenge will suppress intake as a result of the release of specific cytokines and as a result the 'requirements' will have altered significantly from non-challenged animals. While some diseases are induced and thus described in the scientific article, in some situations a subclinical disease may afflict a flock, which may or may not go unnoticed. If unseen, then the performance of the flock will be compromised and the subsequent data generated will not be so relevant for an unchallenged flock. If the disease is treated with an antibiotic, whether it was subclinical or not, this very treatment will alter the response to the treatments employed. Even when perfectly healthy animals are fed growth-promoting antibiotics, the response to other nutrients or additives will be affected. Rosen (2001) noted in a substantial review of the antibiotic and enzyme literature that, while both products improved performance to a similar extent, the presence of the one muted the response to the other. The implication is that health status and the intestinal challenge that an animal experiences will influence the requirements for optimum performance. Given the wide-ranging effects of drugs and coccidiostats, and more recently enzymes, probiotics and prebiotics, it is essential that such additives are clearly reported in all nutrition experiments for context.

Even when all the conditions above are taken into consideration there are several additional points to note, as follows.

Is the performance obtained typical of the breeder standards?

If not, the results obtained may not reflect commercial reality unless, of course, the test is designed to represent a deficiency or stress. Stress can be presented in many ways, but clearly if the level of stress under which the animals used in determination of requirements does not represent the stress under which animals are raised commercially, the results obtained need to be interpreted with caution.

Has the hypothesis to be tested been clearly set?

Digestibility experiments do not necessarily reflect subsequent performance and as a result the hypothesis needs to be clearly stated and examined. Indeed, if the additive or the nutrient influences the intake as well as digestibility, then consideration should be given to the value of the digestibility data in the absence of intake data. Moreover, the desired nutrient/additive concentration may differ with the desired goal. For example, the optimum for growth rate and efficiency may differ substantially from that for optimum carcass yield, bone density or longevity.

Are the statistical models and interpretation correct?

Application of the correct model and parameterization is important if the data are to be interpreted correctly. If a regression model is to be used, the model should accurately reflect the biological effect of the test ingredient employed. Application of a quadratic model makes the assumption that there is a definitive optimum, above and below which there is a loss of performance. If there is not such a response, use of such models is inappropriate or needs careful consideration. Phytase research has shown that the response to these additives is log-linear, i.e. performance increases in a linear fashion with log increments of phytase dose (Rosen, 2002b). In many subsequent studies, the application of a quadratic model incorrectly implies that there is a defined optimum and introduces confusion into the literature.

With simple factorial experiments, the most common mistake is the discussion of main effects when an interaction is significant or the interaction terms when only the main effects are significant. Use of words such as increased, reduced, enhanced, etc. relating to a treatment effect when the statistics do not support such comments is also far too common. If such comments survive in the text when they are not justified, future reference to such work immortalizes an incorrect interpretation of the data.

Replication is often not sufficient; and while this may limit the ability of the experiment to determine the requirement of a nutrient, poor replication is even more of an issue if the goal of an experiment is to show no difference between two treatments, e.g. comparison of two amino acid sources.

Statistical models and data interpretation are discussed in much more detail in Chapter 2.

Do the measured nutrients agree with your calculated values?

The diet and test article should always be measured for the nutrient or additive of interest, e.g. amino acids, fats, energy or enzymes. Failure to determine the actual dietary content of the target nutrient/additive reduces the ability of the experiment to declare a 'requirement'. Moreover, those nutrients that influence the utilization of the nutrient of choice must also be declared, and better still measured, in the trial diets so that the data and results can be put into context. An example would be a phytase study where it is essential that not only is the level of phytase enzyme in each treatment

feed validated by assay, but also the levels of Ca and P, as these nutrients significantly influence performance of the phytase directly. In addition, confirmation of dietary phytic acid content is desirable as well as a statement of the intended, if not measured, vitamin D content and form in the diet. Clearly, the accuracy of the assay will constrain the precision with which the requirement can be determined.

1.3 Ingredient Nutrient Contents Research

In addition to determination of the nutrient requirements of an animal under whatever conditions are of interest, nutritional research also aims to determine the nutrient contents of ingredients so that diets can be formulated to meet these requirements. These ingredients include cereals, protein sources, fats, vitamins, minerals and a multitude of additives. Each of these will bring points of consideration that need to be taken into account if the data generated are to be of value in more general use. In general, the methods for determining the nutrient contents of an ingredient rely either on digestibility techniques, of which there are variants for both ileal and faecal (or in the case of poultry more commonly excreta), or comparative techniques whereby performance on the test ingredient is compared with a standard. In either case, the idiosyncrasies of the ingredients need to be taken into account when using these techniques. In the most in-depth studies, a range of inclusion levels of the test ingredient are used to determine the nutrient content of the ingredient by regression analysis, so that the effect of inclusion level is eliminated and the effect of ingredient interactions minimized. The inclusion levels chosen are set by the palatability of the ingredient, its nutrient content and the likely imbalances it may cause if fed in excess. It is also assumed that the nutrient under investigation is always below the requirement of the animal, otherwise adaptive responses may reduce digestibility with increasing inclusion level, and the assumed linearity between nutrient absorption and nutrient intake will not be valid. One critical assumption is that when the test ingredient is fed, the contribution from the balance of the diet is proportionately consistent, which may not always be the case. Some basic principles and caveats for each ingredient are given below.

1.3.1 Cereals

Cereals tend to be fed at relatively high proportions of the diet and nutritional evaluations generally attempt to use the test ingredients at as high a level (or a range of levels) as possible in order to ensure that the response is evident, measurable and attributable to the test material. The inclusion levels should also be realistic, in that there should not be an order of magnitude between the inclusion level under test and the highest inclusion level used in praxis. As a result, the range in inclusion levels for cereals is probably greater

than any other ingredients that undergo such tests. Nevertheless, care should be taken to ensure that the change in the inclusion level of both the balance of the diet and the test cereal does not pass a threshold that may alter the digestive status of the animal. An example can be seen with more viscous cereals such as wheat and, in particular, barley and rye. With such grains, the intestinal viscosity that results from feeding commercially relevant levels, for example 65–70%, can be orders of magnitude lower than when these grains are fed at 80–90%, which is not uncommon in some experimental procedures (Allen *et al*., 1996a,b). At such high levels, the digestibility of the entire ration, fat in particular, can be markedly reduced, with the consequence that the proportionality of the effects of both the test and balance ingredients is lost. The value of these cereals is thus underestimated if they are to be used at more conventional levels.

1.3.2 Oilseed meals

Oilseed meals are commercially used at moderate levels of inclusion (up to 35–40% maximum) but if tested at higher levels some problems may become apparent which are not normally relevant. Examples include trypsin inhibitors, lectins, erucic acid, gossypol and alkaloids, but there are many others. If dose–response methodology is employed, deviation from linearity at higher inclusion levels should be considered as a potential indicator that such problems might be evident. The inclusion level beyond which such deviation occurs should be viewed against commercial practice to determine whether such issues have any practical implications.

1.3.3 Fats

Fats cannot be fed at too high an inclusion level before they reduce pellet quality (if pelleted diets are to be fed) and thus influence performance (Thomas *et al*., 1998; Abdollahi *et al*., 2013). The quality of the fat also needs to be considered. Highly saturated fats need to be emulsified prior to absorption, more so than unsaturated or medium-chain fatty acids. As a result, any factors that reduce the ability of the bird to emulsify fats will disproportionately devalue saturated fats compared with their counterparts. Such factors include viscous grains, bacterial challenges and, related to this, the lack of use of an antibiotic and/or coccidiostat (Bedford, 2000). Conversely, inclusion of NSPases, emulsifiers such as lecithin, and antibiotics can increase the determined energy value of the fats. Conditions of the test need therefore to be considered if commercial application is to rank saturated and unsaturated sources appropriately.

Oxidative status of fats also needs to be considered as this clearly influences the maximum level that can be tolerated and the energy value determined.

1.3.4 Vitamins and minerals

Vitamins are obviously fed at lower doses than feed ingredients, and several have heat and storage stability constraints that must be accounted for in any study. Some have lower toxicity thresholds than others, so dosages need to be considered carefully and, in the case of the fat-soluble vitamins, the basal diet needs to have an adequate level and quality of fat to assist in their absorption (Dänicke *et al*., 1999).

Several minerals are subject to the same constraints with regard to optimum dose and toxicity effects. In the case of some minerals, there are benefits to inclusion at levels well above the 'requirement' as a result of their apparent antimicrobial activity. Examples include copper and zinc, but care must be taken, as too high an inclusion level can lead to toxicity and poorer performance (Karimi, 2011). Separation of these responses is essential if the correct conclusion is to be drawn. Several minerals also interact with regards to solubility and transporter usage and, as a consequence, an excess of one mineral can drive a deficiency of another (see Section 1.2.6).

1.3.5 Additives

Additives include a multitude of products such as enzymes, probiotics, antibiotics, prebiotics, emulsifiers and organic acids. All will have specific considerations, but the premise to consider is that the basal diet must be relevant for the end user. For example, in general, a phytase would not be used in a diet that contains no phytate. Similarly, antibiotics and other microbial modulators will generate apparent nutritional responses that depend upon the microbial challenge of the test.

1.3.6 Digestibility studies

Digestibility studies, particularly short-term tests, offer the best opportunity to feed the ingredients being tested at levels that markedly exceed those used commercially and, as a result, the caveats noted above are particularly relevant. Furthermore, it must be noted that digestibility trials are really only relevant if there is a measure of intake as well. Knowledge of the AME of an ingredient is all very well, but if the same ingredient is an appetite suppressant or stimulant, the practical value of that ingredient will be markedly lower or higher than the digestibility test alone will have indicated. Unfortunately, most digestibility tests do not allow for a relevant growth and intake measurement and, in some situations, where semi-purified diets are used, the animals actually lose weight during the test, which questions the applicability of the data generated.

1.4 Summary

Growth rate has been used as the example 'response variable of interest' in this chapter. While this is valuable for most commercial operations it may well be that the economics of a particular operation may mean that the operator is more interested in the FCR, breast meat yield, calories/kg meat, mortality, or days to a specific weight rather than the rate of gain. If this is the case, it should be understood that the conditions and nutrient densities that optimize gain could be significantly divergent from those that optimize the end point of real interest. Optimum growth rates occur at lower levels of dietary Ca and P than maximum bone density, for example, though it is not clear whether maximum bone density represents an optimum even for the chicken. Similarly, lysine and energy levels to optimize gain are lower than those to optimize feed efficiency (Han and Baker, 1993).

Commercial chicken production varies significantly around the world, with respect to not only husbandry, environment and nutrition, but also breeds used, slaughter age and ingredients employed. Even within one company the performance between flocks can vary markedly, by as much as 40 points in FCR between best and worst farms. Optimizing performance of the best flocks is a very different task compared with that of optimizing the poorest. Given that the environment has such an overwhelming effect on the performance of the bird, it is surprising that nutrition is of much influence. But clearly it is, and it is only through experience and attention to detail that the commercial nutritionist is able to adjust the information available in the literature and apply it to their specific circumstances to achieve good performance most of the time. This explains why attention to detail in designing and, most importantly, in reporting nutrition experiments is essential if the data is to be of value to the scientific and commercial audiences, respectively.

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2 Most Common Designs and Understanding Their Limits

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2.1 Introduction

Animal and poultry sciences are applied sciences whose practitioners' questions ultimately involve economic applications. In their simplest forms the questions researchers ask are most often, 'How much of something needs be administered to maximize performance (and profits)?' or 'How much of something can be administered without inhibiting performance (and profits)?' Monogastric animal research then often involves administering or feeding a series of different levels of something and observing how it affects performance. The independent factors may be things like nutrients or environmental temperatures and the response (output) variables may be things like growth and egg production, feed intake and efficiency, carcass composition, egg size and composition, behaviours, bone quality, etc.

In most cases, the responses to a series of levels of inputs are dependent on other environmental or genetic factors. The responses to a drug or varying nutrient levels may depend on factors such as environmental temperature or the genetics of the animal being studied. Questions such as 'Do females respond differently than males?' require more complicated experimental designs, meant to determine if interactions exist and how important they are to responses and profits.

Poultry and swine producers need to know how their animals will respond in different situations to discover the right set of conditions to maximize profits. However, no two animals are expected to respond the same. Researchers have to test many animals to see what the average responses will be. The most important question they have to ask is, 'How many animals must be observed to obtain an accurate estimate of averages?' (Aaron and

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Hays, 2004; Shim and Pesti, 2012). Once that answer is known, then researchers can attempt to determine responses and conditions or levels required to maximize profit. Under normal situations, the variation in younger and lighter birds and animals is expected to be lower than in older and heavier ones. So the variances within response studies must be carefully observed and corrections made when necessary. When dealing with nutritional deficiencies or any treatments depressing growth, some individuals may be affected more than others and the variances affected even more by the imposed treatments.

2.2 What is the Goal of Simple Research Trials?

Experimenters often administer (feed) levels of some supplement or nutrient to determine the level that results in maximum profits. To determine profitability, producers need to know the costs of the inputs, the costs of the outputs and the technical relationship between inputs and outputs. The goal of experimentation is usually to determine the technical relationships between inputs and outputs so that producers can predict the most profitable level of inputs for their flocks. The conclusions drawn will depend on how researchers analyse the data and display their results. The methods of analysis used will be influenced by how they regard the data philosophically, and apply their particular mental outlook or model to their analyses.

2.3 Typical Interpretations of Response Data

Consider a simple experiment with six levels of a factor (like a drug, nutrient or health-promoting ingredient) administered to three replicates per treatment (Table 2.1). The replicates may be three individual animals or birds or three pens of individuals.

Seven different interpretations of the results displayed in this table can be given by applying different models or methods of analyses. These interpretations are discussed below.

Table 2.1. Example of a simple research trial. Inputs could be nutrient or drug levels, etc. Responses could be growth, body composition, metabolite levels, etc.

	y (Observed responses)		
x (Input level)	Replicate 1	Replicate 2	Replicate 3
8	60	70	80
9	76	86	96
10	90	100	110
11	91	98	109
12	103	110	121
13	105	113	124

Interpretation 1: Independent level interpretation of typical response data

Most researchers have presented results of such experiments as if the input levels were independent of each other as in Figure 2.1a. They ask questions like 'Is the response from 8 units the same as from 9 units?' and 'Is the response from 10 the same as from 11 units?'

Researchers have typically applied paired t-tests or multiple range tests (Tukey, 1949; Duncan, 1955) to their results to try to distinguish if the various input levels could be expected to give different results. If their conclusion is that feeding 10 units gives the same results as 11 units, yet feeding 10 units is better than 8 units, then it must be most prudent to feed 10 units to achieve maximum output. The more powerful the experiment, the better the chance of finding high input levels to maximize profits. Powerful in this sense means lots of replication and uniform conditions. The less powerful the experiment, the better are the chances of not declaring differences significant and concluding that lower levels are perfectly fine. In this case, it could be concluded that 10 units of x will yield the maximum response, since it is not 'significantly different' at a probability level of 5% (or $P < 0.05$). Giving 9 units yields the same results as giving 10 units, so according to this one experiment, the best answer to what level of x results in maximum levels of y can only be 'between 9 and 11'.

Some researchers prefer to calculate orthogonal contrasts (Billard *et al.*, 2014) and actually determine the probabilities that the various input levels might give less than the maximum response. From a philosophical perspective, this approach seems superior to just declaring whether means are statistically significantly different at, for example, $P < 0.05$ or 0.01. In this example, different conclusions may be drawn from using the two approaches. From Fig. 2.1a it appears that between 9 and 11 input units gives the same output result as 13 input units (not significantly different). However, from Table 2.2 below, it appears that more than 10 units are indeed needed to not have a statistically significantly difference from the output from 13 input units.

Table 2.2. Orthogonal contrast results of comparing responses from different input levels in Table 2.1.

Input level (units)	Probability that the response is different from the highest level fed (13 units)
8	0.0001
9	0.0042
10	0.0179
11	0.5107
12	0.7718

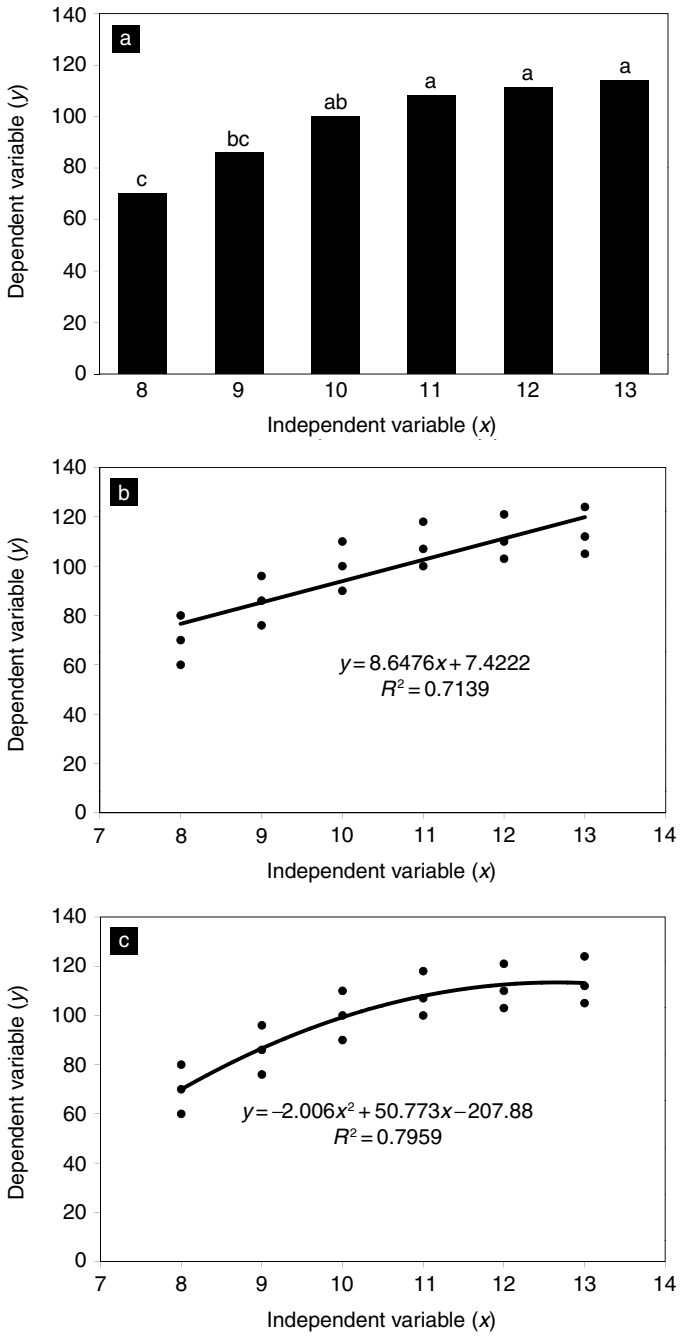

Fig. 2.1. Several approaches used for modelling responses to different input levels for the data in Table 2.1. (a) The bar graph approach (means with different superscripts are significantly different at $P<0.05$ as separated by Duncan's New Multiple Range Test). (b) The linear model. (c) The quadratic polynomial model.

continued

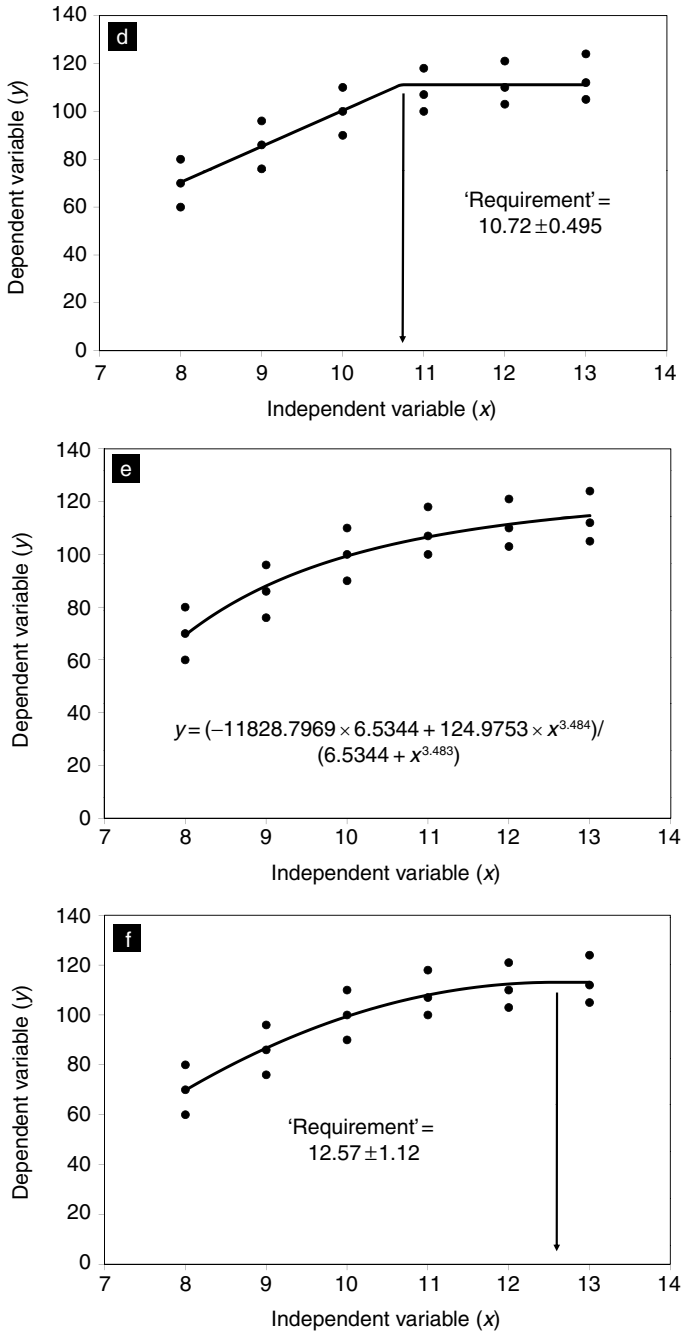

Fig. 2.1. *continued*.
(d) The broken-line linear model. (e) The saturation kinetics models (a superset of Michaelis-Menten enzyme kinetics). (f) The broken-line quadratic model.

Interpretation 2: Simple regression interpretation of typical response data

An alternative way to interpret the same data is to fit a simple regression (Shim *et al*., 2014) of the form: $y = b_0 + b_1 x$.

The question asked is, 'What is the expected value of y for any value of x?' If the data have a simple linear interpretation, there is one unique response assumed for each unique x value (Fig. 2.1b). Contrary to testing the hypothesis 'Do 8 input units give the same response as 9?', the hypothesis tested is whether the slope b_1 is different from zero. If the slope of the line is concluded to be different from zero, it is assumed that 8.49 input units give a different response than 8.50 input units, etc. When only a straight line is fitted to data, there is no obvious optimum level to choose. No input level that yields a maximum response can be determined in the range studied, except perhaps to say that the highest input level resulted in the highest output (positive slope), the lowest output level (negative slope) or that it does not matter what input level is administered (slope = 0).

Interpretation 3: Higher order regression interpretation of typical response data

If the data are best fitted by a quadratic or even higher order polynomial, then there may be two unique y values for each x (Shim *et al*., 2014). When the quadratic model is applied, it is implicitly assumed that there may be two input levels that give the same response (y), but they are not necessarily in the range of input levels being studied. With the quadratic polynomial model, the input level that gives the maximum/minimum response can be determined by setting the first derivative equal to zero. In the quadratic example (Fig. 2.1c), 12.66 input units result in 113.39 output units, so 12.66 units might be called the 'requirement' for maximum technical performance if it was referring to some essential substance like a vitamin or amino acid. The 12.66 units may or may not be the most economical level to administer, as discussed below.

Interpretation 4: The simple spline approach to estimating levels that optimize responses

For most responses of the type in Fig. 2.1a, there is not a maximum response as suggested by Fig. 2.1c, but a range of inputs where the responses are equal (plateau) (Vedenov and Pesti, 2008; Pesti *et al*., 2009). Theory holds that homeostatic mechanisms allow a bird or animal to perform at the maximum rate when excess units of the input (independent) variable are administered. The so-called 'broken-line linear model' (BLL; see Fig. 2.1d) has been applied to such responses, and the breakpoint is taken to represent the required level to reach the maximum response.

With this interpretation, there is a linear response up until the point where the maximum response is reached. Each additional unit of input (cost)

yields the same additional response of output (returns) up until the plateau is reached. From an economic perspective, there are really only two choices in input levels to administer, one each at either end of the ascending line. The substance is administered at the breakpoint, or not administered at all.

Interpretation 5: A theoretical approach to modelling responses based on metabolic phenomena

It seems most unlikely that a flock of birds would respond in a perfectly linear fashion to increasing levels of an input. As animals and birds approach their genetic potential, the response to any given unit of input is expected to decrease. This phenomenon has been called the 'Law of Marginal Returns' or the 'Law of Diminishing Marginal Productivity'.

Enzyme theory holds that enzyme-catalysed reactions follow this pattern: the first unit of substrate results in a big increase in the reactions' velocity. Subsequent equal additions of substrate result in progressively smaller increases in enzyme velocity. Since the metabolism of higher organisms is based on enzyme-catalysed reactions, it follows that the kinetics of growth and drug, supplement, nutrient, etc. administration should follow the same pattern, at least up to the point where toxicity is reached.

Enzyme-catalysed reactions only approach a maximum. In the case of living organisms, maximums or plateaus seem to be reached and eventually adding more of most or many additives results in toxicity. Being sure that levels used to determine the plateau are not excessive is another practical problem facing researchers. When determining animal performance responses, a maximum really is reached before the substance being administered becomes toxic. Thus it may be necessary to apply an analysis like the ones detailed below before applying models to determine input/output relationships for determining supplementation levels (López *et al*., 2000; Aggrey, 2002; Zuidhof, 2005; Vedenov and Pesti, 2008; Pesti *et al*., 2009).

The quadratic polynomial model has a maximum response that could be considered an optimal level and the broken-line linear model has a breakpoint that can be considered an optimal level or 'requirement' in some cases. In contrast, the Saturation Kinetics model's response just heads off into infinity. Because there is some level where anything (including water) diminishes responses, this model seems impractical, although it may theoretically be the best response model in normal ranges. The problem is in determining just what the normal ranges are; and it shares that shortcoming with the other models.

Models without a clear breakpoint between the ascending and plateau or asymptote have no objective way to estimate a 'requirement'. Nutrient levels resulting in perhaps 90% or 95% of the maximum response are sometimes called the 'requirement'. Any percentage of the maximum response is arbitrary. But if the researcher believes that is the shape of the response curve then diminishing returns economics can be applied and practical feeding levels can be objectively determined. Nutritionists setting requirements for

humans are not comfortable using economics as their criteria for daily allowances and so often resort to some arbitrary standard.

Interpretation 6: A practical approach to modelling responses based on goals

A model with a second-order ascending portion and plateau may also be used to represent the diminishing returns phenomena that transition to a plateau. This has been referred to as the broken-line quadratic (BLQ) model (Pesti *et al.*, 2009). It may be less theoretically sound than the saturation kinetics model, but it has the nice feature of having a point that can be used to represent the optimal administration level to pharmacologists, or the 'requirement' to nutritionists. The BLQ model can be interpreted in two different ways. The first is to find the point where the ascending quadratic portion intersects the plateau. This point can be interpreted as the requirement for nutritional studies, the same as for the broken-line linear (BLL) model. It could be considered to be the point of maximum technical efficiency. The second is to find the point of maximum economic efficiency, as detailed below. The 'requirement' or optimal administration level estimated from the BLQ model is always necessarily higher than for the BLL model and has a wider confidence interval.

Interpretation 7: Mechanistic modelling

The BLL or BLQ models may be used for determining optimum feeding levels of nutrients or compounds when cost is not particularly high or important and wide margins of safety are usually given. Examples of such compounds are trace minerals and vitamins and some enzymes and probiotics. When costs are particularly important, as for nutrients like protein, amino acids or phosphorus, or additives like phytase, an economic analysis is most appropriate.

Tables 2.3 and 2.4 illustrate how administration levels to maximize profits could be determined using the various models. Notice how changing the cost of the inputs from \$3/unit to \$4/unit changes the input level that maximizes returns on investment (ROI). Changing the value of the outputs has similar effects: if the value of the outputs were to increase, higher input levels would maximize ROI; and conversely. These examples also show the importance of choosing a model wisely. While the models in Tables 2.3 and 2.4 fit these particular data very well, they give different estimates of administration levels that maximize profits (returns on investment).

This is a very simplified example, but the principles illustrated should be applied to all aspects of production agriculture, simple and quite complex alike (Pesti and Vedenov, 2011). The fact that prices sometimes change even several times per day illustrates just how important it can be to have good data and good models with which to make decisions. With different input prices, levels that maximize profits change.

Table 2.3. Returns on investment (ROI) using the Quadratic Model for the data in Table 2.1.

Input level (units)	Input cost = \$3/unit, Output value = \$1/unit				Input cost = \$4/unit, Output value = \$1/unit			
	Cost (\$)	Output level (units)	Value (\$)	ROI (\$/unit)	Cost (\$)	Output level (units)	Value (\$)	ROI (\$/unit)
11.5	34.5	110.7130	110.7130	76.2130	46.0	110.7130	110.7130	64.7130
11.6	34.8	111.1564	111.1564	76.3564	46.4	111.1564	111.1564	64.7564
11.7	35.1	111.5598	111.5598	76.4598	46.8	111.5598	111.5598	64.7598
11.8	35.4	111.9230	111.9230	76.5230	47.2	111.9230	111.9230	64.7230
11.9	35.7	112.2460	112.2460	76.5460	47.6	112.2460	112.2460	64.6460
12.0	36.0	112.5290	112.5290	76.5290	48.0	112.5290	112.5290	64.5290
12.1	36.3	112.7718	112.7718	76.4718	48.4	112.7718	112.7718	64.3718
12.2	36.6	112.9746	112.9746	76.3746	48.8	112.9746	112.9746	64.1746
12.3	36.9	113.1372	113.1372	76.2372	49.2	113.1372	113.1372	63.9372
12.4	37.2	113.2596	113.2596	76.0596	49.6	113.2596	113.2596	63.6596
12.5	37.5	113.3420	113.3420	75.8420	50.0	113.3420	113.3420	63.3420
12.6	37.8	113.3842	113.3842	75.5842	50.4	113.3842	113.3842	62.9842
12.7	38.1	113.3864	113.3864	75.2864	50.8	113.3864	113.3864	62.5864
12.8	38.4	113.3484	113.3484	74.9484	51.2	113.3484	113.3484	62.1484
12.9	38.7	113.2702	113.2702	74.5702	51.6	113.2702	113.2702	61.6702
13.0	39.0	113.1520	113.1520	74.1520	52.0	113.1520	113.1520	61.1520

Columns 1 (Input level) and 3 (Output level) are from the model in Figure 2.1c. Column 2 (Cost) is Column 1 times the input cost. Column 4 (Value) is Column 1 times the output value. Column 5 (ROI) is the value in Column 4 minus Column 2, etc.

2.4 Choosing an Adequate (or the Best) Model to Use

The adequacy of a given model can be determined from a chi-square goodness of fit test. For each model, a statistic $Q = \Sigma[(O_y - E_y)^2/E_y]$ is calculated where O_y is the observed response y and E_y is the expected response for y obtained from the model; this Q is compared with the table chi-square value with v degrees of freedom (where v = [number of observations] − 1 − [number of parameters estimated by the model]). The data Q_m values and the critical table χ^2_m values for several models sometimes used in nutrition research are displayed in Table 2.5. If $Q_m > \chi^2_m$, then the model is not a good fit.

For the data in Table 2.1, the linear regression model and the Robins, Norton and Baker (RNB) Model 2 model do not fit the data adequately. To choose between the remaining seven models, we look at the sum of residuals, R^2 and Q_m values. The smaller the sum of residuals and Q_m values, the better, while the larger R^2 values are better. Based on these, the quadratic regression model and the broken-line quadratic ascending line model would be eliminated. The remaining five models are all good, with little to choose between them. However, the diagnostic values for the 4-parameter logistic model and the RNB Model 1 are effectively equally adequate. A final decision could be made on the basis that having fewer parameters is usually preferable (RNB Model 1).

Table 2.4. Returns on investment (ROI) using the Saturation Kinetics Model for the data in Table 2.1.

Input level (units)	Input cost = $3/unit, Output value = $1/unit				Input cost = $4/unit, Output value = $1/unit			
	Cost ($)	Output level (units)	Value ($)	ROI ($/unit)	Cost ($)	Output level (units)	Value ($)	ROI ($/unit)
11.5	34.5	109.2088	109.2088	74.7088	46.0	109.2088	109.2088	63.2088
11.6	34.8	109.6766	109.6766	74.8766	46.4	109.6766	109.6766	63.2766
11.7	35.1	110.1266	110.1266	75.0266	46.8	110.1266	110.1266	63.3266
11.8	35.4	110.5598	110.5598	75.1598	47.2	110.5598	110.5598	63.3598
11.9	35.7	110.9768	110.9768	75.2768	47.6	110.9768	110.9768	63.3768
12.0	36.0	111.3785	111.3785	75.3785	48.0	111.3785	111.3785	63.3785
12.1	36.3	111.7654	111.7654	75.4654	48.4	111.7654	111.7654	63.3654
12.2	36.6	112.1383	112.1383	75.5383	48.8	112.1383	112.1383	63.3383
12.3	36.9	112.4978	112.4978	75.5978	49.2	112.4978	112.4978	63.2978
12.4	37.2	112.8444	112.8444	75.6444	49.6	112.8444	112.8444	63.2444
12.5	37.5	113.1787	113.1787	75.6787	50.0	113.1787	113.1787	63.1787
12.6	37.8	113.5013	113.5013	75.7013	50.4	113.5013	113.5013	63.1013
12.7	38.1	113.8126	113.8126	75.7126	50.8	113.8126	113.8126	63.0126
12.8	38.4	114.1132	114.1132	75.7132	51.2	114.1132	114.1132	62.9132
12.9	38.7	114.4034	114.4034	75.7034	51.6	114.4034	114.4034	62.8034
13.0	39.0	114.6837	114.6837	75.6837	52.0	114.6837	114.6837	62.6837

Columns 1 (Input level) and 3 (Output level) are from the model in Fig. 2.1e. Column 2 (Cost) is Column 1 times the input cost. Column 4 (Value) is Column 1 times the output value. Column 5 (ROI) is the value in Column 4 minus Column 2, etc.

Table 2.5. Comparisons for several models (detailed in Vedenov and Pesti, 2008) for fitting nutritional responses. (See also Robbins *et al.* (1979) for details of RNB models.)

Model	Parameters	Sum of residuals	R^2	Q_m	Critical χ^2
Linear Regression Model	1	15068.702	76.13%	89.969	15.507
Quadratic Regression Model	2	1652.331	97.38%	8.853	14.067
Broken-line Linear Model	3	768.439	98.78%	4.290	14.067
Broken-line Quadratic Model	3	1030.092	98.37%	8.621	14.067
Saturation Kinetics	4	609.407	99.03%	3.387	12.592
Logistics, 3 Parameters	3	616.406	99.02%	3.545	14.067
Logistics, 4 Parameters	4	534.412	99.15%	3.545	12.592
RNB, Model 1	3	534.412	99.15%	2.541	12.592
RNB, Model 2	4	2204.653	96.51%	17.837	14.067

2.5 How Much of a Good Thing is Too Much?

Another very common design for experiments with poultry asks the question, 'How much of something can be fed before a decrease in performance can be detected?' This is the objective of experiments: (i) with feed ingredients with non-nutritional factors; and (ii) with some medications when a

dose that is as high as possible, but will not affect the host, needs to be determined. The most common model to be used may be a one-way analysis of variance with a multiple-range test. Another approach is to use a mirror image of one of the models discussed above to determine inputs giving maximal responses. All the various interpretations considered for the requirement threshold models could be made. However, with the multiple-range approach, weaker, less precise experimental replication would overestimate the maximum safe feeding level prediction (Boardman and Moffit, 1971).

An example of an ingredient known for a high content of anti-nutritional factors is pennycress meal. Pennycress (*Thlaspi arvense* L.) is an annual winter plant found in North America and contains two factors (glucosinolates and erucic acid) known to cause deterioration in the performance (weight gain, egg production, etc.) of poultry. In a feeding trial (R.A. Alhotan *et al*., unpublished), pennycress was fed to broiler chicks at 0%, 5%, 10% and 15% of the diet for 18 days. Growth performance was measured and analysed as a response variable to pennycress meal level (Fig. 2.2). As discussed previously, there are several ways to define the maximum safe level of an ingredient. When Tukey's multiple range test was employed, there were no detectable differences in responses to the different levels, suggesting the maximum safe level to be at least 15%. The BLL and BLQ models estimated the maximum level to be 6.57%±0.01 and 1.87%±1.73, respectively. The philosophical approach of the person evaluating the data certainly has a large influence on what might be considered the maximum safe level.

2.6 Variation in Bird Growth and Morphology

Unfortunately, all birds do not perform the same, even when kept under identical environmental conditions. The responses of birds (or mammals, etc.), even when kept under identical conditions, are assumed to be normally distributed. 'Assumed' is the key word here. In reality, illness within a flock (or pen) may cause the distribution to be skewed to the less than average side. While subclinical or clinical illness could cause birds to perform below their genetic potential, there is nothing to cause birds to perform above their genetic potential. So while we theoretically expect the distribution to be slightly or even somewhat skewed, it is usually not possible to declare such skewedness to be significant and normality is assumed. If non-normality is proven, then the data should be adjusted through an appropriate normalizing transformation.

Table 2.6 shows the variances of individual traits within a flock. The birds were raised together in a single pen until they were 34 days old. Then they were moved to individual cages for the individual measurements and calculation of variation amongst them. When birds are housed in a pen, the genetic variance of the pen mean can be estimated by dividing the individual variance by the number of birds in the pen. Some additional pen-to-pen variation within a house is expected due to differences in the microclimates of the different pens. In our experience, this variation is small in the houses we

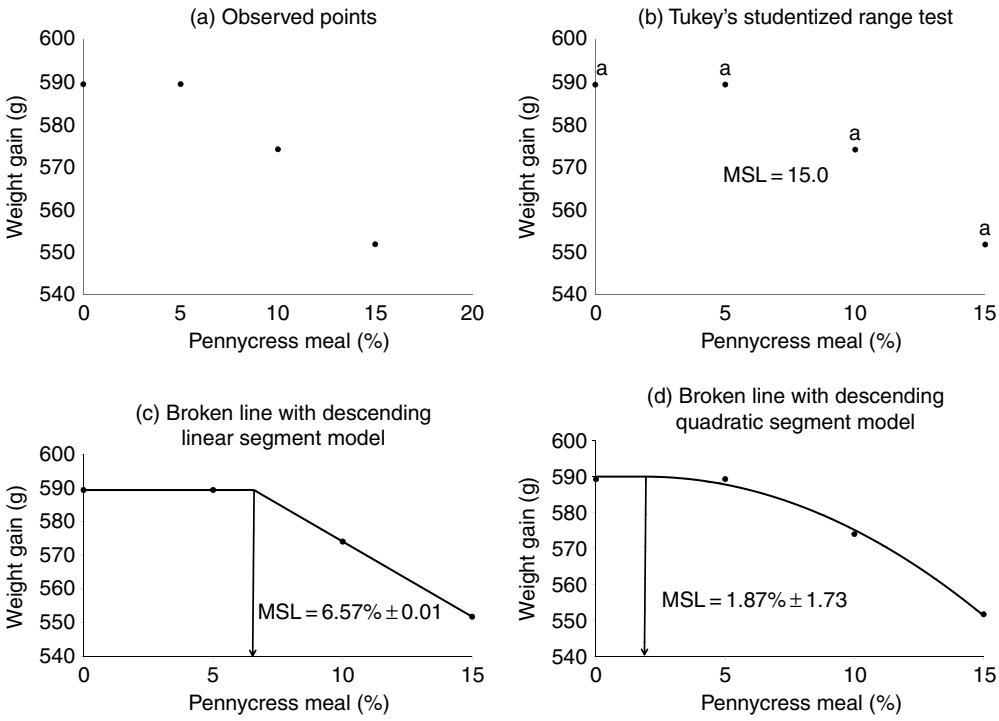

Fig. 2.2. Growth response of broiler chicks to pennycress meal as: (a) presented as points; (b) analysed with Tukey's studentized range (HSD) test; (c) broken line with descending linear segment model; and (d) broken line with descending quadratic segment mode. Maximum safe level (MSL) = mean in (b) and mean +/– SE in (c) and (d).

use, perhaps on the order of 1%. It should be beneficial to determine this value for the particular house(s) being used. Figure 2.3 shows some recent results from our experimental farm showing how standard deviations in individual and between-pen body weights increase with the age of the flock.

The usual distribution of bird body weights in a flock is not 'normal' in a statistical sense, but perhaps consists of a mixture of two normal distributions: one for males and one for females. The higher the variation in a trait, the more replication is needed to detect a desired difference in responses. This phenomenon is illustrated by determining the number of male, female and straight run (as hatched) broilers necessary to detect differences in body weight.

2.7 The Choice of an Experimental Unit

Experiments can be conducted on individual birds, meaning measurements are made on each individual randomly assigned to the treatments (Festing and Altman, 2002). Each bird has unique housing and microclimate conditions and represents one degree of freedom in the analysis of variance. Housing

Table 2.6. Performance and yields of Heritage broilers raised together on stock diets until 34 days of age. At 34 days of age they were moved to individual cages for feed intake measurements.

	Body weight			Gain/Feed			Yield				Pectoralis	
							Hot yield		Chilled yield			
	Day 34	Day 41	Day 48	Day 27–34	Day 34–41	Day 41–48	(g)	(%)	(g)	(%)	Major (g)	Minor (g)
Females												
Count	79	79	65	79	79	79	65	64	64	64	64	63
Average	1434	2011	2335	1.65	1.82	2.20	1762	72.8	1835	79	432	97
SD	89	121	146	0.17	0.17	0.22	118	1.5	119	2.1	49	10
CV	6.2	6.0	6.3	10.50	9.45	10.01	6.7	2.0	6.5	2.6	11.3	9.8
Minimum	1262	1787	2028	1.35	1.58	1.64	1531	66.8	1600	73.4	300	75
Maximum	1636	2284	2671	2.25	2.51	2.69	2040	75.4	2114	88.6	571	123
Males												
Count	81	81	71	81	81	81	71	58	58	58	58	55
Average	1590	2287	34919	0.01	0.03	0.05	23117	3.7	23453	4.0	485	102
SD	123	174	187	0.10	0.18	0.21	152	1.9	153	2.0	56	12
CV	7.7	7.6	6.9	6.38	10.40	10.81	7.5	2.7	7.3	2.6	11.6	11.8
Minimum	1310	1882	2216	1.35	1.42	1.51	1624	68.8	1680	74.2	367	73
Maximum	1845	2580	3105	1.90	2.49	2.51	2383	81.9	2451	86.7	625	124

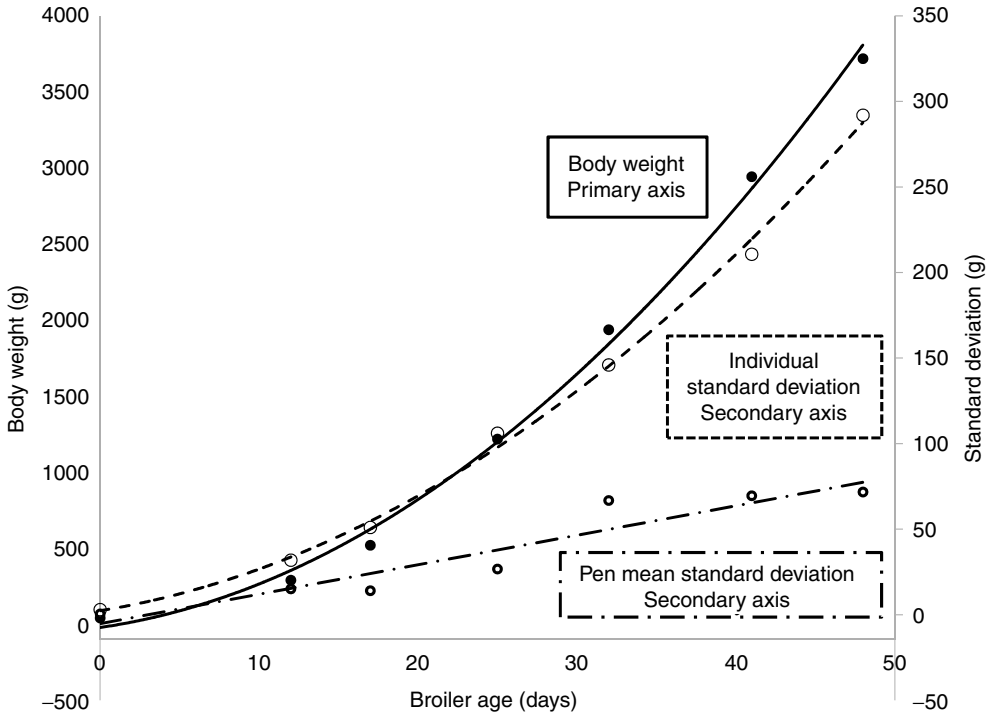

Fig. 2.3. Individual and between-pen variation in a flock of male Ross 708 broilers from an experiment with 40 broilers housed in each of 8 pens.

birds individually in production settings is rare, so using observations on individuals is not always appropriate. For instance, the heat production (metabolic rate) of individually and colony-housed birds may be different due to huddling, particularly under cool or cold conditions. Thus housing birds individually has the potential to compromise the application of results to field conditions where the birds are raised together in a house.

2.8 Experimental Power

Most researchers are primarily concerned with Type I error (α), the probability that they will declare a difference significant when none really exists (reject the null hypothesis when it is true). By tradition, the chance of declaring differences to be significant when they are not is 1 in 20, or $P<0.05$. Researchers should more often be concerned with another type of error, Type II error (β). This error occurs when something is not declared different when it really is (fail to reject the null hypothesis when it is false). Answers to typical questions that poultry scientists ask are more dependent on Type II error than Type I. Questions like 'How much of an additive can be added before there is no longer any significant increase in response?', or 'How much of an

alternative ingredient can be fed before there is no significant decrease in response?', require more powerful experiments to find differences of importance to producers. The convention is to be content with not declaring something different that really is only one out of five times, or $P > 0.80$. Unfortunately, if the chance of committing a Type I error (declaring something different when it is not) is decreased by increasing the critical probability value, the chance of committing a Type II error (not declaring a real difference) is increased for a given sample size, n. To decrease both, the sample size (n) must be increased.

Table 2.7 shows some different pen configuration possibilities for a broiler house and the number of birds of different sizes that could be used when the birds' traits have different variances. In the example, the dependent variable of interest is body weight and so body weight variation was used to determine the upper limit of differences that would be declared different 80% of the time. If an experiment's purpose was to determine differences in carcass or breast meat yield, then the variances for those traits could be used to determine the appropriate number of birds to use in an experiment.

This principle can be illustrated by the following example. If a company were producing 4090 g birds and a 50 g change in body weight per bird would

Table 2.7. Comparison of experimental power with different sizes of birds and different numbers of birds per pen and pens per treatment, based on 10,000 simulations and assuming CVs of 10% (As hatched), 6% (Male) and 7% (Female).

					Difference likely to be declared significant 80% of the time					
					Small pens (1.22 m × 1.37 m)					
		Age at market weight (days)			12 treatments with 6 pens/treatment			6 treatments with 12 pens/treatment		
Market weight (kg)	Birds per pen	As hatched	Male	Female	As hatched	Male	Female	As hatched	Male	Female
1.82	27	32	31	33	57	27	42	36	14	16
2.50	23	39	38	42	72	43	60	35	24	26
4.09	16	56	53	60	118	66	75	73	46	56
					Large pens (1.22 m × 2.74 m)					
					6 treatments with 6 pens/treatment			4 treatments with 8 pens/treatment		
Market weight (kg)	Birds per pen	As hatched	Male	Female	As hatched	Male	Female	As hatched	Male	Female
1.82	54	32	31	33	29	18	20	19	13	15
2.50	46	39	38	42	42	16	25	30	18	21
4.09	32	56	53	60	83	52	58	69	42	49

be an economic disaster, using 6 small pens per treatment with 16 birds per treatment would clearly not be sufficient, since even if all males were used, the smallest difference expected to be declared significant would be 66 g. Even using 8 large pens with 32 female birds per treatment would only give an 80% chance of declaring a real 49 g difference, if there were one. Using males would somewhat increase the chances of finding a real 50 g difference. However, such relatively small differences require large numbers of birds per replicate and replicates. Calculations like those illustrated in Fig. 2.4 are necessary to determine what an acceptable sample size for an experimental comparison could be.

Experimental power is an indication of the probability that an experiment will arrive at a proper conclusion (Zar, 1981; Baker-Bausell and Li, 2002; Aaron and Hays, 2004; Shim and Pesti, 2012; Pesti and Shim, 2012). The detectable difference is a measure of experimental power, but there are certainly no guarantees that the appropriate conclusion will be reached from one experiment. Increasing experimental costs (number of replicates per treatment and birds per replicate) merely increases the odds that appropriate conclusions can be made. Whenever the experimental objective is

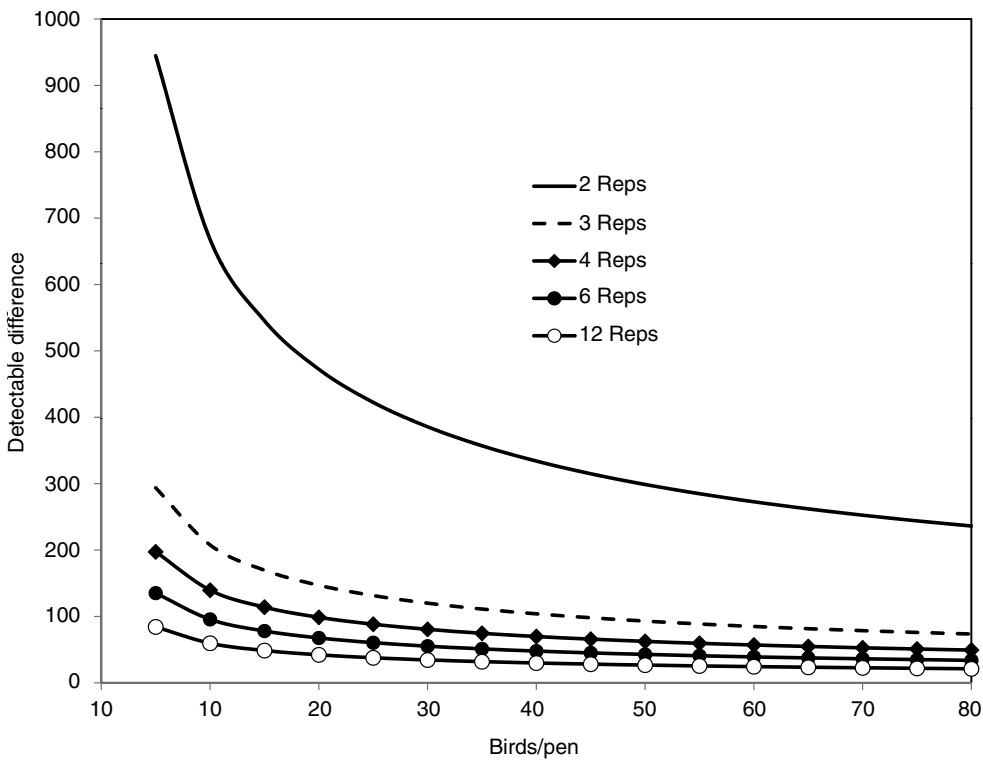

Fig. 2.4. Trade-offs between birds per pen, pens per treatment and detectable difference (80% level) when raising 2.5 kg straight-run broilers.

determining a response line, as in the example in Section 2.3, the reliability in the line is what is important and requires a much more detailed analysis.

2.9 More Complex Designs for More Complex Questions

Very often, there is more than one factor that affects the experimental results (and performance in the poultry house). When this happens, treatments are set up in a factorial arrangement and instead of obtaining a response line from the results, there is a response surface (Myers, 1971). The factors can be arranged in any combination deemed appropriate by the experimenter. The most frequent design used is a simple factorial design (Atencio *et al*., 2005a,b, 2006) as seen in the first four columns in Table 2.8. The independent variables may be things like dietary protein and energy levels, or dietary calcium and phosphorus levels, or maternal versus progeny dietary vitamin D levels as in Fig. 2.5.

The levels of independent variables may also be in a different configuration such as the Central Composite Rotatable Design (CCRD) in Fig. 2.6 and Table 2.8 (Box and Wilson, 1951; Roush et al., 1979; Liem *et al*., 2009). The CCRD is borrowed from designs for maximizing chemical syntheses and increased reaction efficiency. It would seem best suited for designs without a

Table 2.8. Experimental design possibilities for demonstrating the effects of two independent variables on responses. CCRD, Central Composite Rotatable Design.

		Design type			
		Factorial		CCRD	
Treatment	Block	x_1	x_2	x_1	x_2
1	A	–1	–1	–1.414	0
2	A	–1	0	–1	–1
3	A	–1	1	–1	1
4	A	0	–1	0	–1.414
5	A	0	0	0	0
6	A	0	1	0	1.414
7	A	1	–1	1	1
8	A	1	0	1	–1
9	A	1	1	1.414	0
1	B	–1	–1	–1.414	0
2	B	–1	0	–1	–1
3	B	–1	1	–1	1
4	B	0	–1	0	–1.414
5	B	0	0	0	0
6	B	0	1	0	1.414
7	B	1	–1	1	1
8	B	1	0	1	–1
9	B	1	1	1.414	0

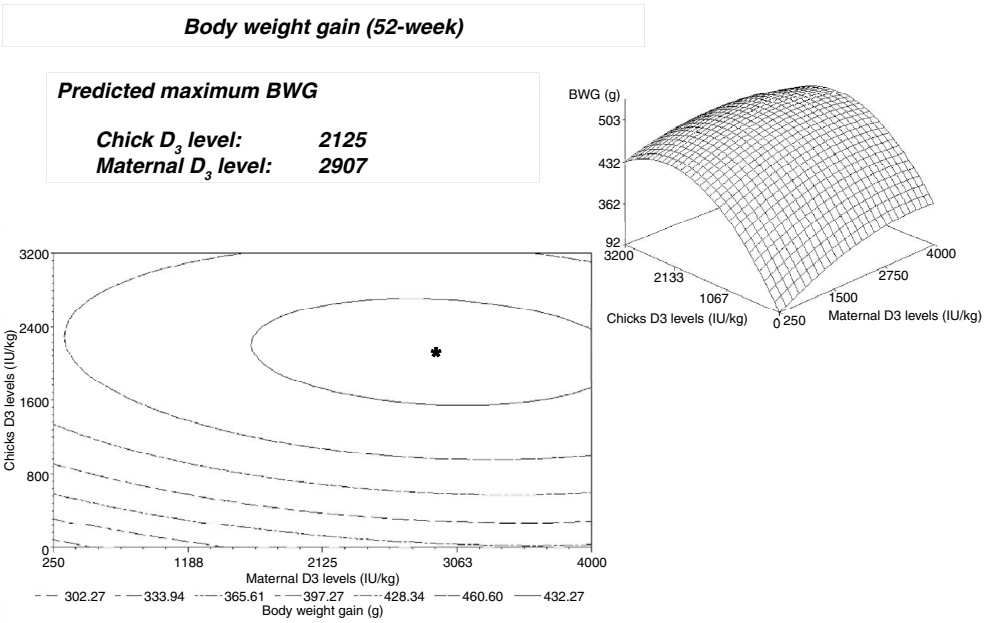

Fig. 2.5. Examples of results of a response surface experiment showing the effects of feeding 52-week-old broiler breeders, and their progeny, different levels of vitamin D.

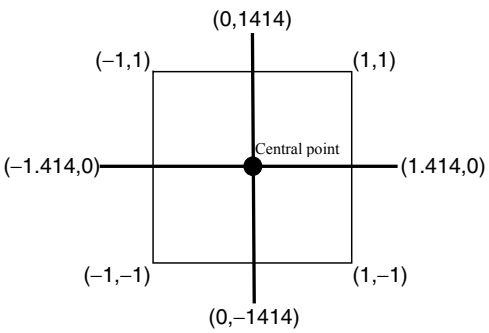

Fig. 2.6. The Central Composite Rotatable Design (CCRD) with treatments placed 'equidistant' from the central point.

plateau. Second-order polynomials reach a maximum with no feature to represent a plateau, yet higher organisms seem to exhibit one.

Sometimes there are factors that lead to variation in an experiment's outcomes that should be considered (Titus and Harshaw, 1935). In poultry experiments, these entities may be houses or rooms with individual microclimates that should be included in the analysis. If an experimental house has two rooms with different heating and ventilating systems, any differences in outcomes in the two different rooms should be removed from the total variation (subtracted from the mean square error). Or, for instance, the

experiment may be conducted in stages with differences in months removed from the total variation.

Table 2.8 shows an experiment that has been divided into blocks: A and B. Each treatment is represented in each block. If the intended outcome is to be applied to field production systems, then the appropriate blocking coefficient must be determined to relate the results to field conditions. Blocks can be any number of factors; for example, day that chemical analyses are conducted, season of the year, etc.

There are many more complex arrangements of treatments in ever more complicated experimental designs. They have descriptive names to help understand their significance: Split-Plot, Split-Split Plot, Split Block, Crossover and Latin Square designs (Cochran and Cox, 1957). They all have their usefulness, but it may be difficult to decide how to relate results to production conditions because of the blocks.

2.10 Summary

There are many possible interpretations of experimental designs, but it is the inference from statistical analyses that is really important for researchers. The researcher's goals, and especially the degree of precision deemed necessary, are particularly important when choosing how many animals should be used, should more than one be put into each pen, how many pens should be used for each treatment, etc. After these things have been chosen and the experiment conducted, the data must be properly interpreted. Dunn (1929) listed some important admonitions, which have also been interpreted as the 12 Commandments of Biostatistics. They are admonitions: counsel, advice and cautions, and they are all as important today as when they were written.

I. *Do not analyse frequency distributions whose elements are not independent.*
II. *Fix no arbitrary standard of probability as an indication of significance.*
III. *Do not make the statement that no difference exists because no significant difference can be demonstrated.*
IV. *Do not use the correlation coefficient in bi-variant data which are non-linear.*
V. *Do not interpret the scale of the correlation coefficient as a percentage scale.*
VI. *Do not confuse degree of relationship with cause and effect.*
VII. *Be sure there is no correlation between errors in the application of difference formula.*
VIII. *Do not confuse per cent of quantity with probability.*
IX. *Be sure to describe relations of variables before computing ratios or indices.*
X. *Never use chi square, χ^2, except upon frequency observations.*
XI. *Never try to explain why differences occur by any method designed purely to test the significance of differences.*
XII. *Do not abuse the application of the probable error concept.*

The most important of these may be numbers II and III. Researchers making conclusions like to have some guides to use in their decision-making

process. They often forget that the guides they use are old tables with F- or t-statistics that are relics from the days before modern computers made calculating actual probabilities easy. The values in the tables are like comfortable old crutches that make thinking about the probabilities quite simplistic. If some treatments cause 'significant' differences then it is easy to draw the conclusion that cause and effect is involved (in violation of Admonition VI). Some researchers like to assign words like 'trending', 'significant' and 'highly significant' to probabilities of 0.10, 0.05 and 0.01. Others may even object to stating that 15 is greater than 10, for example, if the variances make the difference 'not significant'. It is better just to report actual *P* values without adding subjective adjectives.

If something is improved, enhanced, increased, reduced or decreased by a particular treatment versus another, then the implied comparison is between means. The probability that one mean is different from another is an entirely different question. It begs the question of whether the observed improvement, enhancement, increase, reduction or decrease was just due to chance or not. The inference is that the increase or decrease, etc. is repeatable under identical experimental conditions (not just due to chance). In applied agriculture, probabilities should just be guides for determining if further experimentation is appropriate to quantitate differences and decide on the best, most economical conditions for food production.

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3 Practical Relevance of Test Diets

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3.1 Introduction

Most animal nutrition research belongs to applied science and as such its outcomes should be relevant to industry. This means the selection of ingredients, the nutrient specifications used for formulating the diet, the types of feed additives commonly used, the physical quality and the form of the diet should be appropriate for the age and class of the animal to which it is to be fed. Ignoring any of these factors may render the study results irrelevant to practice. However, despite the best efforts of the researcher, it is sometimes difficult to meet these criteria. When this happens, the most important parts, such as the nutrient balance of the diet, should be considered and areas that cannot be accommodated should be clearly stated and justified.

Preceding chapters detail all the basics for conducting proper nutritional experiments for monogastric animals. This chapter will focus on the production aspects of nutrition experiments, discussing how a practical diet can be formulated that will support animal performance relevant to commercial targets. Obviously there are many cases where the objective of the experiment is not to determine growth performance, even if it were possible to do so. For instance, the determination of the energy value of individual ingredients (such as vegetable protein sources or fats and oils) may require multiple inclusion levels of the same ingredients. This may make it difficult to balance all the nutrients, including the energy and protein contents of the test diets. Other examples include trials designed to measure endogenous secretions, or the presence or absence of a single nutrient. Under these circumstances the control diet will not support a commercial standard animal performance, as in a dose-response study measuring the metabolizable energy (ME) value of a protein source, where some of the

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diets will supply excessive amino acid. Again, such diets are difficult to balance and therefore it is not reasonable to expect commercially relevant animal performance. It is incumbent on the author to clearly outline the objectives of the research and to refrain from presenting performance data from such experiments unless it is of particular relevance to the trial in question.

3.2 Commercially Relevant Animal Performance

3.2.1 Indices for measuring animal performance

The term 'performance' in relation to nutrition research refers to different parameters in different classes of animals. In broiler chickens and growing pigs, it covers the growth rate (weight gain, average daily gain), feed intake, feed conversion ratio and mortality. In many countries, feed conversion ratio (or its inverse, feed conversion efficiency, FCE) is used as a performance measure because it takes into account growth, feed intake and, often, mortality. Feed conversion ratio (FCR) is calculated over a given period, for instance, week 1, as follows:

$$\text{FCR} = \frac{\text{Weekly feed intake}}{(\text{Weekly gain} + \text{Mortality})}$$

Some researchers use feed conversion efficiency:

$$\text{FCE} = \frac{(\text{Weekly gain} + \text{Mortality})}{\text{Weekly feed intake}}$$

In the poultry industry, the breeder companies typically publish performance standards based on FCR, rather than FCE, hence it is much easier for a reviewer to check your performance data against the breed standard if you present FCR rather than FCE figures.

In European countries, broiler performance is measured using the European Production Efficiency Factor (EPEF), which takes into account feed conversion, liveweight, liveability and age at depletion:

$$\text{EPEF} = \frac{\text{Liveweight (kg)} \times \text{Liveability (\%)}}{\text{Age at depletion (days)} \times \text{Feed conversion ratio}} \times 100$$

Although this is a more complicated way to measure performance, it has the advantage of giving a single figure for comparing between different flocks and with the breed standard without referring to the Performance Objectives tables of a given breed for a specific age or the weight of the dead animals.

For laying hens, 'performance' often means the cumulative number of eggs produced, measured as hen-day egg production, i.e. the number of eggs that each hen produced at a particular day since she started to lay. Under experimental conditions, such a figure is hard to obtain unless the experiment has covered the entire laying period. So laying hen performance is usually reported as hen-house egg production, that is, the percentage of hens in the house laying during the experimental period.

For growing pigs (from weaning to sale), measures of practical importance for nutritional studies are feed intake, growth rate (weight change over time) and FCR or FCE. For pig nutrition studies, there is a tendency to use FCE. This is perhaps because, unlike poultry, pigs can lose a significant amount of weight when they are moved to experimental units, especially at the time of weaning. However, there is no particular logic about it; it is just a convention that some researchers use to present their findings.

For nutritional studies in pigs, carcass weight, dressing percentage and carcass measurements such as fat thickness (P2) and loin depth are important, as payment is generally made on carcass weight and carcass lean content and/or P2 fat thickness in many countries. These parameters are all affected by nutrition and differ between genders and genotypes; thus these factors need to be taken into account when designing and analysing nutrition studies using pigs.

Nutritional studies in pigs are more complex than in poultry because many pig producers breed their own special crosses using different sires and dams. However, for modern genotypes, there are generally 'expected' levels of performance, which do not vary widely. Table 3.1 shows the levels of performance expected of modern genotypes as a general guide.

The commercial performance standards for various breeds are readily available online. The breed standards are the levels of performance that can be achieved under reasonable management and environmental conditions and when using feeding nutrient levels recommended by the breeding companies.

It is not uncommon for journals to receive nutrition-related manuscripts that present performance data well below the standard of performance expected of the relevant breeds. Of particular concern is the measurement of the efficacy of feed additives under suboptimal performance. Such studies usually compare the performance of treated animals with a control, where the control is very much below the breed standard in terms of body weight and FCR. When comparisons are made relative to such a control diet, the performance of the treatment group(s) looks impressive, especially when it is presented as percentage gained. Reviewers and prospective commercial end-users will ask the question, 'Had the animals performed to the breed standards, would the treatment have been as effective as it seems?' There cannot be a satisfactory answer to this because the production performance of animals may be influenced by numerous factors, including nutrition, environment, stress, disease and husbandry. It is difficult to determine with confidence which of these factors played a role in the suboptimal

Table 3.1. Expected levels of performance for commercial pigs.

Weight	Phase	Growth rate (g/day)	Feed:gain	Feed intake (kg/day)
6.5–25 kg	Weaners	450–550	1.4–1.8	0.63–1.0
25–55 kg	Growers	750–850	2.0–2.4	1.50–2.1
55–105 kg	Finishers	850–1100	2.6–3.0	2.20–3.1

performance of the control and how the treatment worked to alleviate one or all of the factors associated with it.

3.2.2 Presentation of animal performance results

Poultry

To make results arising from laboratory-based nutritional studies relevant to industry, it is helpful to follow commercial practice in terms of implementing changeover of diets and taking performance measurements. This is particularly relevant in the broiler and pork industries where diets are formulated based on the average feed intake for growth periods and consideration of commercially viable load sizes, rather than arbitrary division of the growth periods by weeks, e.g. 1–3, 3–6, etc. Therefore experimental designs should attempt to reflect this industry practice when possible. In broiler chicken research, chickens are slaughtered at various live weights to meet market requirements, rather than at various ages. Likewise, the periods of feeding are dictated by feed allocation, rather than by age. Some commercial producers prefer to utilize set feed allocation rather than age of change, as it both accommodates minor changes in growth rate due to variables such as environment or minor health challenges, and is a practical method for manufacture and delivery planning.

An example of some feed allocations for various growth periods for broilers may look like the following: 300–600 g starter feed; 1000–1200 g grower feed; 1500 g finisher feed; balance is withdrawer feed.

Body weight, feed intake and FCR results should be presented for the relevant periods that coincide with the amount of feed allocation, e.g. day 0–10 for starter, day 11–24 for grower, day 25–38 for finisher, and day 39–market for withdrawal. Some breed recommendations do not include a withdrawal period. Some papers present daily gain, average daily gain, and FCE for a non-standard period, e.g. day 5–35. Although a comparable value can be calculated from such data, it is not convenient and adds to the frustration of the reviewer who may be dealing with a number of requests to review manuscripts.

Swine

Pigs are grown out to different live weights (90–140 kg), with 7–8 different diets from weaning to sale. But the same performance measures are used globally regardless of the country of origin or liveweight at slaughter. For growing pigs, feed efficiency declines with weight as protein deposition (per unit energy intake) decreases and body and carcass fat increase with weight and energy intake. This is affected by the energy content of the diet, and hence many researchers present the amount of energy (digestible energy/ME/net energy) per unit of gain when it comes to expressing dietary energy. Of course, as an important economic indicator, FCE is probably the most used measure.

The purpose of this section is to stress the importance of paying attention to industry practice when possible in conducting nutritional studies. The modern breeds are very sensitive to nutrient excess or deficiency and frequent diet changes are designed to match energy and nutrient requirements as closely as possible to avoid excesses and deficiencies. Apart from some fundamental research work requiring purified or semi-purified diets to answer specific questions, for most applied nutritional studies the control diet should contain the appropriate proportions of ingredients and the levels of nutrients that support commercial standard production performance, such as growth, feed intake and feed conversion efficiency. Such a control diet is the benchmark for the study with which the effect of other treatments will be compared.

The starting point for producing a practically relevant control diet, of course, depends on an appropriate feed formulation.

3.3 Feed Formulation

The development of the feed industry is closely associated with the gross domestic product (GDP) increase across the world and the requirement of high-quality animal protein for human consumption. Over the years, the trade of feed mixing has increasingly become the science of both ingredient substitutability and physical composition. This has occurred because: (i) the progress in the better understanding of the nutritional requirements of intensively farmed animals has resulted in increasingly precise requirement tables; (ii) the nutrient composition of most common raw materials has now been well elucidated; (iii) the digestibility values for many common raw materials have been obtained across the various classes and species of animals; and (iv) there is good understanding of key factors that affect the digestibility of various nutrients in different animals. The understanding of the influence of ingredient choice and inclusion constraints on feed manufacturing processes and ration durability has deepened. Furthermore, measurements of nutrient composition and nutritive value, such as digestible amino acids, have become more standardized (Adeola, 2013). This adds to the precision of feed formulation, which, in turn, is essential for sustainable animal production, now and into the future.

Feed formulation in its essence is an economic exercise of how the nutrient requirements of the target class of animals are matched with the nutrient contents of the available raw materials, in the most cost-effective manner. However, in the context of most laboratory-based studies, researchers view feed formulation as a scientific exercise, ignoring the cost and practicality of the control diet. This is frequently justified because there are numerous examples where the hypothesis requires a narrow set of ingredients to be used in order to test either the inclusion level of one of the ingredients or the digestibility of certain nutrients; for example, experiments involving the use of semi-purified diets, or those measuring the digestible energy (DE) or ME content of a minor ingredient. On the other hand, studies aiming to examine commercially relevant problems, such as testing of nutritional feed additives

and values of alternative ingredients, perhaps should not exclude the relative price of the diet from feed formulation.

3.3.1 Nutritional considerations for feed formulation

When applicable, practical relevance of your study should start from the control diet you formulate. To produce a good control diet, there are three things you need to know as a minimum for a nutritional study: (i) the chemical composition and nutritive values of the ingredients that are available in your feed mill; (ii) the nutrient requirements of the animals you are about to use for your experiment; and (iii) the processing needs of the ingredients.

From the outset, nutritional considerations for feed formulation are straightforward. Tables for nutrient specifications for various farm animals are readily available together with the nutrient composition and energy content of ingredients. However, a number of issues need to be carefully considered. The most important aspect is to understand your ingredients.

Understanding your ingredients

For pig and poultry feed formulation, raw materials are typically grouped into major ingredients, minor ingredients and micro ingredients. Major ingredients include cereals, pulses, cereal by-products, protein meals and lipids (feed fats and oils), whereas minor ingredients cover macro minerals such as calcium, phosphorus, sodium chloride (coming from, but not necessarily limited to, limestone, phosphates, salt and sodium bicarbonate). Micro ingredients include synthetic amino acids (lysine, methionine, threonine, valine, arginine, isoleucine, leucine and others becoming available at an affordable price), vitamins, trace minerals (typically supplied in premixes), feed enzymes and any necessary medications such as anticoccidials.

Choosing the ingredients depends on either the region where the study is conducted or the target audience. However, most micro ingredients such as synthetic amino acids, vitamin and trace mineral premixes and enzymes are very similar across the world, and even major ingredients like soybean meal come from only a handful of sources (Argentina, Brazil, the USA and India). The main differences are therefore usually in the use of energy sources, such as cereals, lipids and rendered products (meat and bone meal, blood meal, poultry and feather meal).

Grains such as corn, wheat sorghum and barley, combined with legumes and oilseed meals, not only provide the bulk of energy and amino acids for monogastric animals, but are also the prime source of anti-nutritive components, which usually have significant bearing on how effectively all dietary components are utilized.

Sources of variation in the physical and chemical characteristics of grains used in monogastric animal diets include variety, seasonal growing conditions and locations, and post-harvest treatment, such as storage conditions (duration, temperature, moisture level in storage). The available energy

and protein contents of grains fed to poultry and pigs, which best represent nutritive value, may vary considerably. Some of this variation arises from the differences in a range of anti-nutritive factors such as non-starch polysaccharides (NSPs), enzyme activity, tannins, alkyl resorcinols, protease inhibitors, amylase inhibitors, phytohaemagglutinins, alkaloids, saponins, and lathyrogens. The relative importance of such factors will also differ according to the type of grain in question.

For instance, the NSP content of grains varies widely, which can affect their nutritive value for pigs (Cadogan *et al.*, 2003) and poultry (Choct and Annison, 1990). This argument is strongly supported by the fact that NSP-degrading enzymes are routinely used in monogastric diets with great success throughout the world. Numerous attempts over a long period have failed to provide unequivocal evidence that nutritive value in grains for poultry can be predicted with sufficient accuracy and precision by simple, low-cost physicochemical measurements used singly or in combination. Nevertheless, it is highly desirable to continue to explore these simple measurements in the expectation that useful statistical relationships with more complex measurements will emerge, or that simple measurements can be used to fine-tune prediction equations based on more powerful techniques such as near infra-red spectroscopy.

Finally, the nutritive value of grains for monogastric animals will be determined not only by the chemical and physical properties of grains but also by the way that these interact with the processes of ingestion, digestion, absorption and metabolism in the animal.

In reality, it is not possible to do a complete pre-characterization of the ingredients used for an experiment and nor should you try. But it is highly desirable to have a good understanding of the ingredients through visual assessments for mould, and contaminants such as weed seeds, and physical appearance, e.g. grain fill or typical colour and odour (fats and oils), and by some basic chemical analyses such as moisture, protein and another parameter essential for understanding a particular ingredient (for instance, total ash content for meat meal, starch content for cassava, and calcium for limestone). It is really common sense to apply your nutritionist knowledge carefully so that the ingredients you are about to use for your experiment will produce a control diet that is low in 'background noise' and accurately represents similar commercial diets.

All in all, it is often discouraging for a referee to read a manuscript that has a lot of work involved in its execution and contains some potentially usefully results that are clouded by the lack of characterization of the control diet, i.e. no pre-characterization of the major ingredients, nor any determined values for basic nutrients.

Nutrient requirements

As mentioned in the previous section, there is nothing more distressing than to see a massive amount of good work that is completely based on an

inadequate control diet. Unfortunately this happens very frequently due to the use of out-of-date standards or inappropriate values for nutrient requirements. Chief among these is the National Research Council (NRC) standard for poultry, *Nutrient Requirements of Poultry*, which was last updated in 1994 (NRC, 1994). It was based on excellent scientific work and was relevant to the commercial practice of the day. Indeed, the NRC poultry standard served the global poultry industry well for many years as a credible guideline. However, the updates have not kept up with the development in poultry science and poultry industry practice, hence many parts of the standard are now obsolete. Nevertheless, some researchers use NRC 1994 for poultry in their studies, which makes it difficult to compare the control with the treatment effects, as the control birds perform 20–30% below standard. Under such conditions, it is difficult to attribute any effect on animal performance to the treatment applied because, had the control treatment performed to the breed standard, such an effect may not have been apparent.

Some argue that all the birds were given the same feed, so any enhancement in growth or FCR should have been attributable to the treatment. But such an argument does not stack up, because the treatment may have made a crucial deficiency marginal, alleviating its effect on animal performance. Had the animals been fed an adequate diet, this would not have happened. Applegate and Angel (2014) eloquently discussed the needs for an update for the NRC standards for poultry.

The most sensible starting point for formulating a practically relevant diet is to refer to the standard recommended by the genetics company that is supplying the breed of animals you are about to use in your experiment. There are numerous national, breeding company and other commercial standards for nutrient requirements for farm animals. For instance, the Aviagen and Cobb Vantress companies have comprehensive sets of standards for all the poultry species they breed. Likewise, for pigs, there is an updated NRC standard (NRC, 2012), along with the updated Danish *Nutrient Requirement Standards for Pigs* (Danish Pig Research Centre, 2014) and the Pig Improvement Company's *Nutrient Specifications Manual* (PIC, 2013).

Supply of energy

Energy is the driver of all things, but in animals and humans it comes in the form of certain nutrients. In pigs and poultry, it primarily comes from starch, fat and protein with a small amount coming from NSPs. In practice, the energy level of monogastric feed is determined by economic criteria, rather than nutritional needs, but to achieve the breed standards, the energy level of feed must be properly set. There is some confusion as there are numerous terms used to describe the energy value of feed. But for poultry, the energy value of feed is expressed as metabolizable energy corrected to zero nitrogen (N) retention (AMEn or AME_n) and is often simplified to either ME or apparent ME (AME).

The ME values of feedstuffs are tabulated so that they can be used as the basis of feed formulation. By doing so, the values of different feed

ingredients are treated as if they were completely additive. This would be acceptable if the ME value were the sole characteristic of the feed. Indeed, the ME value is derived from the interaction between a feed ingredient and an animal and therefore it reflects variation arising from both the animal and the feed. In fact, it is incorrect to obtain a diet ME value by adding up the individual ME values of the feed ingredients used to make that diet. Throughout the years, much research has focused on making the ME value of an ingredient 'less variable', and hence 'more consistent' across different ages and classes of poultry. The use of a true metabolizable energy (TME) system or to apply N correction to ME values are two prime examples of such attempts. The biological relevance and the need for various corrections to the ME systems have been questioned (Vohra, 1972; Farrell *et al*., 1991).

For pigs, the energy value of feed is expressed in terms of DE, rather than ME. In the 11th revised edition of *Nutrient Requirements for Swine* (NRC, 2012), the NRC emphasizes the importance of deriving the correct energy content of feed ingredients, in particular of high-fibre raw materials, and recommends the use of net energy (NE) when possible. Although NE values cannot be directly determined for feed ingredients, the prediction equation developed by Noblet *et al*. (1994) has been widely used, as it has been proven to have good practical relevance.

In summary, for poultry, AME_n is the common expression of feed energy value whereas the pig industry uses DE. NE is becoming increasingly common in feed formulation for pig diets.

Protein and amino acids

All monogastric animal diets must contain a sufficient amount of protein and synthetic amino acids to supply both essential and non-essential amino acids for their biological functions and production. Although the term 'amino acid digestibility' is widely used and well understood, in truth there is no such thing as amino acid digestibility. There is only protein digestibility that produces the end products, amino acids, which are then absorbed or degraded by microorganisms.

Like energy values of feed, amino acid values can also be confusing for some researchers. This is because there are a number of different figures for amino acid values of feed and quite often it is difficult to tell in a database what values it contains for digestible amino acids.

Total amino acids represent the amounts of amino acids present in an ingredient as determined by chemical analysis. Since not all of these amino acids are available to the animal for absorption, due to incomplete digestion of the proteins within which they are contained, the concept of using digestible amino acid values for assessing the nutritive value of proteins is applied. The digestibility value for amino acids is specific to species and class of animals used to measure it in the first place. There are numerous terms used for digestible amino acids, including (apparent) faecal digestible amino acids, (apparent) total tract digestible amino acids, true faecal digestible amino acids, (apparent) ileal digestible amino acids, true ileal digestible amino

acids, and standardized ileal digestible amino acids (SID). In the pig industry, the term 'available amino acids' is often used because a proportion of some amino acids, such as lysine, can react with components in the digesta to become absorbable (Moughan and Rutherfurd, 2012) but not usable. Batterham (1992) defined it as 'the proportion of dietary amino acids that are digested and absorbed in a form suitable for protein synthesis'. All these descriptions relate to: (i) the parts of the gastrointestinal tract where the measurement is taken, such as in the ileum or the total tract (faecal); and (ii) whether or not the values are corrected for basal endogenous losses (apparent vs true or standardized).

In the absence of a more accurate or more practical system for presenting amino acids in feed, the SID value for amino acids has become the accepted figure for both pigs and poultry.

Ideal protein profile

The vast majority of proteins contain all 20 different amino acids as their building blocks. To meet the basic needs of an animal, some of these amino acids have to be supplied in the diet because they are not synthesized, or not synthesized rapidly enough, in the gastrointestinal tract and liver. These amino acids are known as the essential or indispensable amino acids. For pigs and poultry, there are ten essential amino acids: methionine, lysine, tryptophan, threonine, isoleucine, leucine, valine, arginine, histidine and phenylalanine.

Although a lack of any of these essential amino acids will impair animal performance, some are more crucial than others in terms of protein synthesis. These crucially important amino acids are known as the limiting amino acids (Mitchell, 1964). For poultry, methionine is the first limiting amino acid, whereas for pigs it is lysine.

To achieve the best growth and feed conversion in animals, each amino acid must be present in the diet in a unique quantity so that no amino acid is limiting or in excess. This is the concept of 'ideal protein' (Mitchell, 1964). A more correct description is 'ideal digestible essential amino acid ratios'. Ideal protein profiles are set by expressing all essential amino acids relative to lysine. There are a number of reasons for using lysine as the basis for the ratios: (i) lysine is the first limiting amino acid for pigs and the second limiting amino acid for poultry; (ii) dietary lysine is used only for protein accretion and maintenance; (iii) lysine analysis in feed ingredients is easy; (iv) reliable lysine requirement data obtained under various dietary, environmental and physiological conditions are readily available; and (v) lysine is one of the first amino acids that became available for supplementation in practical diets (Emmert and Baker, 1997).

The use of an ideal protein profile is for convenience, rather than for an overarching nutritional reason. The assumption is that the requirements of all essential amino acids change in proportion to lysine and therefore all that is required is to set the lysine requirement correctly in relation to dietary energy for feed formulation, and then calculate the ratio of the remaining amino acids to that of lysine (Baker, 2003).

Tables 3.2, 3.3 and 3.4 show a number of ideal protein profiles for pigs and poultry. The ratios for each amino acid are expressed relative to lysine on the digestible basis (SID) for swine and true digestibility (TD) for poultry (Ajinomoto Animal Nutrition Group, Tokyo).

There are recommendations available from all the breeding companies, such as Evonik, for ideal protein ratios for pigs and poultry.

In practical diet formulation, essential amino acid ratios are usually set as minimums only without maximums to limit excesses of some amino acids

Table 3.2. Ideal amino acids profile for growing-pig feeds (in % of lysine SID).

Amino acid	Piglet	Grower	Finisher
Lysine	100	100	100
Methionine + cysteine	60	60	60
Threonine	65	67	68
Tryptophan	22	20	19
Valine	70	>65	>65
Isoleucine	53	53	53
Leucine	100	100	100
Histidine	32	32	32
Phenylalanine + tyrosine	95	95	95
Arginine	42	42	42

Table 3.3. Ideal amino acids profile for sows feeds (in % of lysine SID).

Amino acid	Lactating sow
Lysine	100
Methionine + cysteine	60
Threonine	>70
Tryptophan	24
Valine	85
Isoleucine	55
Arginine	42

Table 3.4. Ideal amino acids profile for poultry feeds (in % of lysine TD).

Amino acid	Broiler chicken and turkey[a]	Layer
Lysine	100	100
Methionine + cysteine	75	85
Threonine	65	70
Valine	80	90
Isoleucine	67	80
Arginine	105	110
Tryptophan	17	24
Histidine	40	
Leucine	105	
Phenylalanine + tyrosine	105	

[a]Values for turkey come from those for broiler chicken; some variations are possible.

(e.g. leucine). Cysteine requirement can be, and often is, met by synthesis from methionine in practical diets. Methionine is not actually converted 1:1 to cysteine but is usually assumed to be in practical formulation.

The next step for amino acid nutrition for monogastric animals is to explore the roles played by the 'non-essential' amino acids in protein synthesis, immunity and animal performance. The concept of conditional or semi-essential amino acids has already been investigated (Moran, 2011). These refer to amino acids that can be synthesized in the gastrointestinal tract and liver of animals but may become limiting under some circumstances. As more amino acids are produced in crystalline-free form, future feed formulation will no doubt include some of the amino acids that are currently deemed non-essential in the ideal protein profile.

The booklet *Amino Acids in Animal Nutrition* (FEFANA, 2014) provides comprehensive information on all aspects of animal acid nutrition and their practical use.

Fibre

Feed formulation does not pay due attention to the importance of fibre in monogastric animal nutrition. There are several reasons for this. First, most nutritionists do not understand fibre and regard it almost like a filler in feed formulation. This is not surprising, because they know that crude fibre (CF) values used in most databases are not accurate. Indeed, CF values represent variable proportions of lignin and cellulose. The true fibre should be measured as the sum of NSPs and lignin. CF only accounts for approximately 25% of the true fibre in cereal grains and for less than 15% of it in vegetable protein sources such as soybean meal (Choct, 2015). Second, there is no NSP database for feed formulation, and any momentum for replacing CF with NSPs encounters resistance, as some countries require CF for feed labelling, and some countries use CF as a criterion for trading feed ingredients. Third, the advent of feed enzyme technology has led to the view that, since the negative impact of soluble NSPs is taken care of by the use of appropriate enzymes, the emerging issues with fibre are no longer relevant in feed formulation.

Of course, the reality is that databases that ignore 15–30% of components for the most important raw materials are used to produce the world's ever increasing volume of animal feed. The viability of such an approach should be questioned.

Fats

Most energy in dietary lipids comes from triglycerides; and feed fats are oils that are typically 90–95% triglyceride. The non-triglyceride components include free fatty acids, mono- and diglycerides and 'MIU' (moisture, insoluble impurities and unsaponifiable matter). Triglycerides must be hydrolysed by lipase to free fatty acids (FFA) and monoglycerides for absorption and metabolism to yield ME and DE. Emulsification and micelle formulation are necessary for solubilization of long-chain fatty acids (>C14) before lipase

hydrolysis can occur. The main factors that determine ME and DE of fats and oils are animal age, free fatty acid content, fatty acid saturation (unsaturated:saturated ratio, U:S) and fatty acid chain length. In general, young animals require diets with lower ME and DE levels, higher FFA and lower U:S ratios with chain lengths below C14.

In addition to their contribution to energy, some fatty acids are required for specific functions in monogastric animals. For instance, linoleic acid (omega-6) is an essential fatty acid and is usually included at around 1% in poultry diets. Despite much in-depth work showing that total lipids rather than linoleic acid per se has an effect on egg size (Whitehead, 1981; Grobas *et al*., 1999), some people still formulate with more than 1% linoleic acid in order to stimulate egg weight, often with increased feed cost.

Minerals

MAJOR MINERALS Significant new understanding is occurring in mineral nutrition for monogastric animals. The first major development is in relation to phosphorus (P). With the recognition of the poor availability of phytate P for monogastric animals, feed formulation moved away from total P to available P (aP). Available P, often interchangeably used with non-phytate phosphorus (NPP), refers to the potential P utilization relative to a reference P source, which is deemed to be 100% available. It does not refer to P that is absorbed and made available to meet the animal's requirements. Thus, an increasing body of research argues for using values that represent the actual amount of P retained by the animal. Leske and Coon (2002) stated, 'feed phosphorus values determined by retention assays that are dependent upon measuring excreta phosphorus should be described as a percentage retainable phosphorus instead of digestible or available phosphorus'. The point is that phytate phosphorus is not 100% unavailable to monogastrics and non-phytate phosphorus is not 100% available. There are physiological, dietary and nutritional factors that impact P availability. However, for practical feed formulation, the aP values are used, though this situation may change in the near future.

The other side of the discussion is related to dietary calcium (Ca). It is obvious that Ca coming from all plant ingredients is not 100% digestible. In fact, digestibility figures of 20–30% for Ca contained in corn and soybean meal and 60–70% for that contained in limestone have been quoted (Angel, 2013). With the use of enzymes, such as phytase and xylanase, as well as the reduction in the level of Ca used, the 'safety margin' for dietary Ca supply is narrowing and hence more accurate figures for dietary Ca are required (Angel, 2013). This accuracy can only come from having values on the actual amount of Ca available for absorption in the gastrointestinal tract. So the scenario in the future is an overall low level of P and Ca in monogastric diets, with a better defined aP (or digestible P) and possibly digestible Ca (dCa) values.

TRACE MINERALS Traditionally, most commercial nutritionists did not mess with the trace mineral contents of their databases for feed formulation. The reason was that trace minerals were included in the premixes, which needed to be included at certain rates to satisfy the trace mineral requirements. In recent years, trace minerals have become a hot topic, firstly because companies started to produce chelated minerals, known as organic minerals, and secondly there was due recognition that trace minerals affect numerous crucial biological functions at minute levels of inclusion.

VITAMINS Vitamins were traditionally included with premixes, but some vitamins are now used at different levels depending on the production environment or stage of the animal. For instance, the vitamin E level can be set at higher than normal for animals under increased oxidative stress and to increase shelf life of meat; likewise, vitamin C may be added to the drinking water and feed in a stabilized form, to reduce heat stress.

NUTRACEUTICALS Nutraceuticals, such as enzymes, prebiotics, probiotics, symbiotics, herbs, spices, essential oils, organic acids and phytobiotics, are often treatments themselves in nutritional studies. In addition, some of these additives have become a normal part of commercial feed, so there is no problem in including them in the control diet as long as they do not compound the effect of treatments that the experiment is aiming to demonstrate.

3.3.2 Health considerations for feed formulation

Satisfying the nutrient requirements of an animal is only part of producing a practical diet. There are numerous considerations that are not strictly nutritional in the traditional sense. Medications, preservatives (mould inhibitors, for instance) and antioxidants are all related to preventive measures to ensure that the formulated feed is safe and devoid of harmful metabolites forming during or after the manufacture. Many of them also have other actions, such as antimicrobial and immune enhancement.

Medications

The use of medications is a complex area of feed formulation. It is dictated by the health situation of the flock or herd as well as by the policies of countries and regions. Fortunately, under experimental conditions, the environment is clean and the health and husbandry of the animals are closely monitored. This leads to minimal health issues, avoiding the use of most medications required under commercial situations. But nutritional experiments often involve the use of commercial feed. Therefore, medications must be looked at very carefully because most medications are antimicrobials, such as antibiotics and anticoccidials, which can affect the experimental outcome.

Preservatives

Whatever the storage condition of your feed, there will be a degree of deterioration in the nutritive quality of feed over time. Commercial feed formulation uses a number of preservatives to prevent formulated feed from spoilage. The commonly used additives in this regard are mould inhibitors and antioxidants.

Mould inhibitors are used to prevent feed from becoming mouldy. Moulds are live fungi that grow on feed and feed ingredients, especially when the moisture level and temperature are suitable. Unfortunately, many fungi produce metabolites that are toxic to animals. Aflatoxins are such metabolites of fungi. The list of preservatives used for food is vaster than it is for feed. Most consist of acids, reducing agents and salts. Many of the acids come as acidifiers, which are used to control microbial contamination of the feed through inhibition of their growth and are also known to have antimicrobial effects within the gastrointestinal tract of animals. Microbial contamination is not just an animal health issue; it often leads to food safety problems, such as *Campylobacter*, *Salmonella* and *Escherichia coli* infections in humans.

Antioxidants

Oxidation is a major issue for feed and feed ingredients, especially under hot and humid conditions. Rancidity of fats, destruction of certain vitamins (A, D and E, for instance), loss of pigments and spoilage of amino acids are some of the consequences of oxidative damage caused by free radicals. This reduces the nutritive value of feed and leads to major economic losses. From an experimental point of view, despite meticulous attention to details in analysing the macro-nutrient composition of the ingredients and in using the most up-to-date specifications for the formulation, the resulting diet can still lead to poor performance, making interpretation of experimental results very difficult.

Antioxidants straddle a number of areas of feed formulation. For example, many antioxidants are also micronutrients, such as vitamins and selenium.

3.3.3 Processing considerations for feed formulation

Non-nutritive additives

Compound feed for monogastric animals comes in pellets, mash, crumbles, whole grain and liquid. Each type of feed has its unique set of processing requirements and the feed produced will have to be palatable to the animals for whom it is intended.

For broiler chickens, most countries feed a starter crumble diet, followed by pellets. For laying hens, most countries use mash feed. For pigs, both pellets and mash are used, but the ratio of the two forms varies from country to country and region to region. In some countries, liquid feeding is also practised alongside pellet and mash diets.

Pellet binders

For pelleted feed, the integrity of the pellets is important because it determines the quality as well as the advantage of pellets. If pelleted feed contains too many loose particles (i.e. fines), nutrient separation can occur, leading to an unbalanced nutrient intake. This is more of a problem for poultry species, particularly 'shovel feeders' like ducks and geese. There are many factors that affect pellet quality, including starch gelatinization, grind size, steam quality, pellet press, oil content, mineral content and the type of grains used. Table 3.5 shows the intrinsic characteristics of common grains that affect pellet quality. Purely from a feed formulation point of view, poor pellet quality is more frequently seen in diets containing a high level of corn, sorghum, millet or cassava than in diets containing wheat, for instance. It appears that a higher gluten content, the presence of soluble NSP and controlling the oil level all help pellet quality. However, it is not always feasible to control these factors. Thus, for some diets, pellet binders, many of which are typically clays or blends of clays, may be added to improve pellet quality.

Flavours/sweeteners

The sensory attributes of feed are extremely important for pigs, because pigs can detect the minutest amount of flavours and odours. For instance, weaner pigs would refuse to eat feed that contains albus lupin because of its alkaloid content, which many precision analytical instruments have difficulty detecting. So it is necessary to include flavouring agents or sweeteners in pig feed.

Pigments

The preference for yolk and meat colour differs depending on the country. Colour requirements for egg yolk and skins of broiler chickens vary widely between countries and even regions within a country. Some countries want egg yolk golden-yellow to orange-red, whereas others are happy with pale yellow yolk. Similarly, some countries like chickens with yellow skins, whereas others prefer broilers with white skins. There is a natural origin to these preferences: countries where yellow corn is the main cereal source are used to seeing yellow broilers, while those where wheat and barley are used

Table 3.5. Physical and chemical characteristics of cereal grains (Rogel, 1985).

Origin	Starch (% DM)	Amylose content (%)	Granule size (nm)	Gelatinization temp. (°C)
Maize	75	28	2–30	62–72
Waxy maize	75	0.8	4–28	63–72
Hi-maize	74	52	4–22	67
Sorghum	68	–	3–27	68–78
Rice	80	18.5	–	68–78
Wheat	65	26	3–35	58–64
Rye	60	–	–	57–70
Barley	55	22	2-40	52–60

as the main cereals have grown to like white broilers. However, due to clever marketing, various market segments are appearing in many countries that are not in line with the availability of the locally grown raw materials. One example is the 'corn-fed' chicken in countries where corn is not used as a poultry feed. To meet such requirements, pigments are added to layer feed in particular, but also to other poultry feed, to satisfy different markets.

Pigments are xanthophylls like zeaxanthin and lutein, which impart orange-red and yellow colours, respectively. To achieve a desired colour of the yolk or the skin, an appropriate ratio of the two is needed. Xanthophylls come in synthetic form or from natural sources. Yellow maize, marigold, alfalfa meal and capsicum are great sources of natural pigments.

3.4 Summary

In commercial operations, pressing the 'Formulate' button is not an irreversible process once activated. Professional commercial nutritionists may review a series of formulation iterations before finally settling on a suitable version for production. Once submitted to the feed mill the end product is produced. At this point, the formulation is no longer reversible.

The final version of the diet must progress through the milling process without causing problems to the machinery or the work flow, producing a product that is uniform, has the desired moisture level, and has the 'look and feel' expected of it. Finally, the most important test is when it is presented to the animals that are intended to consume it, they do and they perform as expected.

This is why commercial (i.e. profitable) feed formulation requires an experienced nutritionist, rather than anyone who can operate a software program and enter ingredient and price data. A commercial nutritionist once said to me, 'When 100 thousand tonnes of feed are produced, no one can unformulate it; at the end, the animals you feed will never lie to you.' So, understanding your ingredients, knowing your target species for your formulation and grasping the processing needs of your feed mills will make your job much easier. Of course, pre-characterization of your ingredients and repeated validation of your test diets will minimize the risk of producing a dud diet. With technologies such as the near-infrared spectroscopy (NIRS), pre-characterization of ingredients has become a lot easier.

All in all, feed does not work in isolation from good husbandry and environmental conditions. It also relies on the most important nutrient of all: water. Without a proper supply of good quality water, nothing else will give you a commercially viable animal performance.

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4 Characterization of the Experimental Diets

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4.1 Introduction

One of the key tenets of the scientific method is the ability for experiments to be reproduced (Blow, 2014). To allow for reproduction, experimental methods, published or presented, must be described in such a way that every stage can be carried out by an independent laboratory (see Chapter 8). The intricate detail of an experimental diet is no exception, as this is likely to impact the outcome greatly and will form the basis of any experimental treatment in a feeding experiment. Clarity is important, not only for scientific rigour in the community, but also to enable the reader to interpret the results and fully understand the experiment. It also follows that the justification for the choice of diet or ingredients should be clear. A literature review is usually performed at the conception of an idea for an experimental study (Johnson and Besselsen, 2002). In order to maximize the likelihood of a successful outcome, scientists need to be able to interpret the literature that went before. Equally, readers wanting to apply the results of that research should be able to identify clearly how such studies relate to their individual 'real life' circumstances. In line with these ideas, Hooijmans *et al*. (2010) proposed a checklist of items that must be included in all animal studies, to enable meta-analyses to follow in the future. Readers are directed towards this publication as well as Chapter 5 as they provide a useful summary of valuable attributes of a study that should be captured.

The aim of this chapter is to discuss some potential pitfalls when designing and characterizing experimental diets and ideas on how to limit their impact on the experiment. It will also touch on the importance of recording such design information. Statistical aspects of experimental design are covered in detail in Chapter 2.

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 Nutrition Experiments in Pigs and Poultry: A Practical Guide (eds M.R. Bedford *et al*.)

4.2 Designing Diets: the Semi-synthetic Conundrum?

When we design an animal feeding experiment, such as one designed to test the effect of a novel feed ingredient, we might intend that the diet we are using as the basis for our ration is as 'normal' as possible. What do we mean by that? Perhaps that it is commercially representative and utilizes commonly used ingredients (Chapter 3). Of course, this will vary depending on, and perhaps being limited by, the region where the study is performed, and we must consider the applicability of the results in that context. For example, if we are testing a new carbohydrase product, the results will be most applicable to diets containing the ingredients that we have used. If we carry out a study in North America using a locally designed corn-based diet, the results will be most relevant there, as most North American commercial diets are corn-based, compared with a Northern European study where the diet will be based on wheat. Our biggest assumption, however, is almost certainly that we are testing our new ingredient on a digestive tract that is healthy and uncompromised by our experimental diet and management practices. Sometimes we are confined in the selection of our ingredients by the aims of our experiment. In the case of an amino acid digestibility study (discussed in detail in Chapter 5), we may need our test ingredient to be the only source of amino acids. This therefore leads us to a problem: what should be used to make up the remainder of the diet? Often, we intend to make this as uncomplicated as possible by using purified ingredients, very much in the model of rodent studies. We make the assumption that these are entirely neutral in their physiological effects on the tract. However, often these have not been well tested and their effects are not well understood. It is suggested that uncharacterized ingredients are not used, where possible, or their inclusion minimized. If this is not possible, consideration must be made when interpreting results. These purified or non-standard ingredients tend to fall into one of four ingredients: sugars and starch, fibre and non-food ingredients. Some considerations and implications of these materials are described with particular emphasis on the purified ingredients as opposed to those inherent in 'normal' ingredients.

4.2.1 Sugars and starch

It is commonly assumed that the use of certain carbohydrates is appropriate in experimental diets as they, or related compounds, would be found in the common cereals and other raw materials. For example, purified starch and dextrose are common in experimental diets; native and/or cooked starch is clearly a key component of all cereals used in animal diets. The assumption is that they are rapidly digested and absorbed, provide energy and therefore do not themselves have an impact upon our experiments. There is, however, little validating evidence of this in the literature. In fact, Bell *et al*. (1950) and Becker *et al*. (1955) published evidence of severe gastrointestinal problems in swine fed high levels of glucose. The latter concluded that this ingredient

should only be used at low levels. Despite this interesting finding, published in a prominent journal, neither of these papers was widely cited nor was any follow-up work completed. Recent work (Masey O'Neill *et al*., 2014) suggested that when a purified glucose-containing diet and a more 'standard' formulation were compared in digestibility studies in poultry, it seemed clear that the former compromised the digestibility results obtained, suggesting some detrimental effect of the sugar. This is supported by work of Liu *et al*. (2014), who demonstrated that in a diet based on canola (but not corn), the apparent total tract digestibility (ATTD) of phosphorus (P) was dramatically reduced in a semi-purified versus 'standard' (low sucrose and starch) diet. Since canola contains almost three times as much P as corn, the levels required to achieve the same total dietary P levels are much lower. This necessitates the use of much more of the semi-purified portion of the diet in the canola diets. The anomalous results obtained with the canola diets may therefore be as much to do with the 'inert' semi-synthetic part of the diet, rather than the more obvious potential problems of the fibre and other anti-nutrients. Furthermore, Adeola and Ileji (2009) suggested that there was an interaction between diet type (semi-synthetic versus practical diets) and the amount of a test ingredient that was used (in this case distillers dried grains with solubles) on metabolizable energy (ME) measurements. Their experiment used a factorial design of increasing test ingredient and semi-synthetic and practical diets. This finding is of concern as it suggests that the nutritive value of the test ingredients will vary depending on the background diet.

Kong and Adeola (2013) described increased endogenous losses with high dextrose diets. This would result in an underestimation of digestibility, if that were the purpose of the experiment, and may indicate irritation of the intestinal epithelium. Manneewan and Yamauchi (2004) suggested that semi-purified diets containing purified starch reduced villus height relative to a formulated diet containing no purified ingredients, suggesting a very different intestinal response to a purified versus practical type diet. The validity of digestibility data generated under such test conditions for application in more practical type diets could be questioned as a result. More fundamental effects of 'purified' ingredients are also apparent, as Shastak *et al*. (2014) showed decreased feed intake with high starch diets, although P retention was increased. Perryman *et al*. (personal communication) have recently shown that with increased dextrose in the diet, titanium (as an inert marker) is dramatically increased in concentration in the proximal tract because the dextrose is so rapidly dissolved and absorbed, leaving very little undigested material to dilute the marker or indeed interact with the gastrointestinal tract. This immediately causes difficulty in assaying upper digestive tract digestibility of nutrients using the marker method and high dextrose diets.

It is also logical that the way in which carbohydrates (and their constituent monosaccharides) are provided to an animal has a dramatic impact upon the glycaemic response. If the same amount of sugar is supplied as starch as opposed to sucrose, there is a huge difference in immediate post-prandial blood glucose and insulin levels in rats, for example (Wright *et al*., 1983), which may affect feeding behaviour and metabolism. The composition of the

diet may also have an impact on pancreatic secretions. Macronutrients, trace elements, fibre and the physical state of the diet affect secretions from the pancreas (Corring *et al*., 1989). Amylase secretion increases with increased starch concentration in the diet (Noirot *et al*., 1981), which may well be a driver of improved starch digestibility. This should be considered, especially if the diets vary in starch level. However, Partridge *et al*. (1982) suggested that, with more purified starch present in the diet, amylase and lipase secretions fall, as does total pancreatic output, suggesting that the response to native and purified starch may differ. They also indicate that mineral ion output is affected, suggesting that conditions in the lumen and absorption of nutrients may be affected.

Palatability may also be affected by diets containing purified sugars or starch. Mutucumarana *et al.* (2014) reported a dramatic decrease in feed intake in broilers when the test ingredient (corn or canola) was replaced by dextrose, which resulted in a decrease in body weight. This is supported by Shastak *et al*. (2014), who reported feed refusal of such diets and decreased overall intake of semi-synthetic diet treatment groups.

In summary, when considering using purified carbohydrates such as those described above as a dietary filler, the type (whether purified starch or monosaccharides), the form and the amount should be carefully thought out and justified. The 'filler' is assumed to be inert but there is clear evidence that this is not the case if too much is utilized. When such problems are anticipated, alternatives should be sought.

4.2.2 Fibre

One of the greatest concerns when providing non-standard feed ingredients is the impact that such ingredients will have on feed intake (FI). This is particularly true for fibrous ingredients and is troublesome, as changes in FI may impact digestibility and growth measurements.

Son and Kim (2015) hypothesized that phosphorus digestibility would be affected by the provision of fibre and designed an experiment with increasing levels of purified cellulose. There was a linear increase of FI, faecal output and faecal phosphorus with incremental cellulose levels in pigs. Presumably this is caused by changes in palatability and passage rate. Similarly, Van der Klis and Van Voorst (1993) demonstrated dramatic linear decreases in FI, BW and increases in water intake with increases in carboxymethyl cellulose, even at levels as low as 2%. Carboxymethyl cellulose is a highly viscous fibre and thus will dramatically slow passage rate, decreasing intake. Latshaw (2008) described similar results in broilers: that increasing total fibres (by way of alfalfa, wheat middlings and oats) was likely to decrease feed intake and therefore the amount of ME intake. However, type and concentration of fibre is crucial.

It is well known that fibre has two faces: the soluble fraction can be antinutritive in pigs and poultry (Choct, 2015) and the insoluble fraction can be highly beneficial for gut development in poultry. For instance, insoluble fibre

may have a considerable impact on digestibility of nutrients, through an *increase* in passage rate and feed intake (Hetland *et al*., 2004) and most likely modulated by changes in gizzard development (Mateos *et al*., 2012). Likewise, oat hulls increase the relative size of the gizzard, increasing its grinding capacity (Jiménez-Moreno *et al*., 2010). This is thought to be due to the hardness and physical structure of oat hulls relative to, say, cellulose, which has less structure and less water-holding capacity and does not exert the same effect (Jiménez-Moreno *et al*., 2010). Many of the polysaccharides included in this group are present in high concentration in cereal cell walls. Therefore, choice of cereal in the basal diet is impactful and should not vary in type or amount between the test diet and the basal diet. This topic is thoroughly reviewed by Hetland *et al*. (2004) and more recently by others such as Mateos *et al*. (2012).

Therefore, fibre should not be considered inert. If a high fibre ingredient is the test ingredient of interest or is used as a filler, it should be noted that it may be impossible to unravel whether it is the ingredient itself or the change in fibre that influences digestibility. To prevent such problems, direct comparisons should only be drawn with diets of equivalent fibre level.

4.2.3 Non-feed ingredients and phytate

Like fibre, the use of non-feed ingredients may also influence experimental outcomes. For example, sand has been shown to dramatically increase the true metabolizable energy (TME) of a test ingredient (Nam *et al*., 1998) and other beneficial effects have also been suggested (Farjo *et al*., 1986). These are clearly not routine ingredients found in commercial diets. However, at low levels of 50 g/kg or less, Sellers *et al*. (1980) found few dramatic impacts of various clays on broiler performance. Intake was affected, but this did not impact body weight or feed conversion. Although FI may affect digestibility in such a case, there was no reported effect on passage rate, which would potentially be one of the mechanisms for changes in digestibility. As described, some hard, insoluble fibres may act as grinding agents and therefore they are not inert, as they may be assumed to be. This may also be the case with some non-food fillers.

When carrying out a digestibility experiment, often the test ingredient is included at high levels, probably higher than it would be in a 'standard' diet. This may skew the results for several reasons, none less so than it may have inherent anti-nutritional factors that maybe impact the results obtained. This could clearly be the case with water-soluble non-starch polysaccharides (NSPs) which increase viscosity and may increase excreta moisture and decrease digestibility of nutrients (Choct and Annison, 1992). In a digestibility assay, wheat, for instance, should not exceed 750 g/kg of diet for the above reason. The question then arises as to whether it is appropriate to use an NSP-degrading enzyme in experimental diets (where the enzyme is not the test ingredient). This can be argued in two ways. In some parts of the world, enzyme use is ubiquitous and testing a novel ingredient without the

expected enzyme in the basal diet would not be representative of common practice. Conversely, if various varieties of a cereal are being compared, for example, the use of an enzyme may dull differences between them. Furthermore, if the test ingredient is likely to have a mechanism that overlaps that of the enzyme (for example, an antimicrobial agent and an NSP-degrading enzyme), would the use of an enzyme affect the outcome of the study? In that case, it may be beneficial to carry out a factorial experiment to test the effect of the antimicrobial agent both with and without an enzyme.

Phytate presents a similar problem as it is well known as a mucosa irritant. This could present an issue if the bulking agent is a plant material. Liu and Ru (2010) showed that a high exogenous phytate diet increased endogenous loss of amino acids and therefore would decrease the apparent digestibility of such amino acids. This could be ameliorated with the use of a phytase, and this could be considered in such digestibility assays. However, in experiments designed to test growth, the same arguments arise as with NSP-degrading enzymes. Whether a phytase is included or not, the conclusion of the experiment must be drawn with that mind.

Of course, there are other ingredients that may be present in a basal formulation of a semi-synthetic diet, such as prebiotics (added for that reason or inherent to the other ingredients), probiotics, palatability agents, coccidiostats, mycotoxin binders or pellet binders. The impact of these should be carefully considered, especially where they will vary in concentration against the test diet. Many of the above interact with one another. For example, prebiotics, coccidiostats and probiotics all work on the same axis, i.e. microbiome manipulation, and, as a result, the presence or absence of these additives in the test relative to industry practice needs to be taken into account when interpreting the results for commercial use.

The use of such ingredients is clearly unavoidable in some circumstances. However, their use and quantities should be carefully considered so that a meaningful comparison can be made between the control and the test diets. Certainly, any results achieved should be considered in the context of their use; and careful research into alternatives for bulking experimental diets is probably justified. When comparing published values for ingredient nutritional value, the method and diet used should be considered.

4.3 Designing Diets: Describing Test Ingredients and an Appropriate Basal Diet

In conjunction with a well-designed basal diet, it is important to consider the way in which the test ingredient is added or the modification made to the diet, to form the experimental treatment. Two examples will be given here to describe these ideas: (i) trial design to compare one enzyme (or other additive) with a control; and (ii) trial design to compare two different enzyme (or other additive) products. Furthermore, processing and diet form are important considerations and will be covered in Chapter 3.

4.3.1 Trial design to compare one additive with a control

When testing the efficacy of an additive, for example a feed enzyme, it is logical to test that additive against at least one control. There is a multitude of factors inherent to experiments involving animals that may influence the outcome, such as genetics, age, environment, etc. It is important to be able to discount these as having influenced the results. As such, we can design our experiment with a simple control treatment that has 'no additive' and for which all other parameters are equal. In simplest terms, we would like to have one basal diet that varies between the control and the treatment only by the addition of the additive such as an enzyme. However, in practice, including an enzyme means including the test additive into the basal diet at the expense of a basal ingredient. We could consider, therefore, that the control is no longer a control as we have made two changes: first, removing a portion of the basal diet; and second, including the additive. In the case of a difference between control and treatment, how can the true reason for that effect be determined? In many cases we can realistically ignore change in basal diet proportion, as the inclusion rate of the additive is so small that the removal of other components is negligible. In the case of an enzyme, for example, the amount may be only 0.01% and will be within the bounds of normal manufacture error. However, in this case it is probably wise to include the additive at the expense of a major ingredient, such as the main cereal, rather than using a 'filler' in the control diet (which is then removed for the test ingredient) to avoid the issues stated above.

In some cases we would like to include two controls. One would be the positive control (PC), which would be considered the optimum diet for the animal during the growth phase investigated; and the other would be the negative control (NC), which takes out the nutrients that the additive is expected to release. The performance of the NC is therefore expected to be significantly worse than that of the PC and the addition of the additive to the NC is expected to return performance to the PC. In the process of making the NC, we may make radical changes to a diet. When such a change may be justified, will it influence interpretation? For example, consider an enzyme product whereby the hypothesized effect is to improve energy availability of the diet and the recommended application of that product is to use it in a mildly energy-compromised diet. To evaluate this we may want a PC that is formulated to a standard energy level and an NC that is formulated to have less energy. We are intending to do several things here: (i) test that 'removal' of energy has a negative impact on our outcome (say, growth) by comparing the PC and NC; and (ii) evaluate whether the enzyme product can regain that lost outcome (by comparing the treatment to both the PC and the NC, separately).

However, this leads to the question as to what is an appropriate method to remove energy from a formulation. There are several different methods by which this can be done and we are assuming that the energy provided by the enzyme is equivalent to the energy provided by ingredients added to the PC diet. This is probably not the case. Say, for example, that we choose to remove

fat from the formulation to create the NC because, being energy dense, removing fat would cause the smallest change in the ingredient composition of the formulation. However, fat has characteristics that may be beneficial to the growth of the animal, beyond providing energy; for example, improving feed physical quality or altering passage rate. Therefore, we are actually considering whether the enzyme can recover two effects when we compare the treatment to the PC: both the removal of fat and the removal of energy. We can somewhat mitigate this by a factorial experimental design, by including the enzyme treatment on both the PC basal diet and the NC basal diet. In the situation where the enzyme does not return the growth of the animal to that of the PC we can draw two possible conclusions: (i) that the enzyme is not effective at recovering the energy that was removed; or (ii) that the enzyme cannot replace fat. It may be possible to consider alternative ways of forming the NC diet to investigate this question fully. For example, Masey O'Neill *et al*. (2012) reported a study using two NC diets, both with 100 kCal less than the PC: one with fat maintained equivalent to the PC and using fibre as a diluent, and one with fat removed. As described above, increasing fibre content of the diet or using purified ingredients such as carboxymethyl cellulose may reduce energy density but also change the effect of the diet on the digestive tract. Care must be taken to ensure that fibre dilution does not exceed the critical concentration (which depends on fibre type), at which point it may exert an effect beyond that of decreasing energy.

These are clear examples with an enzyme product but we could also consider a more straightforward feeding study where we are testing the effect of including a novel ingredient. In this case, we may need to exchange a larger proportion of the NC with the test ingredient, maybe 5% or 10% and upwards of the total diet, in which case have we so radically changed the diet that the NC versus treatment is no longer simply investigating the inclusion of the test ingredient but could also be considered to be investigating the effect of removal of the exchanged part of the NC? This is probably particularly pertinent when we are considering making exchanges with dietary components that have dramatic effects on passage rate, gastric conditions and satiety, such as fat, fibre and protein.

4.3.2 Trial design to compare two different additive products

There are two different types of comparison we could be undertaking, described by two scenarios. The first scenario compares two products that are similar in nature, for example two enzymes of the same class but different formulation (annotated as A1 and A2). The second scenario compares two distinctly different products (identified as A and B).

To discuss the first scenario, imagine a class of product that, chemically, is known to have the same attributes and is likely to exert an effect through the same mechanism. In that case, once the experimental diet has been decided upon, the design is simple. The research question we are asking is probably, 'Do these two similar products have the same effect on animal

performance?' For a straightforward comparison, the two (or multiple) products should be included using the same basal diet. However, they should also be included at comparable dose rates and the dosing should be explicit from the methodology. If product A1 is dosed at 10,000 units/kg of feed, then so must A2 be. The unit definition should be provided and should be the same for both products, i.e. the units dosed are equivalent. Following on from this, the recovered dose should also be provided and this is now required by some publications in the field. For example, *Poultry Science* (2015) requires that analysed values for ingredients crucial to the experiment are included in all manuscripts. If, say, the ingredient being tested is an enzyme, the recovery of that product in the diet should be included. The full chemical definition of the products should also be included, such as the type, class and unit definition. If the doses cannot be readily matched, this must be explicit in the methodology and may lead to the second scenario. In the case that product A1 is superior, we can realistically conclude that A1 is superior to A2 on a unitary basis and make conclusions about the reasons behind this. In short, we should try as much as possible to compare the active ingredient of an enzyme product on an equal basis.

The second scenario is perhaps more complicated and asks a different experimental question. Whereas in the above we can carry out a transparent comparison with easy interpretation, the second scenario leads us to a more broad research question: 'Does product A lead to a different experimental outcome than product B?' This is more oblique and, depending on the nature of the products, may be difficult to interpret. Imagine the situation whereby product A is a purified enzyme product containing only one chemical activity and product B contains a multitude of activities. Such a comparison may be useful and required by the market place when these are commercially available products. In the event that product A outperforms product B, we can only say that product A as a whole is superior; we cannot make a judgement on the enzymatic activities within that product. Product A may be fundamentally different in its formulation, for example. We could only say that product A, at this dose and under these conditions, is superior to product B. This may be all that is required. If both products are providing a similar nutrient or activity, then for ease of comparison, and wherever possible, we should control for dose to ensure we are not providing twice as much of A than B or, if this is the case, that we make this clear. For example, all activities within a combination product should be explicit and the in-feed recoveries reported. There are clear justifications for this kind of comparison, but caution must be exercised when drawing conclusions about the chemical nature of the products when like-for-like comparisons are not complete. For example, a very relevant experiment would be to compare a suite of different products, each at the manufacturers' recommended doses. However, this does not allow us to make any inference about efficacy or mechanism of the products themselves. If we wanted to be able to draw the conclusion that A was superior to B and to say *how* and *why*, we would need to complete a fully factorial experiment. For example, if A is a purified product and B contains

three different activities, we would need to compare A with all of the individual activities of B and all combinations of the activities in B. This would allow us to draw conclusions about which components of product B are relevant in exerting its effects. If the primary component of B is the same as that in A, we cannot know the supplemental benefit of the further activities without this factorial design. Furthermore, attempts should be made to equilibrate the doses of the components common to the two products to ensure that the differences noted between products A and B are not a result of different doses of the 'actives' between the two products.

A similar question arises if we want to look at two different product types and how they interact. If we have an antimicrobial product, for example C, then to test the combination of A and C we must also test each ingredient, at a consistent dose, separately as well as the combination. Imagine a study testing A and A+C, where A+C is superior. We could not claim it was the combination per se that was superior; it may simply have been the addition of C alone. Or indeed the response to A could have been greater at a higher dose. A full factorial experiment, using several doses of each product, would be far more meaningful a comparison in this regard. Nevertheless, the study does not tell us *how* or *why* the superior treatment is so.

Although these are enzyme examples, of which there are many thousands in the non-ruminant literature, the same would be true for any other chemically active additives, such as amino acids and their analogues, probiotics, prebiotics, yeast cell wall products and antimicrobials.

In summary, whatever additive we are using, we should be explicit about its nature and provide enough information for the trial to be replicated. We should also be clear in the question we are able to address and conclusions we are able to make, from the experiment we have designed.

4.4 Summary

The above discussion highlights potential pitfalls and important considerations when designing animal feeding trials. Proper reporting of experimental design and characterization of diets can mitigate much of this, and allow thorough interrogation of the data by the reader and reproduction where necessary, as described above. It is also proposed that there is a minimum of analysed values that should be presented for experimental diets. As a minimum, analysed values for calcium (Ca), P, gross energy and lysine should be included alongside calculated values for the entire diet. These parameters would help to address some of the issues above. Certainly, *Poultry Science* requires crude protein (CP) and ME levels to be reported (if not meeting the NRC Standards in the case of *Poultry Science*) and Ca and P in laying-hen diets. Interestingly, they also require reporting of analysed levels of graded nutrients (*Poultry Science*, 2015). Hooijmans *et al*. (2010) suggested that water supply is also fully described as part of the dietary design. In our experience, these details are not commonly included.

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5 Measurements of Nutrients and Nutritive Value

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5.1 Introduction

With an ever increasing volume of information to digest, it is essential that you present your research findings in a concise and meaningful manner. In scientific writing, brevity is preferred over long-windedness and strict adherence to technical terms is preferred over elegant variation. Being concise and meaningful is not just about writing; it has its base in the design of an experiment and the testing of the hypothesis. Which measurements are required should be dictated by the hypothesis. A very common oversight with some researchers is to measure what their laboratory is equipped for, or what others in the same field usually measure. One researcher once said to me that such research was like 'a blind person throwing a rock into the ocean and hoping to hit a fish'. Such an approach bulks up manuscripts with irrelevant measurements without an overarching hypothesis, which in turn leads to irrelevant discussion and misleading conclusions. It is imperative to consider what your hypothesis is and then find the tools to test it. The tools in this case refer to the methods and equipment required to carry out the measurements.

This chapter will discuss the areas of measurements that require rethinking. The topic will be covered in two subsections: *in vitro* and *in vivo* measurements.

5.2 *In Vitro* Measurements

There is a saying that no amount of good measurements can save a bad design. Likewise, bad measurements can easily destroy a good design. To a

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very large extent, the quality of data, and hence your research, will depend on a meticulous approach to every measurement you carry out.

Nutritional research without laboratory analyses (*in vitro* measurements) will remain a 'feed and weigh' type of work, which will not advance science. Thus, laboratory analyses are essential in nutrition research. In fact, as challenges facing animal production industries are becoming more complex, nutrition research will increasingly require cross-disciplinary and multi-pronged approaches to find applicable solutions to industry problems. This means the traditional nutrition laboratory will no longer be able to cater for the needs of future nutrition experiments. Future nutrition studies will, to an extent, rely on all emerging technologies such as high-throughput sequencing and spectroscopic techniques. In the context of the current book, emphasis will be placed on the correct use of traditional nutrition research methods. But this section is not about cataloguing all of the methods related to nutrient analyses as outlined in the Official Methods of Analysis of the AOAC International (AOAC International, 2000). The aim here is to highlight pitfalls and issues related to some analyses that have relevance to conducting applied animal nutrition experiments. For a comprehensive coverage of the topic, please read *Laboratory Procedures in Animal Nutrition Research* (Galyean, 2010).

5.2.1 Proximate analyses

The basic nutrient composition of feedstuffs has been in use since the mid-19th century when the Weende Experimental Station in Germany published its methods for proximate analysis. Proximate analysis includes dry matter, ash, crude fat (often referred to as ether extract), crude protein and crude fibre. When all these components are added together and then subtracted from 100, it yields nitrogen-free extract (NFE). NFE in grains is supposed to represent starch, monosaccharides, disaccharides and oligosaccharides. Despite its many shortcomings, this system of analysis has been highly valuable to the progress made in nutrition research over the past 150 years. However, with the advent of technology and the deepening of our knowledge of nutrition, we now understand the deficiencies of the proximate analysis system.

Moisture

Dry matter, or moisture determination, is perhaps the most basic of all nutrient measurements and the correct determination of dry matter is of paramount importance to the accuracy of the rest of the nutrients in a feed ingredient. This is because all nutrients in an ingredient are expressed relative to their dry matter content. From the outset, moisture determination sounds simple, i.e. dry off the water from a given weight of sample and weigh what remains. In reality, it is complex because there are different types of ingredients that require proper handling in order to get an accurate moisture level. For instance, soft-moist, semi-moist and high-moisture ingredients

as well as raw materials that contain volatile matter require different procedures when determining dry matter. For instance, drying pig faeces and poultry excreta using an oven versus a freeze-dryer produces vastly different nitrogen losses, let alone volatiles (Jacobs, 2011).

Minerals

Burning off organic matter at high temperatures and weighing the remainder is a simple method of obtaining total mineral content of feedstuffs, known as 'ashing'. Substantial errors occur in some cases where samples with high moisture content and high lipid content are not properly pre-treated. High-moisture samples should be dried first; and high-lipid samples should be solvent extracted before ashing. Ashing unextracted high-lipid samples will lead to rapid burning, resulting in loss of mineral content through spillage of the sample. In addition to ash, individual elements are routinely measured using inductively coupled plasma (ICP) machines as well as atomic absorption spectrophotometry.

Lipids

Crude fat analysis yields a mixture of compounds that are soluble in non-polar solvents such as ether, and this does not represent unique chemical entities in the form of lipids. A feed formulation matrix requires a 'fat value' (important for laying hens), as fats provide linoleic acid and high energy levels. However, crude fat analysis has very limited value as a tool for nutrition research because the nutritional function of fats depends on their fatty acid profile. For example, the short, medium and long chain fatty acids differ in their numerous roles; saturated and unsaturated fatty acids differ vastly in their nutritional attributes, including energy contribution to monogastric animal feed. Thus, when it comes to analysis of the lipid content of feed, it is essential that the fatty acid composition be properly determined. Measurement of fatty acids is complex, because separate analytical methods are required to determine: (i) volatile fatty acids (AOAC 969.33); and (ii) medium and long chain fatty acids (Outen *et al.*, 1976). There is a wide choice of reasonably reliable methods for fatty acid analysis, and hence the references quoted here are based on personal experience only.

Protein

Animal nutrition research does not really need an analysis of crude protein, because today's feed formulations only require the amino acid composition of raw materials. However, the crude protein content of raw materials still dictates feed trade through government regulations and policies in many countries. The crude protein method has not changed a great deal since its first establishment as part of the proximate analysis system. Crude protein analysis is based on the nitrogen content of an ingredient, which is then multiplied by a factor of 6.25 to derive the protein content. This factor of 6.25 is obtained from the 'average' nitrogen content of a range of different proteins

present in feed ingredients, which is calculated to be 16% (1/0.16 = 6.25). This approach has a number of problems. First, not all nitrogen in feed comes from proteins: there are numerous non-protein nitrogen sources present, such as free amino acids, nucleotides, creatine and choline. Second, not all proteins contain 16% of nitrogen and a small error in nitrogen content will translate into a much larger error in crude protein content. Table 5.1 presents a list of proteins and their nitrogen contents.

There are numerous studies surrounding this subject and there are slight variations in the conversion factors for various ingredients. Thus, this example serves to prompt researchers to pay attention to the fact that the average factor of 6.25 does not apply to many feed ingredients. The appropriate factors for individual ingredients should be applied when calculating their crude protein contents.

It goes without saying that protein nutrition is in fact amino acid nutrition. Therefore, measuring the amino acid contents of feed ingredients is essential for any nutrition research for monogastric animals. There are well-established methods for determining all the amino acids in feed ingredients.

Fibre

In the context of animal nutrition, fibre is probably the most confusing and ill-defined nutrient. This arises from the fact that there are numerous terms used to describe fibre, but in effect many of the terms refer to a variable proportion of the same chemical entities depending on their extraction methods. Among all the terms used, perhaps crude fibre is the longest standing one and it has little or no distinct value to pig and poultry nutrition research. The next section will discuss fibre and carbohydrates in detail.

5.2.2 Fibre and carbohydrates in feed

Crude fibre

Crude fibre (CF) refers to the organic remnant of feedstuffs insoluble in hot, dilute sulphuric acid and sodium hydroxide (Henneberg and Stohmann,

Table 5.1. Factors for converting nitrogen content to protein content (common feed ingredients used in pig and poultry feed formulations). (From Jones, 1931.)

Ingredient	Factor	Ingredient	Factor
Corn	6.25	Soybean	5.71
Wheat	5.83	Meats	6.25
Sorghum	6.25	Milk	6.38
Barley	5.83	Eggs	6.25
Rye	5.83	Beans	6.25
Oats	5.83	Cottonseed	5.30
Rice	5.95	Sunflower seed	5.30
Millets	5.83	Peanut	5.46

1859). Choct (2015) highlighted the deficiency of the crude fibre system by giving an example of the major nutrient values for wheat, sorghum and soybean meal (Table 5.2). As seen below, the nutrient values only add up to 92% for wheat, 93% for sorghum and 70% for soybean meal. This prompts the question, 'What is the missing nutrient?'

The missing nutrient, by and large, is part of the fibre that is unaccounted for in the crude fibre determination. This missing 'fibre' consists almost exclusively of non-starch polysaccharides (NSPs) because crude fibre, more or less, represents the cellulose and lignin components of feed ingredients. The description 'more or less' is important because the proportion of cellulose, and to a lesser extent lignin, extracted can be highly variable depending on the ingredient. It is possible that some esterified xylans or other neutral polysaccharides may remain in crude fibre, again depending on the sample source. Thus, it is not possible to calculate crude fibre as a set percentage of NSP for all plant ingredients.

Detergent fibres

The problem associated with crude fibre determination was recognized in the early 1960s. Van Soest (1963) proposed the detergent fibre system to fractionate the fibre in feed into two components: acid detergent fibre (ADF) and neutral detergent fibre (NDF). This is a significant improvement on crude fibre, but it is still based on extraction processes that do not produce distinct chemical entities. NDF and ADF are not able to account for the soluble NSPs present in feed. So what do ADF and NDF values actually represent? ADF accounts for most of cellulose and lignin, whereas NDF covers cellulose, lignin and various proportions of the insoluble non-cellulosic polymers, such as insoluble xylans, insoluble mannans and insoluble pectic polysaccharides. From common components, cellulose and lignin in both fractions, the following approximation is often used to obtain a value for hemicellulose:

$$\text{NDF} - \text{ADF} = \text{hemicellulose}$$

This poses a problem because there is no such molecule as hemicellulose. The word 'hemicellulose' (half cellulose) came into existence in the late 19th century. Schulze (1891) thought that plant cell wall components soluble in

Table 5.2. The amounts of fibre unaccounted for in wheat, sorghum and soybean meal.

Nutrient (%)	Wheat	Sorghum	SBM
Protein	13	9	47
Starch	60	65	1
Fat	2	3	1
Crude fibre	3	2	5
Water	12	12	10
Ash	2	2	6
TOTAL	92	93	70
Missing	10	7	24

alkali were precursors to cellulose, which is now known to be incorrect. This alkali-soluble fraction covers arabinoxylans, mixed linked β-glucans, xyloglucans, mannans, galactomannans, galactans, arabinans and any other neutral polysaccharides other than cellulose. Unfortunately, the hemicellulose value obtained by subtracting the ADF value from the NDF value does not really represent the real value of the alkali-soluble fraction. This is because, as mentioned earlier, the NDF procedure does not account for the soluble fractions of various polysaccharides.

Dietary fibre

From a monogastric animal nutrition perspective, dietary fibre (DF) represents the sum of NSPs and lignin, the latter being a polyphenolic compound. There are two well-established methods for measuring DF. One is the series of enzymatic–gravimetric methods provided by the AOAC Total Dietary Analysis (Methods 985.20; 993.19; 991.42; 991.43; 992.16), which uses enzymatic removal of non-cell wall organic materials and then gravimetrically measures the residue corrected for ash. The other technique is known as the Uppsala Method. This method quantifies each individual sugar residue by converting them into alditol acetates and measuring them using a gas chromatograph (Theander *et al.*, 1995). Lignin and uronic acids are determined separately in the Uppsala Method. There are a number of advantages in using the Uppsala Method, including the separation of the individual sugar composition of dietary fibre that gives an idea of the type of polysaccharides present in an ingredient, and the ability to fractionate NSPs based on their solubility in water (the other method also offers this option).

Understanding the interrelationships between CF, ADF, NDF and NSP values is essential. Table 5.3 presents a comparison between them for the three most important plant ingredients for pig and poultry diets: corn (maize), wheat and soybean meal.

Table 5.3. Composition of maize, wheat and soybean meal (% dry matter). (From Graham and Åman, 2014.)

Analytical component (% DM)	Corn	Wheat	Soybean meal
Ash	1.4	1.7	6.6
Crude protein	9.1	11.0	53.3
Crude fat	4.6	2.4	2.8
Sugars	2.6	3.5	3.5
Oligosaccharides	0.3	0.2	5.3
Fructans	0.6	1.8	0.9
Starch	69.0	66.5	0.0
Crude fibre (CF)	2.3	2.5	4.2
Acid detergent fibre (ADF)	2.5	3.4	4.9
Neutral detergent fibre (NDF)	9.2	10.0	8.4
Non-starch polysaccharides (NSPs) + lignin	10.0	11.0	20.8

From this particular example, it is clear that the CF value represents a very small proportion of the total fibre present in these ingredients. On the other hand, it can be seen that the values for NDF and NSPs are similar for corn and wheat – cereal grains with little or no pectic polysaccharides. For soybean meal, a vegetable protein source rich in pectic polysaccharides, the NDF value is less than half of the NSP content.

Figure 5.1 shows the overlaps and interrelationships between CF, NDF, ADF and NSPs.

Starch and other carbohydrates

STARCH Starch is the most important energy source for pig and poultry diets as it makes up 60–70% of cereal grains on a dry matter basis. Two types of molecules, amylose and amylopectin, make up starch. Amylose is a linear α(1→4) glucan and accounts for 20–25% of starch in common feed ingredients. Amylopectin is the α(1→4), α(1→6) branched glucan with α(1→6) branches occurring every 24 to 30 glucose units.

The physicochemical properties of starch, however, are extremely complex and are affected by a number of factors (BeMiller and Whistler, 2009). These include botanical source, harvest condition, geographical location, storage and processing. Such effects will also influence the nutritive value of starch for monogastric animals, such as the formation of resistant starch, the change in the amylose-to-amylopectin ratio, and the rate of digestion. There are two methods used for starch analysis: one for total starch and another for resistant starch. The AOAC Method 996.11 for total starch assay is straightforward and is used throughout the world. Likewise, the AOAC Method 2002.02 for resistant starch assay is readily available.

LOW-MOLECULAR-WEIGHT CARBOHYDRATES Here, the term low-molecular-weight carbohydrates is used to cover carbohydrates other than NSP and starch. There are numerous terms, some highly ambiguous, for these carbohydrates. Monosaccharides and disaccharides are sometimes called sugars, whereas

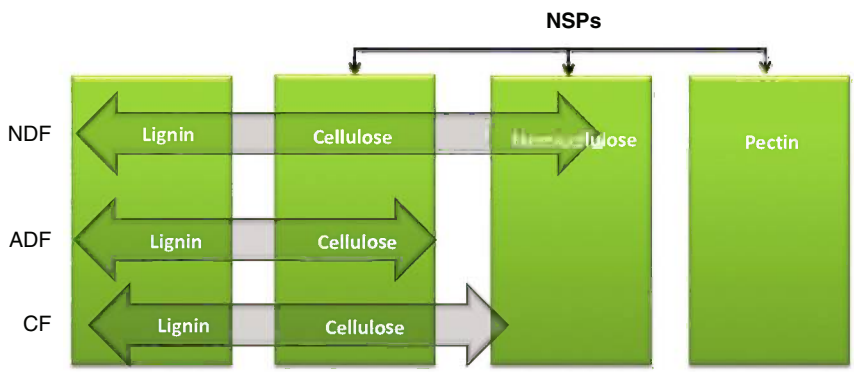

Fig. 5.1. The overlaps and interrelationships between crude fibre (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF) and non-starch polysaccharides (NSPs).

oligosaccharides and inulin, etc., are often called prebiotics. The term 'available carbohydrates' is used in human nutrition and sometimes in pig nutrition. It was originally coined to include starch and soluble sugars for human nutrition, and later it was applied in pig nutrition to describe carbohydrates that can be digested. Purely from a research point of view, the use of this term in animal nutrition is not recommended.

For common feed ingredients, low-molecular-weight carbohydrates include monosaccharides, disaccharides, oligosaccharides and short-chain carbohydrates such as inulin. In addition to these low-molecular-weight carbohydrates that occur naturally in monogastric feed ingredients, there are numerous other such carbohydrates that may be used in pig and poultry diets as prebiotics. For instance, inulin is a fructan that has a degree of polymerization ranging from 2 to 60. Inulin is widespread in nature, with very high concentrations in plants such as chicory and Jerusalem artichoke.

There are numerous methods for measuring monosaccharides, disaccharides, oligosaccharides and other specific low-molecular-weight carbohydrates. For instance, the Steegmans *et al.* (2004) method uses an enzymatic and spectrophotometric procedure to determine glucose, fructose, sucrose and inulin.

In the proximate analysis system, starch together with some low-molecular-weight carbohydrates is covered under an entity called nitrogen-free extract (NFE). It is defined as follows:

$$\text{NFE} = 100 - (\text{CP} + \text{EE} + \text{ash} + \text{CF} + \text{water})$$

where CP = crude protein, EE = ether extract and CF = crude fibre.

In cereal grains and tubers such as cassava and potatoes, NFE consists mainly of starch, whereas in vegetable protein ingredients such soybean meal, canola meal, lupins and sunflower meal, it represents mostly NSPs (other than cellulose plus free sugars), such as monosaccharides, disaccharides and oligosaccharides.

NFE is a meaningless entity that is completely redundant when feed carbohydrates can be fully characterized with starch, NSPs and other carbohydrate measurements.

5.2.3 Summary

Proper analysis starts with careful sample collection, sample preparation and understanding the nature of the sample to be analysed. The second step is to find the most appropriate method for measuring the nutrient you want to determine. Third, you will need to have access to (and know how to operate) the equipment necessary for the analysis. This final step usually makes or breaks an analysis, because not all laboratories have the appropriate equipment with trained operators. It is your responsibility to decide on what analyses are best for your experiment and whether or not they can be done in-house. Good analyses are essential for the characterization of your ingredients and for answering the questions posed by your research hypothesis.

Measuring only those nutrients for which your laboratory is equipped, or simply taking measurements that you are comfortable in setting up, is not necessarily the best way to test new ideas.

5.3 Determining Nutritive Value of Ingredients

While laboratory analyses produce the 'nutrient composition' of an ingredient, they will not give you the 'nutritive value' of that ingredient. This is because nutritive value is the reflection of how nutrients contained in an ingredient are utilized by an animal under a given environment, ignoring the effects of anti-nutrients present in certain ingredients. It is a value that has come into existence as the result of the interaction between the ingredient and the animal consuming it. One of the most commonly used measurements of nutritive value of feed ingredients is digestibility of nutrients, or energy. Theoretically speaking, the digestibility of all nutrients may be determined. There are four ways to determine digestibility of nutrients or energy: (i) *in vivo* work; (ii) *in vitro* techniques; (iii) prediction equations; and (iv) spectroscopic technologies, such as near infrared spectroscopy (NIRS) and Raman spectroscopy.

The most reliable method is the *in vivo* technique, that is, feeding the nutrient of interest to the animal for which it is intended. However, it can be tedious, is costly and, for some nutrients, may be inapplicable. Thus, *in vitro* techniques may be more suitable to determine digestibility in some instances. *In vitro* techniques mimic the physiological conditions of the digestive tract of animals under laboratory situations. Unfortunately, *in vitro* techniques work well only under some circumstances and their application is limited to only a few nutrients under set conditions. So the third method, employing a prediction equation, is sometimes preferred. Prediction equations can be developed based on the physical characteristics, chemical components or nutritive values of feed ingredients. The fourth method is based on the use of spectroscopic technologies, which, in turn, are calibrated using *in vivo* data, to determine a wide range of chemical constituents as well as some nutritive values for different animals. The range of nutrients and nutritive values included in NIRS, for instance, is widening at a very rapid rate as both speed and accuracy have increased over time.

5.3.1 *In vivo* experiments

Measuring nutrient digestibility values is one of the most fundamental nutritional studies in monogastric animals. The reason is simple: feed constitutes the largest cost for pig and poultry production throughout the world and any gain in nutrient digestibility has the potential to reduce cost.

The digestibility experiments described here will cover nutrients as well as energy. In regard to energy, poultry experiments will involve 'metabolizability', because poultry excrete urine and faeces together and hence it is

convenient to measure metabolizable energy (ME), rather than digestible energy (DE) as used for pigs.

Animal nutrition research is expensive and time-consuming and may have ethical implications. The cost of an experiment depends on sample size. However, animal experiments without including an optimal sample size, containing an appropriate number of replicates and meeting the basic requirements for health and wellbeing of the animals not only waste resources but also have the potential to produce misleading conclusions. Properly designing your experiments requires characterization of the feed ingredients that make up the diets, and statistical consideration. The latter is covered in Chapter 2 of this volume.

Furthermore, for any animal experiment to be successful, a number of common conditions must be considered. Many of these relate to animal care and husbandry and are clearly outlined in the management guide relevant to the animals you are about to use. Thus, this section only describes the very basics of preparation for a successional nutrition experiment using pigs or poultry.

Pre-experimental preparations

QUALITY OF THE EXPERIMENTAL ANIMALS To minimize the effect of any known factors that affect uniformity, the animals chosen for an experiment should be healthy and come from a donor herd or flock similar in age (for poultry) or parity (for pigs). It is preferable, but not always possible, to avoid chicks from very young breeding hens or piglets from young sows in their first parity. The aim is to obtain animals with a uniform body weight and a robust immune status that are able to consume sufficient amounts of the proposed feed.

TRANSPORTATION Many institutes do not have the luxury of having a breeder facility on site. This means that the experimental animals, often young animals, have to be transported from one location to another. Transportation of animals requires great care in terms of air quality, temperature and overall comfort. Care should also be taken between the animal house and the transport vehicle because, on a hot day, young animals can easily be dehydrated, and likewise on a cold day, they can be chilled. Stress during transportation, loading and off-loading can cause lasting problems for your experimental animals, which in turn, will affect your experimental precision.

HUSBANDRY AND MANAGEMENT A reminder is given to researchers that, when placing your animals into experimental units, the appropriate management guide relevant to the animals chosen for the experiment must be followed with respect to ventilation, temperature, lighting, stocking density, floor comfort and water quality.

FEED Feed form affects feed intake in both pigs and poultry. In poultry, feed form can also affect nutrient digestibility, possibly through altered

levels of crop and gizzard holding and subsequent effects on digesta transit in the small intestine (Svihus and Hetland, 2001). Thus, the same form of feed must be fed during adaptation and collection.

Types of in vivo *experiments*

Digestibility experiments using animals include: (i) the total collection method, involving the measurement of feed intake and quantitative collection of excreta over a period of time; and (ii) the ileal digestibility assay, which requires the inclusion of a digestibility marker in the feed and collection of a representative digesta sample from the ileum (this can be from other parts of the intestine) either through the slaughter of the experimental animals (usually the case in poultry) or via ileal cannula (usually in pigs). Digestibility of nutrients, such as amino acids, is seldom determined using the total collection method, because of the influence of endogenous secretions and microbial activity in the hindgut. There are other techniques, such as the rapid metabolizable energy bioassay in poultry, that involve force-feeding of a known amount of feed followed by a quantitative collection of excreta; however, these techniques are no longer widely used due to concerns with inaccuracy (Härtel, 1986) as well as animal welfare considerations.

TOTAL COLLECTION METHOD As the name suggests, the total collection method determines the apparent digestibility of nutrients or energy between the mouth/beak and the anus of the animal. For poultry, there is a European Reference Method that was originally designed for the determination of metabolizable energy (ME) (Bourdillon *et al.*, 1990a,b). For the determination of apparent ME (AME) in birds fed *at libitum*, the procedure involves a 4-day adaptation period followed by an overnight fasting, and a 3–4-day period of *ad libitum* feeding and excreta collection after overnight fasting (Fig. 5.2). The term *ad libitum* infers that the experimental diet must be palatable to the animals to enable a proper level of intake.

The key elements of this protocol, i.e. the adaptation and excreta collection periods, are quite standard although some institutes may opt to have a 3-day adaptation period, instead of a more commonly used period of 4 days. Similarly, the fasting periods – one before the start and one at the end of excreta collection – vary from institute to institute. Typically, the initial fast lasts around 16 h (overnight from 5pm prior to the start day to 9am on the

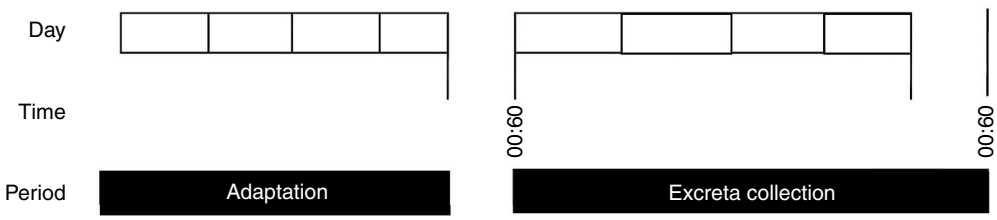

Fig. 5.2. Graphic presentation of an apparent metabolizable energy experiment in poultry.

start day). Whatever the period you choose for the two fasting periods, it is important that your experiment complies with the codes of conducting animal experiments set by the organization where the work is to be done.

Although Bourdillon's work gives a good start for standardizing the ME procedures for poultry, there are numerous areas that vary in published literature. The birds used in the original work for developing the Reference Method were adult cockerels. Later, an inter-laboratory study used both adult and young birds. Today broiler chickens reach slaughter weights at or around 30–35 days under most production systems. Thus, it is very common to see ME trials conducted between day 20 and day 28. In published literature, there is no uniformity in fasting procedures and many laboratories do not routinely apply fasting, either before or after a trial. Then there is the issue of drying and processing excreta. Despite it being well documented that drying excreta at 80°C seems to offer the best compromise in terms of minimal loss of volatiles and maximum speed of drying, there is no clear consensus for this and many papers do not even mention this in their materials and methods. Another source of error is moisture re-equilibration for the collected excreta stored in non-sealed bags. The amount of moisture reabsorbed depends on the environment where the excreta is kept. Thus, for a properly run ME trial, the dry matter content of the excreta at the time of gross energy determination should be measured to make sure the moisture level of the excreta has not changed drastically between the last weighing at the time of collection and drying and at the time its gross energy content is determined.

For pig experiments, both the adaptation and collection periods are usually longer than that in poultry. Typically, an adaptation period of 5–7 days is followed by a total collection period over 4–6 days (Adeola, 2001). The pigs are given meals two to three times a day and there is no fasting period applied. The same issues with standardization apply, together with the collection and processing of faeces.

Nutrient digestibility using the total collection method is calculated using the following equation:

$$\text{Nutrient digestibility } \% = \frac{\text{Nutrient intake} - \text{Nutrient in excreta}}{\text{Nutrient intake}} \times 100$$

THE MARKER, INDICATOR OR INDEX TECHNIQUE Various indicators, often known as markers, are used in digestibility studies for many *in vivo* experiments. Indigestible markers are used by researchers as their measurement allows an estimate of the amount of excreta or faeces corresponding to a given amount of feed intake. This is especially important when total collection is not possible, such as in the case of ileal digesta. The marker technique assumes that the marker is intimately mixed with the rest of the feed and it goes through the digestive tract without having any effect on the animal or its microflora or being affected itself throughout the digestive process. Thus, in principle, the fraction of a nutrient digested in the gut can be calculated relative to the marker included at a known level in the diet.

For the total collection method, the inclusion of a marker in the diet can negate the need to quantitatively collect excreta (faeces, urine or both). In pig experiments, some researchers include markers in the diets in order to determine digestibility in free-moving animals, instead of animals in pens.

The use of markers in the case of total collection experiments saves time and effort. More importantly, markers enable the determination of digestibility throughout the digestive tract of animals.

Ideal markers are:

- inert with no adverse effects on the animal, including physical, physiological, psychological or microbiological;
- neither digestible nor absorbable, both by the animal and its microflora, and totally recoverable;
- able to mix well with feed and distribute uniformly in the digesta; and
- easy to determine but not too bulky, i.e. take up a large proportion of the diet.

Unfortunately, an ideal marker is yet to be found. This is because there are at least three phases in the digesta: the solid; the liquid; and semi-soluble. The three phases of digesta do not always move together, leading to nutrient separation along the gastrointestinal tract depending on the physicochemical nature of the nutrients, such as solubility in water or lipid or at different pH and ionic strengths, the physiological condition of the animal, such as crop and gizzard holding in chickens and stomach retention in pigs, and the nature of feed constituents, such as the presence of viscous polysaccharides that change gut dynamics.

Common markers, such as chromic oxide, titanium dioxide and acid-insoluble ash, associate themselves with the solid phase of digesta as they are insoluble. Thus, they are not always distributed uniformly in the digesta. This means any nutrients associated with the soluble phase are not properly marked and hence digestibility calculations may not represent the true values for the nutrients. To address this issue, nutritionists use soluble (chromium EDTA, polyethylene glycol) and semi-soluble markers (long-chain hydrocarbons) in addition to the traditional solid phase markers.

Despite these imperfections, the marker technique is a very valuable tool for animal nutritionists and has been reviewed by various researchers (Kotb and Luckey, 1972; Khan *et al.*, 2003). The technique gives a good estimation of digestibility (Scott and Boldaji, 1997) and the values often agree well with those obtained using the total collection *in vivo* methods (Han *et al.*, 1976).

Nutrient digestibility using the marker technique is calculated using the following equation:

$$\text{Nutrient digestibility coefficent} = 1 - \left[\frac{\%\ \text{Marker in diet}}{\%\ \text{Marker in excreta}} \times \frac{\%\ \text{Nutrient in excreta}}{\%\ \text{Nutrient in diet}} \right]$$

MEASURING TRUE DIGESTIBILITY When apparent digestibility values are corrected for endogenous losses consisting mainly of proteins, they are called true digestibility values. Thus, the term true digestibility applies to protein

digestibility (often referred to as amino acid digestibility) and occasionally to energy, such as true digestible energy or true metabolizable energy.

As hind-gut fermentation has a marked effect on protein digestion, true digestibility of amino acids is usually determined in the ileum for both pigs and poultry. Estimation of endogenous protein losses is not simple. In fact, an ideal method is yet to be found. The list of methods below, reviewed by Ravindran and Bryden (1999), shows the key techniques used for estimating endogenous amino acid losses in poultry:

- fasting of birds for 24–48 h (used only to measure flows in the excreta);
- feeding of a protein-free diet;
- applying linear regression, following feeding of diets containing graded levels of protein;
- using guanidinated dietary protein;
- using enzyme hydrolysed casein and ultrafiltration; and
- feeding of highly digestible protein, e.g. wheat gluten.

Stein *et al.* (2007a), while summarizing the various terms used for measuring amino acid digestibility in pigs, presented a similar list of methods used to determine endogenous losses in pigs.

STANDARDIZED ILEAL DIGESTIBILITY Proteins in the digesta originate from three sources: (i) feed proteins that remain undigested; (ii) basal endogenous losses that result from the renewal of the digestive tract regardless of the nature of the diet; and (iii) specific endogenous losses that relate to the characteristics of the feed the animal consumes. While the apparent digestibility value ignores both basal and specific losses, the true digestibility assay tries to take both into account. This borders on impracticality, because factors affecting specific endogenous losses such as feed intake, anti-nutrients and stress are broad and complex. Therefore, there are arguments for and against the use of either the apparent or the true digestibility values for feed formulation. The main argument for using apparent digestibility values is that the apparent digestibility bioassay is straightforward and easy to conduct. However, apparent ileal amino acid digestibility values obtained in individual ingredients are not always additive in mixed diets (Stein *et al.*, 2005), making the very essence of practical feed formulation, additivity, questionable. On the other hand, the difficulty, and often inaccuracy, of measuring endogenous losses of protein has rendered the true digestibility bioassay of limited practical use.

To overcome the deficiencies of the apparent and true digestibility bioassays, the concept of the 'standardized ileal digestibility' (SID) assay was proposed. The idea was to correct apparent ileal digestibility values of amino acids for basal endogenous losses, which are characteristics of the animal and the feed intake but are independent of diet characteristics. It was demonstrated that the standardized digestible amino acid values are more additive compared with apparent digestibility values in pigs (Furuya and Kaji, 1991). Adeola (2013) reviewed the topic and concluded that SID values better represent the amino acid digestibility values of feed ingredients for poultry

species, including broilers, layers and turkeys. Stein *et al.* (2007a) reached a similar conclusion for pigs and emphasized the importance of deriving SID values from experiments where feed consumption is close to voluntary intake. This is because basal endogenous losses are affected by feed intake (Stein *et al.*, 2007b). However, it is worth noting that the accuracy of SID values depends on the basal diets used to generate them as well as the age of the animal. In many cases, the use of a semi-purified diet is highly problematic as it affects feed intake (see Chapter 4). Sometimes, nutrient imbalance caused by such diets may also affect young animals that are sensitive to deficiency. Having said this, there is no such thing as a flawless technique as far as animal digestibility techniques are concerned, and at least SID values reduce or even eliminate one source of variability. Therefore the SID values are recommended by major amino acid databases for use in practical feed formulation for both pigs and poultry. The method of calculating SID values is shown below:

$$\text{SID }\% = \left[\frac{\text{Amino acid intake} - (\text{Ileal amino acid flow} - \text{Basal ileal endogenous amino acid losses})}{\text{Amino acid intake}} \right] \times 100$$

DETERMINING THE NUTRITIVE VALUE OF SINGLE INGREDIENTS Determining the nutritive value of individual raw materials is one of the most fundamental types of animal nutrition experiments. The reason is that all feed formulation databases require nutritive value data for each and every ingredient. The method chosen depends on the nutrient to be measured. The following section will discuss the most commonly used techniques for measuring the nutritive value of individual ingredients.

Dose–response/regression method The response to including a nutrient, an additive, or an ingredient at various levels in a basal diet, in terms of animal performance measures such as growth or feed conversion ratio (FCR), and digestibility of nutrients and energy, may be measured using a dose–response experiment. Due to the simplicity of the concept, dose–response experiments are used widely in animal nutrition studies. Poor designs of such studies are perhaps responsible for numerous misleading conclusions, and hence added variability of nutritive values of feed ingredients for pigs and poultry.

Dose–response experiments are costly because they require more treatments than single-treatment experiments. However, a dose–response experiment without an optimal sample size, containing an appropriate choice of dose levels and an adequate number of replicates, not only weakens the power of your experiment, but also has the possibility of producing misleading results. The design of dose–response studies and the interpretation of results obtained using this method have been the subject of many papers and book chapters (Morris, 1983, 1999). Chapter 2 gives numerous examples for interpreting results obtained from dose–response studies.

Substitution (difference) technique At least two nutritionally adequate diets must be formulated in order to use this technique. The first one, a reference diet, must be well characterized in terms of digestibility. The second diet, the test diet, will be the reference diet substituted with a known level of the test ingredient. This should contain the appropriate proportions of the same minor ingredients (minerals and vitamins), i.e. the test diet and reference diet share the same minor ingredients in terms of volume and type (Sharma *et al.*, 1979), so that any difference in energy or protein or lipid content comes from the 'substitution' ingredient. The key assumption with this model is that, in any diet, the total amount of energy or a particular nutrient is the sum of that contributed by individual nutrient-bearing ingredients; that is, they are additive in diet formulation. In practice, this is not strictly true, because of the complex interactions between nutrients, between ingredients and processing (physical and chemical), and between a diet and the animal consuming it (physiological, microbial and immunological status of the animal). But we would not be able to use the difference technique unless we took the assumption of additivity as true.

Single-ingredient replacement assay For cereal grains, this assay often means that all 'energy-bearing components' of the reference diet may be replaced. This is also known as the single-ingredient replacement assay. Mollah *et al.* (1983) used a diet based on 80% cereal grain (such as corn or sorghum), 13.3% casein and 6.7% minor ingredients (vitamins and minerals) to test the AME value of wheat varieties for broiler chickens. Annison *et al.* (1994) implemented slight modifications to this technique, including a determined ME value for casein-HCl for the purpose of measuring the ME value of different wheat varieties. In this protocol, all the corn is replaced with either different varieties of wheat or another cereal to be tested. This method can also be used to determine the energy value of protein ingredients by using a pre-determined value for the cereal grain, such as corn or sorghum. For instance, if the AME value of a new batch of soybean meal is to be required, a test diet consisting of corn and soy together with the appropriate minor ingredients can be formulated. The calculation uses the following equation:

$$\mathrm{AME}_{\mathrm{cereal}} = \frac{\left(\mathrm{AME}_{\mathrm{diet}} - \mathrm{AME}_{\mathrm{casein}} \times 13.3\%\right)}{\text{Cereal inclusion level \%}}$$

The key advantage of this approach is the 'like replacing like' argument. Since ingredients with similar profiles replace each other, there is less complication of ingredient interactions, such as replacing cereal with a protein ingredient or vice versa. There is also the very high inclusion level of one cereal source in the diet that could accentuate the impact of anti-nutrients present in the grain. In addition, due to the use of a very limited number of ingredients, the diet is somewhat artificial and some amino acids can become marginal, depending on the two main ingredients used. Thus, questions may be asked whether ME, DE or digestibility values obtained using this technique accurately mimic how an ingredient behaves in a more practical diet.

The reliability of the single-ingredient replacement assay depends on the use of the same batch of the test ingredients for repeat runs. It is recommended that a large batch of the reference ingredient is first obtained and characterized including its nutritive value, for instance, ME or DE value. This allows the determination of a number of test ingredients in subsequent trials. In Annison *et al.* (1994), casein-HCl was the reference ingredient, which was obtained in a large quantity and was stored in a rodent-proof room for subsequent assays. In other cases where the cereal, such as corn, was the reference ingredient, and the ME value of the protein sources was required, a large quantity of corn was sourced and stored properly. This is important because different batches of the same ingredients, such as casein or corn, can vary in nutritive value. For ingredients like wheat or barley, batch-to-batch variation in ME or DE value can be very large.

To overcome these limitations, some researchers advocate the use of the practical diet replacement assay where a reference diet composed of practical ingredients is used and the test diet is prepared by replacing a portion of the reference diet with the test ingredient. Then the two diets are assayed simultaneously, negating the necessity to have specific ingredients assayed and stored for subsequent trials because the reference diet acts as a control.

Practical diet replacement assay This assay was first proposed by Sibbald *et al.* (1960) for poultry. It has since been modified extensively and has been applied in experiments using other species, such as the pig. The basic tenet of the assay is as follows:

1. A reference diet is prepared, which may contain several ingredients and is balanced to meet the nutrient requirements of the test animal. For the sake of simplicity, the reference diet is the sum of a basal component (BC) such as grains and protein sources, and a minor component that consists primarily of minerals and vitamins (MV) although in many cases it also includes markers, enzymes, synthetic amino acids, etc. It is obvious that BC + MV = 100%.
2. To obtain DE, ME or digestibility of a test ingredient, the common approach is to substitute only the BC of the reference diet with the test ingredient and keep a constant level of MV between the two reference and test diets. For the determination of ME value in poultry, for instance, the ME value of the test ingredient (AME_{ingr}) is obtained using the ME value of test diet (AME_{test}) and the reference diet (AME_{ref}) as well as the inclusion level of the reference diet (%Ref) and the substitution level of the ingredient (%Subs). The assumption here is '%Ref + %Subs = 100%' and the calculation for AME_{ingr} is:

$$AME_{ingr} = \frac{\left(AME_{test} - AME_{ref} \times \%\ Ref\right)}{\%\ Subs}$$

Issues with the practical diet replacement assay

Nutritional imbalance Two common mistakes that can be seen in published papers are: (i) the substitution of the reference diet with a test ingredient, without any distinction being made between the BC and MV parts of the diet (leading to massive imbalances in vitamins and minerals, as well as in energy

and amino acids); and (ii) imbalance issues caused even when due consideration has been given to minerals and vitamins (usually due to an energy ingredient replacing part of a protein source or vice versa). Keeping the ratios between various ingredients constant across substitution levels is meant to alleviate ingredient interactions, and hence keep the energy or nutrient contributions coming from the reference diet consistent. However, ingredient ratios are no substitutes for nutrient ratios, and so constant ratios maintained between ingredients as they appear in the original reference diet will have little or no effect on addressing nutrient imbalance.

At least in the modern genotypes of poultry, young birds are sensitive to nutrient imbalance. This raises questions as to whether the use of this method to determine the nutritive value of ingredients in young animals is appropriate.

Confusion regarding minor ingredients (MV) There is a great deal of confusion as to what constitutes the minor ingredients in relation to the substitution method. Minor ingredients often refer to all constituents other than the BC. The assumption is that MV does not contribute to energy and protein. This is not true, because synthetic amino acids, for instance, contribute to energy like any other protein source in the diet. The phrase 'keeping the minor ingredients constant' means that the same level and type of MV are used in the reference and test diets, pointing to the end products, that is, when the two diets are formulated. Unfortunately, many researchers set the level of test ingredient first and then add the MV at the same level as is in the reference diet.

As an example, consider the following. Say a researcher decides to use a test ingredient of 40% sorghum and the same level of MV as in the reference diet, i.e. 5%. This will create a calculation error. The error comes from the fact that the % MV in the reference diet is kept unchanged, so the 40% sorghum comes out of BC, i.e. 95% rather than 100% of reference diet. So the percentage of the reference diet substituted is: $(100-40-5)/95\times100=57.9\%$, rather than 60%. Thus, the assumption '%Ref + % Subs = 100%' is no longer true because $57.9\%+40\%\neq100\%$. The equation, therefore, is as follows:

$$\mathrm{AME}_{\mathrm{sorghum}}=\frac{\left(\mathrm{AME}_{\mathrm{test}}-\mathrm{AME}_{\mathrm{ref}}\times57.9\%\right)}{40\%}$$

So, why fuss about a difference of 2.1 percentage points in substitution rate? Based on the above equation, if the test diet contained 3200 kcal ME, and the reference diet had 3100 kcal ME, the error in calculation would be equivalent to 162.75 kcal for sorghum ME. When the MV level is high, such as in laying-hen feed (where limestone and phosphates are included in excess of 7–8%), the error rate renders any value generated meaningless.

Recommendations for improvement

'Like replacing like' approach The practical diet replacement method may be improved if we take the 'like replacing like' approach. For cereals, one cereal replaces another, and likewise for vegetable protein sources. For instance,

imagine a situation where a corn–soy-based broiler chicken diet is used as the reference diet to evaluate the ME value of a new variety of sorghum. The test diet includes 40% sorghum to replace the same amount of corn only in the reference diet. Due to the fact that nutritional profiles of cereals are relatively similar, this should avoid large nutritional imbalances. This is achieved through the fact that the reference diet and the test diet are determined together and, apart from the cereal in this case, everything else is left identical. This allows greater flexibility in balancing the diet than with the single-ingredient replacement assay. Let's take the example that we have used earlier to test the AME value of a new sorghum sample. We use a corn sample with a pre-determined AME value (AME_c) to formulate the reference diet. The reference diet looks like: 65% corn, 30% other (proteins, amino acids, lipids, etc.) and 5% minerals and vitamins. Sorghum only replaces the corn part of the reference diet at 40%, so the test diet will be 25% corn, 40% sorghum, 30% other and 5% minerals and vitamins.

Then the calculation would look like:

$$AME_{sorghum} = AME_c + \frac{(AME_{test} - AME_{ref})}{\text{Sorghum inclusion \%}}$$

Of course, such an approach should be limited to testing ingredients that are similar in nutritional profiles to the ones they are replacing in the reference diet.

Test diet, instead of test ingredient

In an example, a reference diet contains 65% corn, 30% protein sources and 5% MV. We want to determine the ME value of sorghum and decide that our test diet will substitute 40% in the reference diet. The key is to establish the test diet. It is assumed that the reference diet is mixed homogenously, so a 40% test diet will replace: (65% corn + 30% protein sources + 5% MV) × 40% = 26% corn + 12% protein sources + 2% MV. Since both the reference and test diets need to meet the requirements for minerals and vitamins, our test diet must replenish the 2% MV in the reference diet being displaced by the 40% substitution. Thus, our test diet will be: sorghum + 2% = 40% and therefore the inclusion rate of the test ingredient, sorghum, is 38%. Thus, the appropriate equation is:

$$AME_{sorghum} = \frac{(AME_{test} - AME_{ref} \times 60\%)}{38\%}$$

This approach will still keep the MV constant between the two diets with the complication in the calculation.

Multiple levels of substitution

To increase robustness of the method, multiple levels of substitution are recommended. The advantage of such an approach is to avoid any effect that level of inclusion has on the nutritive value.

5.3.2 Determining the digestibility of specific nutrients

This section will focus on phosphorus and lipid, two nutrients that have received a great deal of coverage in both research and industry application, and yet methods determining their digestibility are prone to errors.

Measuring phosphorus digestibility in poultry

Phosphorus (P) is a much studied nutrient but more work is still required in this area in the future. It is also a nutrient that has many peculiarities as far as digestibility studies are concerned. Firstly, feed P comes from phytate and non-phytate sources (NPP). The amount of P released from phytate in the gastrointestinal tract of pigs and poultry, with or without the addition of phytase, is variable. Secondly, P digestibility is affected by numerous factors: the type of the reference diet in relation to the ingredients used, the Ca:P ratio, the level of NPP, age and period of measurement, and where in the intestine the measurement is taken. There are numerous publications elucidating the pitfalls of measuring P digestibility (Rodehutscord, 2013; Mutucumarana *et al*., 2015) and the response to phytase (Bedford *et al*., 2015). Thus, this section is only meant to serve as a reminder that when designing an experiment for P digestibility or phytase response, it is essential to pay close attention to each of the key factors that affects P digestibility.

Measuring fat digestibility

Fats and oils, collectively termed lipids, make up only a small proportion of pig and poultry diets, but are important contributors to dietary energy and provide essential fatty acids. They also increase palatability of feed, reduce dustiness of mash diets and improve pellet mill output by lubrication. As the energy value of lipids is more than twice that of starch, even a small error at relatively low levels of inclusion can translate into significant costs in feed formulation. The complexity associated with lipid digestibility is more pronounced in poultry than in pigs. For instance, lipid digestibility, and hence the ME value, for poultry is affected by: age of animal; background ingredients in reference diet; and chemical characteristics of lipids.

AGE OF ANIMAL In poultry, the ME of lipids increases with age irrespective of type. This is particularly pronounced in young birds, where the ME value is low in week 1 and markedly higher by week 3. This means that lipid digestibility values determined before and after week 3 in broiler chickens, for instance, can be vastly different. Thus, in commercial poultry feed formulation, there are usually two separate ME values for lipids.

BACKGROUND INGREDIENTS IN REFERENCE DIET As with any other digestibility experiment, lipid digestibility studies must pay close attention to the composition

and the types of ingredients used for the reference diet. High levels of soluble NSPs reduce the digestibility of lipids through elevated gut viscosity that affects the mixing of enzymes and their target substrates in the digesta, reduces the effectiveness of micelle formation (particularly important for saturated lipids) and changes the gut microflora, leading to increased deconjugation of bile salts. Also, if excess amounts of minerals, such as calcium, are present, they can result in the formation of soaps, which will render certain fatty acids unavailable for digestion and absorption.

CHEMICAL CHARACTERISTICS OF LIPIDS The ME value of lipids is affected by a number of intrinsic factors related to their chemical characteristics. These include degree of saturation, change length, positions of the double bonds, ester linkages, ratio between saturated and unsaturated fatty acids, and the level of free fatty acids present in the lipid. It is important to understand these characteristics because sometimes a blend of lipids, rather than a single type of fat, is the product of interest in digestibility experiments. For instance, every 1% increase in free fatty acids can reduce the ME value of lipids by approximately 0.1%; xylanase responses in diets based on viscous grains are very different between saturated and unsaturated lipids as well as between medium-chain versus long-chain lipids.

5.3.3 Indirect measurements of digestibility

Animal experiments are costly and time consuming. Therefore, simulating digestibility in a laboratory situation (*in vitro* digestibility methods) using mathematical modelling (prediction equations, for instance) or applying 'black box technologies' such as NIRS has many attractions including the provision of more rapid, less expensive tests for measuring the nutritive value of feed ingredients. In addition, ethical considerations regarding the use of animals for scientific experiments mean that the use of indirect techniques not involving animal experimentation for measuring nutrient digestibility will continue to be explored. The main challenge for any indirect technique is the ability to produce nutritive value data that correlates well with *in vivo* values.

In vitro *digestibility methods*

The ability to mimic the digestive processes in the gastrointestinal tract of animals and humans is of great interest from the perspectives of both cost and operation (speed and ease of measurement). Protein and dry matter are two items that have received significant attention in developing *in vitro* methods. The assays range from a simple one-stage digestion to multi-stage processes that have numerous steps and require specialized equipment and technicians to carry them out. In the end, such assays turn out to be neither inexpensive nor rapid.

In vitro techniques were the subject of an entire symposium (Fuller, 1991) and there are many other great reviews available on the topic (Sibbald, 1987;

Farrell, 1999; Moughan, 1999; Ravindran and Bryden, 1999). It is sufficient to say that nutrient digestion in pigs and poultry is extremely complex. It involves enzymatic, microbial and mechanical processes. These processes are dynamic and are affected by numerous intrinsic and extrinsic factors. Thus, obtaining digestibility values that mirror values obtained using *in vivo* methods is difficult, if not impossible.

It is apparent that numerous attempts over a long period have failed to provide unequivocal evidence that nutritive value for pigs and poultry can be predicted with sufficient accuracy and precision by simple, low-cost physicochemical measurements, used either singly or in combination. Nevertheless, it is highly desirable to continue to explore these simple measurements in the expectation that useful statistical relationships with more complex measurements will emerge, or that simple measurements can be used to fine-tune prediction equations based on more powerful mechanistic modelling.

Simulation models

When there are reliable *in vivo* data, there is a possibility of modelling them mathematically. Modern agriculture would struggle to function without simulation models and monogastric animal nutrition is no exception. Indeed, mechanistic models are used widely in pig (Black *et al.*, 1986; Noblet *et al.*, 1994, 2004) and poultry nutrition (Gous, 2014) and the topic is exhaustively reviewed (Gous *et al*., 2006; Sakomura *et al*., 2014).

Spectroscopic technologies

There is a wide range of spectroscopic techniques, covering the electromagnetic spectrum of light. All of these techniques use light to interact with matter, and explore features of a sample to learn about its consistency or structure. One such technology that is of great interest to the animal nutrition industry is near-infrared spectroscopy (NIRS). Its application in animal nutrition research and industry includes the measurement of not only nutrients but also nutritive value indicators, such as DE in pigs and ME in poultry. It is rapid and non-destructive for samples. With time, even more powerful spectroscopic technologies will become applicable in agricultural industries. One such example is Raman spectroscopy, which is currently used in the field of chemistry to fingerprint molecules.

5.3.4 Summary

Determining 'nutritive value' requires two elements: the feed ingredient and the animal to which it is fed. Nutrient digestion and metabolism are highly complex and hence nutritional science has to make certain assumptions. First, the nutritive values obtained for individual ingredients are regarded as additive when two or more ingredients are combined. In reality this is not true, due to interactions among ingredients during processing and in the gut, and the physiological and immunological and gut microflora status of the

animal at the time of feeding. But without this assumption, it is not possible to formulate feed. Second, the digestibility of a nutrient is assumed to be constant, irrespective of the inclusion level of the ingredient containing that nutrient, which again is not always true. This is the basis of much published work that employs various levels of inclusion for a given test ingredient. The database values used in our day-to-day feed formulation come from such work. Third, nutritional imbalance is ignored when measuring the energy or digestibility of individual ingredients, as discussed earlier in the case of the practical diet replacement assay. Some imbalances are unavoidable for certain ingredients because it is not always possible to find a reference diet that remains nutritionally balanced after a portion of it is substituted by a test ingredient (for example, as with a high-fibre by-product).

The reality is that applied research is, in many aspects, like an art rather than an exact science. However, every effort should be made to minimize errors where possible through meticulous planning and operation. You should pay attention to the design of the experiment, health and husbandry of the animals, thorough characterization of ingredients used, formulation of the feed, cross-checking of final diets, and collection and analysis of data. These factors are all discussed throughout this book. A simple determination of dry matter contents of your ingredients, both at the time of sourcing and at the time of diet mixing, can avoid costly errors in calculation. Also, measuring the chemical composition of the final diet and comparing it with calculated values provides you with a cross-check that controls inaccuracy in feed formulation, ensures the quality of diet mixing and, more importantly, could help avoid the cost of running a failed experiment.

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6 Designing, Conducting and Reporting Swine and Poultry Nutrition Research

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6.1 Introduction

To be successful, an experiment needs to be properly designed with a clear objective in mind, executed with efficiency and appropriate attention to detail, correctly analysed and interpreted and then presented with clarity and comprehensiveness (Festing and Altman, 2002). This chapter will address all of these aspects, but the primary objective is to assist the reader to produce reports from studies that are complete and detailed. For reasons explained below, it is becoming increasingly important to be able to compare different experiments that have been conducted on the same or similar topics. This can only be done when the individual experiments are completed correctly and when the reports of the experiments contain sufficient detail as to allow such comparison.

The pig and poultry industries have evolved at a very rapid rate. While some of the changes are structural in nature, many of them are the consequences of developments in production technologies driven by a strong global research and development sector. In other words, these industries have a strong interest in science and utilize research as an important basis for management decisions. When new technology presents itself, assuming that it makes sense practically and financially, it will be rapidly adopted.

Because science is so important, most large pork and poultry producers, as well as feed and genetics companies, maintain internal research capabilities that allow them to develop and/or evaluate new technologies under conditions that reflect their own commercial enterprise or market conditions. Consequently, an increasing proportion of research is conducted in proprietary facilities; however, there is still a great need for, and much interest in, research that is carried out in the public domain.

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Research in the public domain fulfils many important roles and in general it serves:

- as the scientific foundation for innovations in the future;
- a very important role in establishing modes of action: when a mode of action of a new technology is understood, the technology can be applied in commercial production in a more focused and effective manner;
- as an independent venue for evaluating new or evolving technologies, providing more credibility than might be the case if all of the data were generated in proprietary laboratories;
- to provide a valuable venue for research for those companies that choose not to maintain their own internal facilities;
- as a platform for training graduate students. Because science is so critical to the future evolution of the pork and poultry industries, an adequate supply of new graduates is necessary to support research and development activities, as well as associated sales efforts, in the private sector. It also replenishes researchers who retire or move into other roles in their careers. As a consequence, the number of positions requiring advanced degrees in nutrition has increased and will continue to do so.

While the pork and poultry industries are rapidly evolving at an increasing technical level, so too is the world of research. Improved and almost instantaneous communications facilitate a much greater level of collaboration. Faculties at universities are no longer restricted to collaborations with colleagues within a geographical area, but can now just as easily work with others halfway around the world. Partnerships between the private and public sectors in research are becoming increasingly common and will no doubt continue to grow. The tools of research are becoming much more sophisticated; procedures that used to take weeks or months and cost perhaps thousands or even tens of thousands of dollars can often be done in a few days at a fraction of the former cost. New capabilities in the laboratory are arriving with increasing frequency, making research more productive and more precise along the way.

Recent developments in global communications not only make it easier to collaborate, they also permit the communication of research results to be completed at lower cost and in less time than in the past. In other words, new technologies are great friends of both the farming sector and the research community. However, to meet the needs of increasingly technically focused pork and poultry industries, expectations of the research community are changing. The following are two typical examples.

First, consider the manner in which industry makes decisions on new technologies. The research community ideally carries out careful statistical evaluation of its data, determining if the reported outcomes are likely to be 'real' or are simply the consequence of random chance. The research community has long accepted that a P-value of 0.05 is sufficiently stringent to lead to reasonably well-founded conclusions. Loosely translated, this means that there is only 1 chance in 20 that the differences among treatments reported in the experiment were due to chance, and that there are 19 chances

out of 20 that the differences were not due to chance. However, the industry may view this differently than public institutions in at least two ways. The industry may be willing to take more risk than 1 chance in 20; if the rewards of adopting a new technology are sufficiently great, and the cost is modest, it may accept a level of risk of 1 out of 10 or maybe even 1 out of 5. Industry logic would be that the new technology is worth a certain rate of return; and if there is 90% or even 80% certainty that the benefit is real, the risk may be acceptable in order to benefit from the reward. Consequently researchers should report the actual P-value, rather than a range such as $P<0.05$ or $P>0.10$. When the actual P-value is reported, readers can make their own decisions about whether the potential risk is worth the perceived reward.

In this way, it can be seen that the two communities view risk differently. Researchers need to be certain that their conclusions are well founded and based on sound experimental and statistical procedures. Their 'reward' is confidence in their data and avoidance of inappropriate conclusions. However, pork and poultry producers, while also wanting a degree of certainty in their decision-making processes, realize that there is also risk in not adopting a valuable new technology as well as the risk of adopting one that is not real. To researchers, the motivation is to avoid concluding that a technology is beneficial when it is not, while producers want to avoid missing a technology that might be beneficial, while at the same time avoiding technology that is not beneficial. There is less risk to researchers being very conservative in their conclusions, whereas producers pay a price for being too conservative.

Second, there is the question of repeatability. People unfamiliar with research are surprised to learn how difficult it is to repeat the results of a single experiment. People in the industry are more likely to put greater faith in multiple experiments reporting the same outcome with a higher P-value, than a single experiment reporting the outcome with a lower P-value. Therefore, both the research community and producers should place greater emphasis on the repeatability of research results as the way to achieve the greatest confidence in a particular outcome, such as the efficacy of a new technology.

Even when there is confidence in the outcome based on sound statistical analysis, there is also a desire to understand the mode of action of new technology or novel research results. If a mode of action is not understood, it is much more difficult to adopt a new technology correctly; if the critical conditions required for a new technology to work are unknown, repeatability will be more difficult to achieve. What works on one farm may not work on another. For example, a new technology may only be effective under a particular dietary regime, or with a particular health status. Thus, the design of the experiment(s) becomes much more critical, so that underlying conditions of the experiment are considered in both the conduct and reporting of individual experiments and in the drawing of conclusions.

Given that mode of action and repeatability of experimental outcomes are important in the development and adoption of new technology, the ability to compare the results of multiple experiments is extremely valuable. The objective of this chapter, then, is to consolidate thoughts on the proper design,

conduct and reporting of nutrition studies for the benefit of researchers but also for the users of research results.

6.2 Planning the Experiment

6.2.1 Defining objectives

By far the most critical step in designing an experiment is defining the objective. This is much easier said than done, as it is often difficult to clearly enunciate the objective in a statement that leaves no doubt as to what is expected to be achieved. However, a clear and concise experimental objective makes it much easier to define methodology and, interestingly, also simplifies the process of presenting and discussing results as well as drawing conclusions. Since the conclusion obtained from an experiment should refer back to the original objective and the manner in which data are presented and discussed should also have the same focus, the importance of a well-defined objective becomes apparent.

Similarly, there should be a hypothesis that puts the objective into perspective, perhaps based on previous research in the same research group or in publications by others that appear in the literature. A clear hypothesis helps to validate the objective, by providing a solid scientific basis or expectation. It clearly demonstrates that the researchers have an image in their minds of how the experiment should turn out and why. At the end of the experiment, the hypothesis may not be supported by the data, but it still provides a focus for discussing experimental results. It may also reveal, if the hypothesis is based on current dogma in the field, that the subject may be less well understood than previously expected. As such, the hypothesis, along with the objective, points to the next step in experimental planning.

6.2.2 Written protocol

A written protocol is essential to a successful experiment. It should contain enough detail so that those who plan the experiment and those who implement it are in complete alignment. The protocol can also be used to ensure that all required regulatory requirements are met before the study starts, such as animal care approval.

A wide variety of protocol formats are successfully employed, but they should include a detailed description of the experimental treatments, the selection of animals from the herd and how they will be assigned to the experiment and to the dietary treatments, the timing and methodology of the collection of data and biological samples, the handling of data and biological samples, the methods of assay, the statistical methods to be employed, the disposition of the animals, and unused feed and other materials at the completion of the experiment.

If the facility is undertaking research on a regular or continuous basis, the development of standard operating procedures (SOPs) is advised. SOPs

are a key part of communication in the laboratory, ensuring consistency of everything from animal handling and sample collection to sample assay. When SOPs are in place, the protocol for individual experiments can be abbreviated, as it is no longer necessary to repeat this level of detail for each experiment. SOPs are particularly helpful in laboratories involved in student training and can be useful in training new staff as well.

6.2.3 Review of facility capabilities

Prior to developing the experiment protocol, it is important to take stock of the capabilities of available facilities as they relate to the experimental objectives. Such capabilities will include the following.

- Animal genotype
  - Relevance: is the genotype typical of what is utilized by the target audience of the research?
- Animals: are performance outcomes relevant to the target audience?
  - One of the challenges of all research facilities is their phenotypic relevance. If the average pig in the research facility is growing at the rate of 1000 g/day, and the typical pig on the farm is growing at 845 g/day, translating research outcomes to commercial practice becomes much more difficult.
  - This challenge applies as much to research facilities operated by pork producers as it does to public research facilities.
- Facility capacity
  - Number of pens and number of pigs per pen.
  - Please see discussion below on proper sizing of experiments.
- Flooring and penning
  - Flooring and penning materials should recognize both the comfort of the animals and the particular needs of the research. Many animal care documents provide guidance on this topic (FASS, 2010).
  - In growth trials, the materials should be appropriate to the age of the pig and free from edges or protrusions that could cause injury.
  - In digestibility trials, the same requirements apply as for growth trials, with the addition of the ability to collect urine and faeces, quantitatively and/or separately, as required by the protocol. In all instances, urine and faeces need to be collected free from contamination, especially with feed.
  - In trials utilizing animals surgically prepared with collection devices, such as catheters or cannulae, an additional requirement arises, that is, freedom from any materials that could result in damage to the device or the surgical incision.
  - When animal movement must be restricted to facilitate sample collection (e.g. blood, urine, faeces) or physiological observation (e.g. heart rate, respiration), the crate should have a high degree of comfort combined with great flexibility in its dimensions (height, width

and length) to accommodate the variation in size among animals, as well as their increase in body weight as the study progresses.

- Ventilation design and capacity
  - Ideally, the facility should be able to maintain a consistent thermal environment for the pigs for the duration of the experiment, so that it does not compromise and confound experimental outcomes.
    - This level of control is rarely available for summer months, due to the cost of installing and operating air conditioning. It is less of a problem in the winter, when heating capacity should be sufficient to maintain a constant facility temperature independent of the outside ambient temperature.
    - If air conditioning is not available in the summer months, optimal management of the ventilation system is essential to minimize excessive facility temperatures. As a general rule, the temperature within the facility should never be more than 2°C above the outside ambient temperature during the summer months.
  - In addition to maintaining a healthy and consistent environment, there is the need for monitoring, ensuring that target conditions are achieved for the pigs or birds, at least to the extent possible given ambient conditions outdoors. This capability in turn establishes the ability to report the environmental conditions in resultant reports and manuscripts.
- Animal and bird handling capability
  - Proper facilities for animal handling are as important as facilities for animal housing. Included in this are facilities for procedures as simple as collecting body and feed weights to more complex capabilities for surgical preparation of animals, as well as for the collection of biological samples, such as blood, urine and faeces.
  - Such facilities should be designed for the safety and wellbeing of the animals but also for the people involved.
- Sample and data collection
  - Data integrity, to be discussed in more detail below, is critical to the success of any experiment. It is also an essential part of the institution's and researcher's credibility.
  - Collection of quality data, such as that related to body weight or feed intake, or physiological measurements, can only occur with the proper equipment and properly trained personnel. The equipment must be properly maintained and must be calibrated before each use.

6.2.4 Statistical plan

Traditionally, nutrition research has been conducted in such a manner as to reduce biological variation in order to maximize the power of the experiment (Festing and Altman, 2002). Thus, animals selected for an experiment are screened to remove those that were much bigger or smaller than average,

or that possessed some uncertain or questionable traits. While this approach has great merit, and indeed does improve the power of the experiment, it also excludes an evaluation of how outliers in the population respond to the experimental regime under evaluation.

To pork producers, for example, treatments that have minimal effects on average performance of a group of pigs, but that reduce variation or provide particular benefit to smaller or poorer performing animals, could be of great value. 'Normal' variation in the body weights of a population of healthy pigs at a market weight of 125 kg can be represented by coefficient of variation (CV). This CV has been defined as 9.7% (Beaulieu *et al.*, 2010). Thus, to encompass 95% of all pigs in a population, the range in body weights will be greater than 47 kg, i.e. from 101 kg to 149 kg! If the herd has health problems that increase body weight variation, the range could be even greater. Consequently, any technology that will reduce body weight variation will have great value to pork producers. Yet, selecting a population of pigs at the beginning of an experiment to eliminate slower-growing or poorly performing pigs prevents an aspect of a study that could have great value. The pork industry has addressed this problem by utilizing large barns for research, with 1200 or 2400 head of pigs, and in which almost all pigs in a group are put on test. Only minimal selection takes place at the start of the experiment. With 40 pens or more available, it is possible to distinguish the response to a treatment based on body weight, if the pigs are blocked by body weight. In this scenario, the performance of smaller and slower-growing pigs can be separated from that of more advantaged animals. To be able to undertake a comparison of response within subsets of a population, a randomized complete block designed experiment is required.

Power test

Chapter 2 covers statistical designs and interpretations of the results in detail. Thus, this section will discuss some statistical aspects in the context of developing the experiment protocol. One of the first considerations in working out an experimental protocol is to run a power test to determine how many observations will be required in order to pick up desired statistically significant differences among treatment outcomes. Many textbooks on statistics include clear instructions about doing a power test, but perhaps the most convenient ones are found on the Internet. To undertake the calculation, the following information will be required: the desired magnitude of treatment differences that can be detected as statistically significant; the standard deviation of the group of pigs (generally derived from previous experiments or published experiments conducted under similar conditions); the P-value desired; and the power of the test. The latter is typically 0.80 but can be as high as 0.95; it refers to the likelihood of detecting a significant difference if one exists.

While a power test should be conducted, often experience in undertaking similar research in the same facility, in other words historical experience, suffices to ensure that adequate replication will be achieved.

The standard deviation of the measured outcomes, such as average daily gain, or feed efficiency, or apparent total tract digestibility of gross energy, or plasma urea nitrogen, is the critical determinant of the power of an experiment. While experience with the same genetics in the same facility conducting similar studies will provide a value for standard deviation, this is not a static number. It can rise or fall with such influences as the level of stress or the health status of the animals. Also, if the diets are deficient in one or more nutrients, population variation may increase.

Statistical models and evaluation

The development of the experimental protocol should also include the selection of the appropriate statistical model that will be used upon the completion of data collection. Perhaps the first decision is the selection of analysis of variance (ANOVA) or regression analysis. A derivative of analysis of variance is analysis of covariance (ANCOVA), which corrects for potential bias in the data. For example, initial body weight is often used as a covariate in growth studies, since it is well known that initial body weight can affect growth rate, feed intake and feed efficiency, independent of the experimental treatments. If analysis of covariance is to be used in this instance, it is critical that the range in initial body weights is such that there is considerable overlap in all treatments. If such overlap does not exist, it is not possible to differentiate differences in performance outcomes between those due to treatment and those due to differences in the covariate.

In growth studies, repeated measures analysis is often required, if intermediate as well as overall 'start-to-end' data are collected. Because the performance of the pigs in time period #2 will be at least somewhat dependent on their performance in time period #1, this must be accounted for. If repeated measures analysis is not conducted on such a dataset, errors in statistical conclusions can occur.

Once the dataset is complete, ANOVA or ANCOVA can be conducted. Most data collected in swine and poultry nutrition studies generate parametric data, so ANOVA and ANCOVA are appropriate. It should be noted that when analysis of variance is undertaken, three assumptions are required to be true: (i) that the data have a more or less normal distribution; (ii) that the variances of all treatment groups are the same; and (iii) that the observations are independent of each other. The latter means that the pig's or bird's response to one treatment is not influenced by the response to another treatment. For example, if one is titrating levels of amino acids, or levels of feed additives, analysis of variance is only appropriate as the basis for subsequent regression analysis through the use of polynomials. However, in this instance, regression analysis is preferred. The use of mean separation tests is not appropriate in this instance.

If the data are not distributed normally, or the variances are not the same across all treatments, some form of transformation will be required. Common examples include logarithmic transformation, logit transformation, or a square root transformation, depending on the nature of the data.

The experimental unit is the smallest unit to which the treatment can be assigned (Easterling, 2015). For example, in a study evaluating the impact of a feed additive, the treatment can only be assigned to the pen or cage for feed intake and the calculation of feed conversion ratio, and not to the individual pig or bird within the cage. Thus, in this case, the pen or cage must be the experimental unit. Since individual body weights can be measured within the pen or cage, the pig or the bird can be considered the observational unit, as distinct from the experimental unit. In mixed model analysis (such as PROC MIXED in SAS), if the individual weights are employed, then the error associated with the pen must be included in the model as a random effect. However, it is more common to utilize average pen or cage weights, eliminating the need to consider within-pen error and consider the observational unit and the experimental unit to be the same.

There may be some exceptions to this definition. For example, some studies may involve an evaluation of nutritional regimen in pigs or birds differing in health status or under different environmental conditions. According to the above definition, since the disease regime or the environmental regimen can only be assigned by barn or isolated room, barn or room should theoretically be the experimental unit. This is logistically impossible, because experiments would have to be very, very large in order to satisfy this definition. Therefore, in this instance, an assumption must be made that the effect of barn is negligible, and it is possible to compare pens within one barn against pens within the other barn, or pens within one room against pens within another room. Sometimes there are data to support this assumption, such as the results of previous experiments that revealed 'no barn effect' or 'no room effect' on pig or bird performance. The problem can be minimized if the animals or birds placed in both barns are randomly selected from the same population.

Once analysis of variance has been completed – and the effect of treatment found to be significant – if more than two treatments were investigated, some method must be used to determine which treatment means differ. If there are only two treatments, a simple t-test will suffice. If there are more than two treatments, a large number of statistical tests, called multiple comparison tests, are available. The selection of the appropriate multiple comparison test is a complex topic and open to much discussion – and consternation. Simply stated, there is concern that some tests are too conservative, leading to Type II errors (finding treatments to be not different when they in fact are different) and others are too generous, leading to Type I errors (finding differences when there are in fact no differences). It is up to the researcher to determine which test(s) they wish to employ and to consider the conclusions of the study in the context of the statistical analysis employed. One of the most common multiple comparison tests is the Least Significant Difference or LSD test; it is one of the least 'rigorous' and most likely to lead to Type I error. Nonetheless, due to its simplicity, it is widely used in animal studies. Other common tests include LSD with Tukey–Kramer adjustment, Tukey, Bonferroni, Scheffe, etc.

A slightly different option is the multiple range test. Whereas the multiple comparison tests define a single interval to test differences among all means,

the multiple range tests use intervals that differ, depending on how close the means are within the array of treatments. Examples include the Duncan and Student–Newman–Keuls methods.

As an alternative to multiple comparison tests or multiple range tests, pre-planned single degree of freedom F-tests, such as orthogonal contrasts, can be employed. To avoid bias, the comparisons should be selected during the planning of the experiment, not after its conclusion.

In some studies, such as when a range of concentrations of a nutrient or an additive is being evaluated, regression analysis may be more appropriate. Regression analysis is particularly useful for estimating the relationship between a dependent variable and an array of independent variables. Correlation analysis can also be useful, by determining to what extent changes in the independent variable might influence the dependent variable. An example is the correlation between dietary fibre and digestible energy content of a feed or feedstuff.

Numerous textbooks are available to provide more detail on the topic of data analysis (Sprinthall, 2011; Montgomery, 2012; Lyman and Longnecker, 2015).

6.2.5 Animal care standards and pig management

Virtually all publicly funded research facilities have animal care committees ensuring that the wellbeing of animals used in experiments is maintained. Many private research facilities also maintain their own internal animal care committees for the same purpose.

Most reputable journals will not accept manuscripts for publication unless it can be shown that the research was conducted in conditions that met or exceeded accepted published standards of animal care. The *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010) defines a standard of care that is accepted at many public research institutions. Of course, individual institutions may have additional requirements above those listed in this document. Other countries or regions have their own publications that serve the same intent.

6.2.6 Data integrity

Critical to achieving success in any experiment is ensuring data integrity, accuracy and precision. Accuracy refers to closeness of observed means to the actual population mean. In other words, accuracy refers to the achievement of measurements that are the same as the true value within a population (van de Pol, 2012).

Precision is different and refers to the proximity of individual observations about the observed mean. Stated another way, precision refers to lack of scatter. It refers to the repeatability of a measurement (van de Pol, 2012). If the same measurement is taken five times, and all measurements are the

same, that is considered highly precise. If the measurement varies each time it is taken, then it is considered less precise or even imprecise. A measurement can be highly precise, but inaccurate. It is very important that a high degree of precision should not be interpreted as accurate. A consistent error is still an error. Just because the same answer is achieved does not mean it is accurate, only precise.

With respect to accuracy and precision, it is possible to have a dataset fall into one of four categories (Fig. 6.1): (1) accurate and precise; (2) accurate and imprecise; (3) inaccurate and precise; or (4) inaccurate and imprecise. Intuitively, the goal of all researchers is to obtain category 1 data. Not only are the data correct, but there is little variation about the mean; this allows the study to identify small differences as statistically significant. Category 2 data still have correct treatment means, but the variation about the means is larger, making it more difficult to detect statistically significant differences. Because both categories 1 and 2 have accurate treatment means, these datasets are considered generally acceptable, although obviously category 1 is preferred. Category 3 and category 4 data are unacceptable because, irrespective of precision, the treatment means will be incorrect, potentially leading to incorrect conclusions. Category 3 datasets may be the most troublesome, because small variation may not only result in incorrectly identified significant differences, but may also lead to false confidence in those incorrect results. In other words, a small degree of variation is often interpreted as reflecting high quality data. Frankly, category 4 data are generally of less concern because wide variation often prevents determination of statistically significant differences.

The construction of many larger-scale research barns has provided the pork and poultry industries with a tremendous opportunity to achieve greater precision in growth studies than was ever possible in smaller facilities typical of many public institutions. For example, a well-run large-scale growth study with pigs from 20 kg to 125 kg should have a standard error for average daily gain (ADG) of about 10 g/day and for average daily feed intake (ADFI) of about 30 g/day. Smaller-scale studies, with fewer pens and fewer pigs per pen, might have standard errors for ADG closer to 15 or even 20 g/day and for ADFI, 50 g/day or greater.

Scale of experiment does not necessarily lead to greater accuracy. In this respect, category 3 datasets (previously described) are the most worrisome for reasons already stated. The increase in large-scale wean-to-finish research barns with 1000 head or more on test, representing perhaps 40 pens, may give the sense of greater accuracy, when it is precision that is improved. Errors are still possible and researchers must be vigilant for such errors.

It is clear then that the achievement of data integrity requires careful attention to detail. The following are important practices that help to achieve dataset integrity.

Standard operating procedures (SOPs) are a first step to ensuring that all experimental activities are carried out correctly. SOPs should be readily available to all persons involved in research. Well-written SOPs are clearly presented, so that all readers can understand their content. They should

Fig. 6.1. Illustration of the four categories of data, as it relates to accuracy and precision.

include the objective of the SOP, so that the reader understands why following a particular SOP is important. Furthermore, SOPs are living documents and should be constantly reviewed and updated as procedures evolve and are improved over time. One of the risks of SOPs is that, after the time spent putting them together, they then sit on a shelf in a binder, or in a computer server, and are never looked at again.

With SOPs in place, an associated critical step is training. All personnel involved in a particular research activity should be trained on the relevant SOPs; training must include why the procedure is important, what the procedure is and what to do if the procedure is not followed. Training can be accomplished in many different ways. One favourite is to have the trainee initially watch the trainer, then have the trainee take on more of the procedure until, at the end, the trainer is watching and the trainee is doing it all. At this point, the trainer can determine if the trainee is capable of conducting the procedure without supervision. Depending on the procedure, training can take an hour or two, or a number of days, or even weeks. A training log should be kept so that the supervisor knows who is properly trained on each procedure. No staff members should be given the responsibility of doing a procedure unless they have been fully trained.

There are other specific procedures that can be included in a trial to improve accuracy and precision. One is to utilize a set of standard weights, ensuring that the scale is operating correctly and accurately. To the greatest extent possible, the standard weights used to calibrate the scale should be similar to that of the weight being measured. Of course, if a pen scale weighing 3000 kg of pigs is being used, then smaller standard weights will be necessary for practical purposes. However, each corner of the pen scale should be tested, to ensure that each load cell is working correctly.

In the instance of growth studies, proper feeder adjustment is more important than may be thought. It is widely known that feed wastage reduces the accuracy of feed intake data. Measuring feed 'consumed' is actually feed disappearance, meaning the sum of feed intake and feed wasted. The greater the wastage, the greater will be the inaccuracy of feed intake data. In addition, variation in feeder adjustment results in greater variation in feed wastage among feeders, which will in turn reveal itself as reduced precision.

Furthermore, within a given experiment, even if wastage is completely avoided, inconsistent feeder adjustment can increase variation in the data, as some animals will have easy access to feed and thus eat to full appetite, while if the feeder is adjusted too tightly, some of the pigs will reduce intake (Smith *et al.*, 2004). Feeder adjustment can also impact feeder capacity; if adjusted too tightly, each pig in the pen will spend more time eating, meaning that the theoretical capacity of the feeder is reduced (Smith *et al.*, 2004).

The same concept applies if water intake is being measured. If flow through a drinker is being measured, the measurement is not really water intake but water disappearance. Like feed, water disappearance is a combination of intake and wastage. Dish drinkers, or so-called wet/dry feeders, are known to reduce water wastage, but their level of accuracy is not well defined.

Water meters must be selected with great care, especially if measuring water on a pen basis. Inexpensive meters often do not measure liquid flow very accurately if the flow rate is small and intermittent. Thus, meters measuring water on a barn basis are likely to be more precise, but provide little information on the response to individual treatments.

In studies of energy or nutrients, it goes without saying that assays of the diets should be undertaken to confirm that assumptions made during formulation were correct and that diet composition targets were met. For example, if an experiment is investigating the requirement for lysine, the diets must be assayed for lysine and other essential amino acids. Assays of lysine are required to ensure that the target treatment levels were achieved in reality. Other amino acids should be assayed to ensure that a deficit in the intake of a secondary amino acid is not impairing the ability of the pig or bird to respond to lysine. In the same manner, if enzymes are being evaluated, the diets should be assayed for enzyme content, to ensure that treatment objectives were being met.

It is also possible to determine the actual energy concentration in diets used in a growth study; markers can be added to the feed and faecal samples and can be collected at specific time points in the experiment. Sometimes, if the diet contains a sufficient concentration of acid-insoluble ash, markers do not need to be added (Hernández *et al.*, 2004; Jurjanz *et al.*, 2014). If there is no suitable endogenous marker present, exogenous markers such as titanium dioxide can be added to the feed at the rate of 0.4% at the time of mixing. In swine, collecting fresh faecal samples over the course of 2 or 3 days from each pen, or a randomly selected subset of pens, will provide sufficient sample to estimate the digestible energy (DE) content of the diet (Holloway and Patience, 2014). If metabolizable energy (ME) or net energy (NE) is desired, appropriate prediction equations can be applied to make the conversion (Noblet, 1994; NRC, 2012). The faecal samples should be fresh and should be placed in cold storage as soon as possible, to minimize further fermentation. Faecal samples can be collected five days after the marker has been added to the feed (Jacobs, 2011).

If feed additives are being evaluated, diets must be appropriately assayed to ensure that the active ingredient(s) are present and at the desired concentration. Sometimes it is not possible to assay for the additive and added nutrient in the finished feed, due to the sensitivity of the assay. In this instance, the premix should be assayed for the nutrient or compound of interest and then for another constituent; the final diet is assayed for this other constituent to confirm addition of the premix at the proper levels to the final diet.

Experimental diets should also be assayed for a cross-section of nutrients, to help confirm that the diets were mixed according to the formulation. The exact assays will depend on the nature of the experiment and of the diets. Finally, mixer efficiency tests should be conducted to confirm mixing accuracy and to ensure that the correct mixing time is being used.

All animals that die during the course of the experiment, or are removed for humane reasons, must be recorded, including their weight, the date of

removal and the reason for removal. This information becomes part of the final report. When deaths occur in group pens, the calculation of ADG and ADFI must be modified to correct for the changing number of animals per pen. There is no perfect way to make such adjustments, but the most common method is to calculate on the basis of pig days. This assumes, probably incorrectly, that the removed pig was growing at the same rate prior to its removal, as it would have if it had not been ill or injured. It also assumes, again probably incorrectly, that the pig was consuming as much feed as it would have, if it had not been ill or injured, up to the day of its removal. Unfortunately, there is no way of knowing the actual growth rate or feed intake of the pig, unless individual feeding stations are employed to correct this error. For this reason, data collected in the presence of a large number of removals will be unavoidably biased.

6.3 Interpreting Experimental Outcomes

The raw data should be carefully scrutinized, to ensure that it is free of error. One useful step is to select one pig or one pen at random and complete all calculations by hand; this will ensure that the spreadsheet or database is making the correct calculations. Another useful step is to draw a scatter plot of the data, to visually identify outliers. However, outliers should only be discarded if there is independent evidence that the observation is incorrect, such as an entry in the log book made at the time of collection expressing uncertainty about this measurement.

In any experiment involving animals, death or euthanasia of sick animals can occur. If the incidence of death or removal is greater on some treatments than others, this should be recorded and noted.

6.4 The Experiment Report

One of the motivations for writing this chapter, and indeed this book, is the desire to improve and standardize reporting of research outcomes, whether in the form of a research report or a journal manuscript. The need is greatest when people are undertaking a meta-analysis of the literature, because missing information undermines its quality. This section will attempt to identify key information that should be included in any research report, to allow comparisons across studies conducted at differing institutions located in different countries or continents.

6.4.1 Introduction

Typically, the introduction should provide a brief description of the current knowledge on the topic of interest, and why the study is important to advancing our understanding. It has been suggested that the references cited should

be as current as possible. When recent or highly relevant publications are missed, the reader is left to wonder if the researchers were themselves current on the topic prior to conducting the research. The introduction should also provide a clearly stated objective(s) of the research as well as a statement of the hypothesis being tested. The statement of the objective is particularly critical, as explained previously.

6.4.2 Materials and methods

Following is a list of information that should be included in the materials and methods section of any manuscript or report:

- Animal ethics
  - Details of approval of the protocol by the local animal care committee should be provided, if available.
- Description of the animals
  - Genotype
    - Definition of the sire and dam lines from which the experimental animals were derived, or the genotypic line in the case of birds.
  - Age and weight
    - If the pigs start the trial 'following weaning', details should be provided. If the animals were given time to adjust to weaning prior to the start of the trial, details should be provided. A pig weaned for 3 or 4 days is a different pig than one that starts a trial on the day of weaning.
    - The weight of the pig or bird should obviously be provided.
    - In the case of sow studies, the parity of animals needs to be reported.
  - Gender
    - The gender of the pigs should be defined; and, if not all of one sex, how they were allocated to treatment.
  - Selection
    - Is the whole population of available pigs or birds included in the experiment, or was a subset selected to achieve greater uniformity?
      - The degree of selection should be explained, since a selected sub-population may perform differently than the total available population.
      - On what basis were the pigs or birds selected? Was it based on age, body weight or pre-test performance?
    - Were the pigs or birds on a previous experiment and, if so, what was the nature of that experiment? Were the pigs balanced across treatments in a manner that would prevent bias due to previous dietary treatment?

- Health status
  - Some description of the health status of the herd or flock during the course of the experiments helps in the interpretation of the results.
  - A description of any vaccination programme would also be helpful.
  - If a medication programme is followed, it should be defined.
  - The number of animals treated for illness or injury should also be recorded.

- Housing
  - The facilities housing the pigs or birds should be clearly described, including stocking density, pen or cage size, as well as the number of pens/cages per treatment.
  - Feeders should be described in sufficient detail for the reader to understand how this might impact performance. This might include the number of feeding spaces per pig or the length of feeder available per pig. It would also include whether the feed was delivered in liquid form, via a dry feeder or via a wet/dry feeder.
  - Water supply should also be described, both in terms of the type of drinker and the number of drinkers per pen or cage.
- Diet composition
  - Tables should provide the ingredient composition of all experimental diets, as well as information on the nutrient composition, relevant to the topic of the study.
  - Aspects of the diet directly related to the study objective should be analysed and the results reported. Examples would be amino acids in studies designed to determine an amino acid requirement, enzymes in studies evaluating the effect or mode of action of enzymes, or chemical composition of the test ingredient if it is being evaluated as a potential feedstuff for pigs or poultry.
- Feed supply
  - In what form was the feed delivered?
    - Mash or pellets; if the latter, specify the size of the pellets.
    - Particle size of the main ingredients such as corn, wheat, barley, soybean meal, DDGS, canola meal, etc. should also be provided. Particle size of the mixed diet is of limited value.
  - How was the feed provided to the pig?
    - *Ad libitum* or according to a scale?
      - If other than *ad libitum*, the method of determining the daily allowance should be explained.
- Water supply
  - If water intake was being measured using flow meters, were they sufficiently accurate to provide meaningful data? Were the meters calibrated?
  - If water intake was being measured, how was wastage accounted for?

- If relevant to the topic of the study, the chemical composition of the water should be provided.

- Environment
  - The seasonality of the study should be reported, along with the mean and range in facility temperature during the course of the experiment.
  - Preferably, the start and end dates of the experiment will be reported.
  - In the case of studies involving environmental stress, humidity would also be required.
- Data collection
  - If weights (body weights, feed, etc.) were collected, how were the scales calibrated and how often were they calibrated?
  - If carcass data were collected from a commercial abattoir, how were the data validated? On high-speed lines, it is sometimes difficult to ensure that each pig is assigned its correct carcass data unless this is somehow validated.
  - If blood or tissue samples were collected, explain how they were collected and describe in detail how they were stored if not analysed immediately.
- Laboratory analysis
  - All analytical procedures should be clearly described so that the results of the analysis can be properly interpreted. There are often different assays for a particular compound or element; for example, nitrogen in feed, faeces or urine can be assayed by the Kjeldahl method or by combustion. References for each assay should be provided.
  - The acceptable level of variation of an assay should be reported. For example, a CV of 1% might be acceptable for one assay but 5% might be acceptable for another.
- Statistics

  Overall, the description of the statistical methods should be in sufficient detail 'to enable a knowledgeable reader with access to the original data to judge its appropriateness for the study and to verify the reported results' (ICMJE, 2015).
  - The experimental unit must be defined.
  - The method of allocating pigs or birds to pens, and pens to treatments, should be clearly explained.
  - If the pigs/birds, or the pens, were blocked, this should be noted and the method explained.
  - Was the experiment a completely randomized design, a randomized complete block design, or other?
  - Were the data tested for normality? If so, provide details on the methodology.
  - Describe the statistical model employed, and the software used.
    - Define the dependent and independent variables used in the model.
    - Define the methods used for mean separation, if applicable.

        - Define the regression model used, and explain why it was selected over other models, e.g. linear, quadratic, cubic, exponential, etc.
        - If ANCOVA, define the covariate.
    - Actual P-values should be reported, rather than ranges such as $P < 0.05$ or $P > 0.10$.
- Economic analysis
    - If an economic analysis was undertaken, all of the assumptions should be reported, and the methods of calculation clearly explained, so that the reader could repeat the analysis.
- Funding
    - The source of funding of the study should be provided.

It is somewhat controversial, but in the instance of growth studies in pigs, readers typically prefer studies based on a common ending weight as opposed to a common ending date. If the latter results in differences among treatments in final weight, it is impossible to extrapolate with a satisfactory degree of accuracy to a common end weight. Since most pigs are sold on the basis of a predetermined body weight, research that is terminated on the basis of date or age rather than weight often fails to meet the needs of most readers.

6.4.3 Results

The results section of a report does not need to describe every measurement outcome. These will be reported in the associated tables and figures. Rather, it should focus on the important outcomes of the experiment – either those that relate to the objectives of the experiment or unexpected findings that might be of interest to the reader. When presenting such results, all statements of differences should be associated with a specific P-value, not a range (e.g. $P = 0.045$ rather than $P < 0.05$).

Tables and figures should be able to be interpreted without reference to the manuscript. Thus, footnotes may be required to explain treatments and other aspects of the experiment. Tables should include not only treatment means, if applicable, but also the standard error of the mean (SEM) for each outcome as well as relevant P-values. It is helpful if each table includes the number of experimental units. Figures should similarly include sufficient information on statistical analysis to explain, for example, standard errors, P-values and values describing fit if regression analysis was employed.

The results section would benefit from a brief description of the overall performance of the animals involved in the study. For example, it would be useful to explain how the pigs or birds on this particular study performed compared with the norm for the facility. If mortality and/or morbidity was an important outcome for the study, it should be accompanied by appropriate statistical analysis. In any case, if it appears that mortality or morbidity

were related to a specific treatment, even if unexpected, this should also be reported.

6.4.4 Discussion

The discussion is the section that can separate good reports from great reports. It is normal to discuss key results of an experiment in the context of related work completed elsewhere, or previously at the same institution. However, since the authors of the manuscript are in the best position to do so, they should 'dig a little deeper' and develop a narrative of the experimental outcomes, seeking to explain the results in a broader context and perhaps with a mode of action in mind. Unlike the results section, the authors are given latitude to speculate on their interpretation of the results, provided of course that they can offer solid scientific support for their statements. Indeed, the reader appreciates this depth of discussion, as it is assumed that the authors are more knowledgeable on the topic of the study than the vast majority of their readers.

The discussion brings into the conversation related literature to expand and solidify the statements made by the authors. It is important that the most recent and relevant literature is included, for to do otherwise attracts scepticism about the validity of the research itself.

6.4.5 Conclusions

The conclusion section should refer directly back to the objectives of the study, and concisely and clearly state whether the objectives were achieved and in what manner. Of course, it is critical that any conclusions drawn from an experiment are fully supported by the data.

6.4.6 Literature cited

The cited literature should be current and reasonably comprehensive. Unrefereed documents should be avoided, or at least minimized, as they will have less scientific stature than refereed articles.

6.5 Summary

To be successful, an experiment needs to be properly designed with a clear objective in mind, executed with efficiency and appropriate attention to detail, correctly analysed and interpreted and then presented with clarity and comprehensiveness. The most important step in the planning process is the development of a clear and concise objective, prepared alongside a hypothesis that is based on the current state of the art. Planning will also

include an assessment of research facilities and their capability, in the context of the study objective(s). A sound statistical plan needs to be in place prior to the conduct of the experiment, to ensure that the data are interpreted correctly, but also to ensure that as much information as possible can be extracted from a given dataset. Animal care standards must be established and adhered to, for the benefit of the pigs and the birds utilized in the experiment, but also to ensure that the highest quality data are being generated. Data integrity is essential in any experiment and can be achieved through careful planning, attention to both the accuracy and the precision of the information, clearly defined procedures and training of all study personnel. Analysis of experimental diets is required to ensure that they have been properly manufactured and that the nutrient/ingredient of interest has been added according to the protocol requirements. Upon completion of the study, data should be carefully scrutinized to ensure freedom from errors due to collection or transcription. Once the data have been suitably analysed using the proper statistical procedures, the final report can be prepared. It should be highly detailed, not only to allow the reader to understand the nature of the experiment, but also to support further interpretation if the reader so desires. This might include the combining of multiple experiments on a similar topic in a meta-analysis, which in itself requires that all aspects of each study are carefully defined and explained. In this way, the value of a given study can be magnified. Finally, the report of the experiment should include a thoughtful discussion that helps the reader to put the outcomes into the context of existing literature, and hopefully to share a greater understanding of the mode of action. When the mode of action of experimental outcomes is understood, new information can be most effectively applied under diverse farm situations.

The world of research is changing in many different ways; the private sector is playing a much more active role, and partnerships between the private and public sector are increasingly common. Simplified global communications facilitate collaboration across great distances. All of this leads to exciting changes in research – changes that enhance its quality and depth and which accelerate its adoption into commercial practice. However, none of this can happen as effectively as it should without the original study being very well planned and described in a final report with clarity and comprehensiveness.

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7 Extending the Value of the Literature: Data Requirements for Holo-analysis and Interpretation of the Outputs

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7.1 Introduction

Individual scientific papers address specific topics and deliver information relevant to the hypothesis being tested. The scope of most papers is necessarily narrow, as the goal is to control all sources of variation so that the variation attributed to the variables/treatments of interest can be isolated and detected. Ideally, each new paper yields information that is incremental to the current knowledge base, with some papers (the exceptions) providing quantum leaps.

In many cases, the response to a set of treatments within any given trial is subject to influence from a multitude of conditions, some of which are known and some unknown. Those conditions that are known to influence the response should be controlled or at least measured, and those that are unknown simply contribute to the variation in the data in the literature. Many of the 'unknown' conditions may have actually been measured in many individual experiments and simply not been recognized as having a role to play in the responses observed. In this regard, a data-driven review of all the information in the literature may enable such conditions to be teased out.

The problem is that the scientific literature is vast: refereed articles that are tagged as addressing 'poultry nutrition' in a Google scholar search exceed 500,000. Even when specific topics are considered, such as broiler methionine requirement, the number of papers can be so large (>5000) that it is not possible to have a working knowledge of all data. As a result, no one individual scientist will be able to give a completely objective interpretation of the literature surrounding a specific topic. Indeed, most reviews are the author's or authors' subjective interpretations of the subset of papers selected.

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Objective reviews of the literature should be based on a numerical rather than subjective analysis of the data available. Such reviews are now becoming more and more evident in animal nutrition due to the value they bring, but they have been a common feature of the medical literature for many years (Rosen, 1995, 2001a,b). As indicated above, such data-driven reviews may well find effects or factors, which influence the response in a given field, that were never recognized in any of the individual trials conducted. An example is the beneficial effect of the inclusion of a coccidiostat in poultry diets on the scale of response to a dietary phytase (Rosen, 2001b). This influence was teased out using a holo-analysis of all the relevant phytase literature even though there were no individual trials where the effect of a coccidiostat on phytase response was investigated. Another example from the same author suggested that the response to an NSPase enzyme was severely reduced if that enzyme was not fed from day of age. Data has emerged that subsequently suggested that such an effect is real and that feeding throughout the life of the animal optimizes performance (Cardoso *et al.*, 2014). It should be noted that in such a comprehensive analysis, all available data are considered for use. Although holo-analysis will be discussed in this chapter for reasons that will become apparent, the principles discussed here apply to meta-analysis as well.

7.2 Holo-analysis – Minimum Requirements

Holo-analysis differs from meta-analysis, where the latter tends to set stringent criteria that must be met for the data to be considered suitable for inclusion in the analysis (Rosen, 2006). The advantage of meta-analysis is that such pre-selection often results in a more homogenous dataset and, as such, the models generated are often more focused and narrow. An example is the review by Letourneau-Montminy *et al.* (2010) where the goal was to investigate the influence of calcium, phosphorus and phytase on performance of broilers. Datasets were restricted to those trials where only an *Aspergillus* phytase was considered and the dose employed was less than 2000 phytase units (FTU)/kg, the diets were only based on corn and soybean (i.e. no other protein or cereal sources) and response criteria considered were limited to intake, gain, feed conversion ratio (FCR) and tibia ash %. The data collected were from papers published between 1996 and 2005, containing eight papers that covered 15 experiments and 203 treatments. This is a significant reduction from all papers relating to phytase use in broilers between these dates that number more than 1000 (Rosen, 2002). Clearly, such a stringent criterion results in only a minority of the total data being considered, but the models generated were strong with regards to the proportion of variation explained and the error term generated, i.e. R-squared (R^2) and root-mean-square error (RMSE). The limitation of such models is that their ability to predict responses under a variety of conditions is limited by the variation in the dataset considered. Holo-analysis, on the other hand, due to its inclusive nature, tends to result in less precise models but models which are applicable under as wide

a variety of conditions as exists in the literature. Perhaps this characteristic of holo-analysis makes it more applicable in 'real-world' situations. As discussed earlier, such a strategy can result in models that predict effects, which have not been directly tested in any of the papers selected, and as a result the models can extend the value of the original papers considerably. There is clearly an opportunity, therefore, to learn more about a subject area with no further animal experimentation needed. There are some criteria, however, that need to be considered when collecting, selecting, utilizing and analysing data from the literature in a holo-analytical model. Much of this has been discussed by Rosen (1995, 2001a,b) but for the purpose of completeness this section covers some of the key aspects of holo-analysis below.

7.2.1 Considerations in use of data for holo-analysis

Data collection

The role of holo-analysis is to quantify the relative contributions and interactions of treatment, feed, genetic, environmental and management independent variable factors on the response variable of interest, with the express intent of enabling a prediction of such responses on manipulation of the significant variables identified. The scientific literature provides a large pool from which the test data are mined and from which it is hoped the desired models provide insight into the field of interest. Initial problems that need addressing include the initial data collection stage. The search criteria employed may miss a number of publications due to poor selection of keywords in the search strategy but also poor or inaccurate titles, keywords and abstracts employed by the authors. Clarity in the title and selected keywords is therefore essential if the published data are to be used to their full extent.

Once collected, the next challenge is to identify the variables that should be included in the analysis. It is not essential that the identity of the variables collected is pre-determined; indeed some surprising associations have been identified with the production variable of interest when 'all available variables' were collected. Thus, it probably pays to dissect a sample number of publications to identify the breadth of variables that could be recovered from each paper, even if the utility of such variables in the analysis is not recognized. A suggestion of some groups is shown in Table 7.1. These suggestions are especially relevant to literature-based data, which may be more limited in detail. However, the list may be extremely extensive, particularly in the case of commercially collected data.

A challenge during the data collection phase is to ensure that the data entered is not biased and is meaningful. Clearly, identification and elimination of duplicated data sets is essential to prevent bias or undue weighting of a particular data set. Detection of duplicated data is often made even more difficult by slight divergences in the data between duplicate publications but their removal is essential if the analysis is to be representative. The 'meaningful' nature of the data relates to the uniformity in the detail of the materials

Table 7.1. Suggested groups of data that may be valuable for holo-analysis.

Group	Examples
Meta data	Author, country, year
Animal information	Strain, age
Husbandry	Lighting regime, pen type, stocking density
Infrastructure	Drinker type, feeder type
Feed and nutrition	Full formulation for each age group, nutritional specification
Results	FCR, body weight, feed intake either on an absolute basis or relative to a control

and methods employed in each study. Although several minimum guidelines have been suggested for general scientific papers, e.g. the Poultry Science Association guidelines or more general guidelines, such as the 'Gold Standard Publication Checklist' (Hooijmans *et al.*, 2010) or the ARRIVE guidelines for biomedical research (Kilkenny *et al.*, 2010, 2014), there is little adherence to such advice in the literature at large. Moreover, the minimum guidelines that a journal may set might not be sufficient to allow for the trial to be repeated, let alone enable inclusion in a systematic review (Hooijmans *et al.*, 2010). In a survey of 271 papers on the subject of biomedical research on laboratory animals, only 13% of the papers reported both the weight and the age of the animals employed (Kilkenny *et al.*, 2009). More specific guidelines for speciality topics, such as feed enzymes (Rosen, 2006) exist but it is clear they are rarely consulted. As a result, the details available for some of the key variables that are known to influence the response to a given factor are patchy. Direct contact with the authors responsible for those papers where details are missing has been attempted (Rosen, 2000) in previous studies but this is both time consuming and not guaranteed to improve the quality of the dataset. An example of such relates to the feed enzyme dataset. The significant number of publications that lack adherence to the standardized name for the products and/or their units of activity means that correct identification and classification of some products is often not possible. In his example, Rosen (2000) states that the enzyme resource yielded 252 different generic descriptors of enzyme activity, with very few being recognized methods of nomenclature. The current status of reporting is still inadequate. Often generic or vernacular terms, such as 'hemicellulase' or 'pentosanase', are used that do not meaningfully define the enzymes used. Simplification of such a huge list into more meaningful groupings would make a significant improvement in the subsequent analysis, not only in terms of biological relevance and accuracy but also in reducing the number of variables within the variable 'enzyme' so that there is a greater likelihood of a significant model being generated. The most significant and glaring problem relates to the identification of the source of the enzyme of interest, i.e. the gene from which the enzyme is transcribed, along with the organism that hosts the gene and produced the enzyme. The source organism and production strain should be considered the bare minimum of reporting, since both (the former in

particular) have such an impact on the characteristics and thus likely efficacy of the enzyme. Even this may not be detailed enough, as the source organism often produces several isozymes of the activity described, and these may also differ considerably. As often as not, the product used is not pure and contains multiple side activities from the production strain which may or may not be related to the enzyme of interest. In some cases, the side activities may well be as important as the main declared activity (if indeed a main activity is identified). Whether or not the presence of these side activities is declared is one problem, the other being that even when they are declared, their units, and hence possible relevance to the outcome, are not. Enzyme assay units themselves are a further point of frustration in that even when perfectly serviceable and internationally accepted methods are available, use of alternate or unpublished methods in some publications renders the data within almost worthless. This is especially so when no attempt is made to provide a clear conversion factor between the established and proprietary assays. Such obfuscation severely limits the ability of the subsequent analysis to extract insights of interest, particularly with regards to determination of optimum dosage and comparison of products.

Although the example above may seem specific to the enzyme database, the principle that any study should be presented in a manner that it could be replicated is not. Inaccurate, inconsistent and deficient reporting as discussed above means that the trial cannot be faithfully repeated and indeed the utility of the data in a holo-analysis is severely limited (see section on selection of variables, below). All fields where the product of interest suffers from poor nomenclature or poor adherence to nomenclature are subject to similar limitations on post hoc analysis. Probiotics, prebiotics, essential oils and plant extracts, mycotoxin binders and a multitude of other feed additives are all subject to inconsistencies in reporting and thus would benefit from stricter editorial demands for compliance.

Data storage and organization

The manner in which the data are stored is important as it influences the ease with which they can be analysed. A spreadsheet or simple relational database is more than sufficient. In readiness for analysis, as with most data analyses, the data are entered in a linear manner, with each row in a spreadsheet representing the information relating to the outcome of one treatment. A suggested format is given in Table 7.2. The output or independent variable of interest is an absolute performance metric, such as FCR. Rosen (2000) first noted that actually entering the data in a manner such that each row represented the effect of the treatment compared with the control, rather than the absolute performance of all treatments, including the control, results in a more robust model, albeit with a poorer R^2 and larger RMSE (an example of this is shown in Table 7.3). The use of the difference between the control and the treatment invariably pulls the control performance into any model as a significant variable, as it is a measure of the performance of the animals in the trial of interest for their given age or weight.

Table 7.2. Example table for the suggested format of data for holo-analysis.

Manuscript	Country	Strain	Treat	Age at start of experiment (days)	Age at end of experiment (days)	Bedding	Housing type	Cumulative 42-day FCR	42-day body weight (kg)
Smith *et al.*, 2015	UK	Ross 308	A	21	42	Shavings	Cage	1.70	3.00
Smith *et al.*, 2015	UK	Ross 308	B	21	42	Shavings	Cage	1.72	3.00
Jones *et al.*, 2016	USA	Cobb 500	A	1	42	Shavings	Floor Pen	1.68	3.20
Jones *et al.*, 2016	USA	Cobb 500	B	1	42	Shavings	Floor Pen	1.70	2.80

Table 7.3. Example table for the suggested format for holo-analysis when each row represents the effect of the treatment compared with the control.

Manuscript	Country	Strain	Treat	Age at start of experiment (days)	Age at end of experiment (days)	Control 42-day FCR	Cumulative 42-day FCR	FCR change vs Control	% change in FCR
Smith *et al.*, 2015	UK	Ross 308	A	21	42	1.80	1.70	0.10	5.56
Smith *et al.*, 2015	UK	Ross 308	B	21	42	1.80	1.72	0.08	4.44
Jones *et al.*, 2016	USA	Cobb 500	A	1	42	1.70	1.68	0.02	1.18
Jones *et al.*, 2016	USA	Cobb 500	B	1	42	1.70	1.70	0	0

Data cleaning

Once the data have been abstracted and entered into an appropriate database, the first challenge is to establish their veracity. A simple and informative first step is to plot out the distributions of each variable, which is a very simple process in most statistical packages. The means and standard deviations of each variable are useful descriptive statistics and the distribution graphs also give an initial indication as to whether the data are normally distributed and whether there are clearly anomalous values in the dataset. Care must also be taken to ensure that the values are correctly aligned with one another. An example would be identification of a 21-day body weight as that of a 42-day body weight in the column for body weights. This would clearly introduce an error into the 42-day analysis but would not necessarily be caught in a simple distributions test where mixed age data are present in the weights column but not sorted by age.

With regards to class variables (discrete or nominal), running a distribution and sorting the list alphabetically will enable simple discovery of incorrectly entered variables. Problems such as a simple misspelling resulting in creation of two or more discrete and distinct variables, where only one is intended, can have significant effects on the analysis. Examples would be names of additives where, say, glucanase and glucannase (a misspelled name) would be considered as different products.

Exclusion of anomalous data should be undertaken with care. In general, animal production data sets are data sparse. Data are at a premium and therefore should only be excluded if it is absolutely clear that the value is erroneous. There is often a temptation to exclude data that seem to be contrary to the mean but exclusion of such data will limit variation in the dataset and may result in the subsequent model failing to detect some associations of interest. The counter to such an observation is that very few erroneous data points are needed to significantly reduce the likelihood of the production of a valid model, or indeed increase the likelihood of generation of an erroneous model. Considerable time should therefore be devoted to this process as the quality of the model is dependent upon the quality of the data.

Selection of variables for use in models

In situations where variables are limited in number, the task of deciding which variables to consider as dependent and independent is very easy. In the majority of such cases it is already known that particular independents are correlated with particular dependents and the only challenge is to ensure that the independents are not correlated with one another when fitting the models. It is also possible that variables appear to be independent but actually are not. For example, is the dependent variable actually calculated from the 'independent' variables? We have seen this in survey-based data and caution should be applied. For example, a European Production Efficiency Factor (EPEF) value is not strictly independent from the values for FCR, body weight, etc. that are used to calculate it. Fortunately, in this example, both

variables are most likely dependents so would probably not be included as variables in a model, but the principle still applies.

If there is a great number of variables, the challenge is to identify those that are most important. When data are sparse, consideration should be given to generation of models with as few variables as possible for two reasons. The first is simply to avoid overfitting; the second is practical and relates to interpretation. Models based on many variables, particularly those with many interactions, can be very difficult to use intuitively. While the model may be a reasonably good interpretation of the data, the inclusion of multiple variables and their interactive terms reduces the consistency of response to a particular variable and thus makes practical understanding or application very difficult. Initial screening of potential variables is therefore encouraged and the selection processes should also endeavour to select only variables that are not correlated with one another. Inclusions of all variables in a principal components analysis is one method of achieving both screening and correlation tests at the same time. Variables that are identified as lying in the same principal component should be considered as correlated and thus the most highly correlated with the principal component selected as a potential variable. If the dependent variable is located in a principal component and its correlation component is high, the most highly correlated independent variable in the same positive control (PC) should also be selected, as by definition it is related to the dependent. Our experience with commercial datasets suggests that the number of principal components should ideally be restricted to ten or fewer to limit the size of the model for the reasons noted above. In such cases, when reviewing and selecting the independent variable of interest it should be noted which other independent variables reside in the same principal component. The reason is that these remaining variables will not be selected for modelling, as they are correlated with the selected. However, the selected variable is possibly not causative and thus could be acting as a proxy for the remaining variables. For example, many dietary amino acids are correlated with one another and as a result only one can be selected. It may be that the amino acid selected is methionine from a statistical viewpoint, while recognizing that, from a biological viewpoint, it is lysine that is most relevant. In such cases, the statistically favoured independent variable could be replaced by the biologically or commercially relevant variable. This is the first in several cases where the biological or commercial relevance of a selected variable may need to take precedence over the variable selected for statistical reasons.

Multivariate analysis can also be employed to determine which variables should be employed in specific models. Those variables that correlate with the dependent are to be immediately considered as independent variables. The same multivariate analysis is then used to select which of the independent variables to use if there are correlated independents. Generally, if several independents are correlated, the independent that is most highly correlated with the dependent is the favoured option and the rest are rejected. Note again, however, that in many cases the biological or commercial significance of the variables considered should be taken into account. In some cases the

variable that is most highly correlated with the dependent may be rejected in favour of a more poorly correlated independent if the latter makes more sense to the end user of the model.

A further consideration is that the above processes are very good at selecting independents that are linearly related with the dependent. In many instances it is well worth plotting all potential independents against the dependents in order to determine if there is a visible relationship which may seem to be based on a more polynomial or non-linear relationship. Although this may seem to be a time-consuming process, we have found this to be of great value in many instances. When variables are selected on this basis, they will need to be tested subsequently for correlation before the model is constructed, as with the methods described above. Clearly, when visual relationships have been established, then the challenge is to identify and subsequently confirm that the appropriate term (e.g. quadratic, non-linear, logarithmic) for the selected variable does indeed predict the dependent in the subsequent models.

7.2.2 What makes a good model?

In simplistic terms, a good model is one that describes a large proportion of the variation (i.e. a high R^2 value) in the dataset and simultaneously has a low error term such that any predicted value is determined with a high degree of confidence. Ideally, the model is populated with variables that are truly causative, but this can never be proven from such methods of data analysis. Correlation does not imply causation and thus models have to be interpreted accordingly. As an example, there is a highly significant relationship between sales of margarine and the divorce rate in Maine, USA, with an R^2 value of 0.98 (Fletcher, 2014). There is no suggestion or likelihood, however, that the one causes the other. It is obviously a coincidence, but it is a cautionary note for the interpretation of the models created.

A further consideration is that, regardless of the size of the dataset, it is likely still a fraction of the total data available (i.e. $n = \text{all}$). As a result, the model generated is simply an estimate of the model that would be generated if all the data were available. Thus the robustness of the model needs to be considered. Robustness is a term that describes the ability of the model to apply to data that it has not been exposed to. Indeed, a robust model would accurately predict the absolute values of a dataset where $n = \text{all}$ but access to all data is a very rare occurrence. In practical terms, robustness is a test of utility of a model over time. For example, if a model generated using this year's data were to be applied to next year's data and found to give a similarly good fit, it can be considered to have some degree of robustness. A truly robust model would not evolve at all in terms of the variables in the model or their coefficients as additional data becomes available. Modern statistical tools allow tests of robustness by randomly segregating the entire dataset collected so far into separate pools: one that is used to generate the model; and a further one or even more random pools that are set aside to test whether

the model generated is indeed a good predictor of the original data. The model generated to describe the first pool of data by standard statistical methods is then tested on the reserved dataset(s) to determine if the variables selected when multiplied by their coefficients can accurately predict the independent from these remaining test data subset(s). If the model generated is robust, the R^2 values and error terms of the generated model on all datasets are all very similar. Such a process of validation is highly recommended, as it is often relatively easy to generate a model that provides a reasonable interpretation of the whole dataset but it is far more difficult to generate one that is robust. The size of the dataset will determine how successful such an approach will be, simply because many datasets are too small to allow for splitting into test and validation datasets and still have enough data for a reasonable model to be generated.

The number of data points required to generate a good and robust model depends upon the dataset at hand. The more the data reflects reality (i.e. few errors), the smaller is the dataset required if a model can be generated from the variables at hand. The most important consideration is whether the variables present in the dataset do have a role to play in the prediction of the independent. Some understanding of potential mechanisms that operate in driving the response of interest helps in the selection of variables for the model. It is thought, for example, that air quality influences performance of each broiler flock (Reece, 1980; Donaldson *et al.*, 1995; Beker *et al.*, 2004) and as such some measure of air quality would be a desirable variable to offer to performance models. Unfortunately, in our experience with commercial data collection, acquisition of such information from each farm is almost impossible. Thus the number of rows of data required to generate a 'good model' depends upon the dataset itself. In some cases, no amount of data will generate a meaningful model as a result of there being no independent variables present that are relevant for estimation of the dependent. In others, relatively few lines of data are needed and in fact reasonable models may be generated with as few as 100 or more data lines. Rosen (2003) noted that 20 data points was only good for reasonable first efficacy appraisal of an additive, 50 was the minimum to establish the key variables influencing the response, and hundreds of data points or more were needed to provide reliable models from which commercial decisions can be made. This clearly suggests that there is a minimum number of rows of data that are needed to generate a 'reasonable model', and indeed suggests that a robust model has been achieved if it does not evolve with addition of further data over time.

7.2.3 Model types

There are many different types of models that can be employed in the investigation of the relationships between the dependent and independent variables. The goal is to establish which independents are most influential on the outcome of interest and to determine what input variable settings result in the optimum output.

Linear and quadratic models

The most commonly used models are based on linear (and sometimes extended to quadratic) terms, with interactions between some, if not all, of the terms included in the model (Rosen, 2002; Hooge *et al.*, 2010; Letourneau-Montminy *et al.*, 2010; Sales, 2014). Some investigations simply determined the difference between treated and control with no other factors in the model (Hooge *et al.*, 2010; Sales, 2014). If only linear terms are to be considered, the model is relatively easy to fit and interpret. Again, the fewer the terms in the model, the easier it is to understand. However, if only linear estimates are considered, clearly the optimum output (i.e. the maximum or minimum of the dependent) will always be at one or other extreme of each of the independent variables. Indeed, for variables that are only linearly related to the independent, the inevitable conclusion is that the dependent continues to improve with each and every increment or decrement in the variable. There is no predicted maximum or minimum with linear models. The maximum output achieved within the confines of the dataset will also be associated with a very large confidence limit as a result of the limitations in data at these extremes. Most biological systems are not linear in their response to a given independent variable if the range employed is wide enough and, as a result, the extension of the model beyond the dataset is clearly not advised.

Implementation of quadratic terms may be advisable for a number of independent variables if it is thought that the response to linear increments of the variable diminish or increase to an asymptote and then decline once the maximum or minimum is achieved (Letourneau-Montminy *et al.*, 2010; Siegert *et al.*, 2015). Such a relationship can be considered a reasonable estimate of the biological effects of nutrients that become toxic when fed to excess. The difficulty in assigning a quadratic term is that the relationship assumes a symmetrical approach to the maximum or minimum from either side. In other words, the rate at which performance improves towards the optimum with increased dose of an independent is the mirror image of the reduction in performance with increments in dose beyond the optimum. The biological justification for such a relationship needs careful consideration in this regard, as many nutrients improve performance up to the point of the optimum but performance may well remain at this optimum with significant further increments in dose. This would be the case if the animal is able to excrete or re-route the excess nutrient elsewhere with little or no consequence on the dependent variable but this is only up and until the point at which the excretion or detoxification process starts to bear on the dependent variable of interest. Alternative modelling considerations are the broken-line or broken-line quadratic models, which are described in more detail in Chapter 2. These models assume that once the asymptote is achieved there is no further increment or reduction in the dependent variable (within the ranges of the independents employed) and thus such models take account of the points raised above. The linear and quadratic versions of these models merely differ in their interpretation of how the optimum is achieved.

Non-linear models

There are many non-linear models available that can be employed and similar considerations regarding the biological relevance of such models apply (Sauer *et al.*, 2008). Exponential and asymptotic models need to be considered against the broken-line models discussed above. The caveat of such models is that they reach the optimum at an infinite dose of the independent variable and, as a result, not only is calculation of the optimum irrelevant but also the biological relevance of such models falls down as the dose of the independent variable increases beyond 'normal' ranges. Such models are biologically flawed at the outset, as there is no biological relationship between an independent and dependent whereby there is no consequence for perpetual increments in the independent. Some specialist models, e.g. Gompertz, are designed to describe particular relationships and should be considered first, ahead of all other functions, as they have been. However, it is clear that the apparent biological relevance of a model does not mean that its statistical relevance can be ignored. If the model does not fit the data well, alternatives should be considered and the authors should consider why the alternative may be more relevant under the circumstances of the dataset.

Partition models

Partition models, whether simple or more sophisticated variants, such as random forest or boosted tree, are certainly worth considering as they allow simple methods of combining what appear to be different modelling types all in one. By virtue of the manner in which the dataset is sequentially split based on improved statistical fit, the shape of the relationship between any variable and the dependent can end up being linear, quadratic, asymptotic or threshold. The coarse nature of the 'lines' created depends upon how many splits are introduced into the model. Such models also implicitly identify interactions between variables that can be almost impossible to predict ahead of analysis. In this regard, the output from partition models should be viewed as being an aid in determining which variables, functions and interactive terms, if any, should be considered in the linear and non-linear model selections.

Neural network models

Neural networks are quite often the most successful method of producing models with the highest R^2 value and lowest error terms when dealing with animal performance datasets or even prediction of nutrient contents of ingredients (Perai *et al.*, 2010; Savegnago *et al.*, 2011; Mehri, 2013). The problem with neural networks is that there is so much choice in the number of nodes and the relationships between them that it is often not clear whether the setting chosen has arrived at the optimum possible model. This is made worse by the fact that, even when settings are fixed, re-running the model will arrive at a different solution as each run is unique in its solution. Added to this is the difficulty encountered in parameterizing an output such that the

solution can be used in a spreadsheet for exploring the interrelationships between the independent and dependent variables. All other forms of modelling are relatively easily transcribed into other media for display of the output, which is often the endpoint for many analyses.

7.2.4 Modelling considerations

Generally, when all data are considered for inclusion in the analysis, as is the case in holo-analysis, the models generated struggle to achieve an R^2 value and/or error term as impressive as those achieved with meta-analysis driven models where data may be highly selected. Where holo-analysis is employed simply to identify and quantify variables that contribute to an outcome of interest, the factors that contribute most to the model are generally not known ahead of the analysis. For example, if a literature review was performed to determine which factors contribute to weight gain of broilers to 21 days of age, the selection criteria for relevant papers is very broad and as a result the dataset could be extensive. This is not necessarily advantageous, since the analysis is somewhat akin to an investigatory process and there may be only a few variables that, together, are present in sufficient papers to generate a significant model.

Using holo-analysis to investigate the effect or value of a specific nutrient or additive added to a diet, however, has the advantage in that most trials will have captured the information relating to the additive of interest and will likely have recorded the performance of an unsupplemented control. This latter point gives such work a distinct advantage in attempting to derive a model, since most often the greatest proportion of variation is represented by the performance of the control animals. Most models reported to date fall into this second category and the significance and presence of control animal performance is almost universal. Inclusion of a dose term for the additive or nutrient of interest is highly desirable if the dataset were collected with the express intention of generating a model to describe the effects of the selected variable, but it is not always the case that this term is as important as those that may have been suspected or indeed significant. It was noted in several models generated by Rosen (2000, 2002, 2003), for example, that the dosage of enzymes employed on the gain or FCR of broilers was not as important a term in describing the response as control animal performance, or indeed other terms that had nothing to do with the additive per se. For example, on weight gain of the broiler, the inclusion of fat in the diet had a larger effect than increasing the dose of an enzyme. Other terms, such as age, stage of growth, breed and nutrient density of the diet, can influence the performance response to the independent of interest. Simply put, often the additive or nutrient being investigated is by no means the most influential factor in describing the performance level of the animals in the dataset collected, even though the data collected in the holo-analysis were derived from trials set up to investigate the effect of the factor concerned. This highlights the need to ensure that each paper reports as much information relating to the diet and

husbandry as possible, since it is not always known ahead of time whether these variables are important in describing the response to the variable being investigated.

The greatest constraint to deriving a successful model from the literature is the lack of consistency with which the experiments are described (Kilkenny *et al.*, 2009). The result is that there are too few data available to construct a model if it combines many independents that are inconsistently reported. There may be 500 data lines available for an analysis, for example, each line having the output variable (e.g. FCR) of interest. If age is found to influence FCR significantly and is included in the model, but it is only reported in 400 lines of the data, then the dataset is reduced to those 400 records. If lysine content of the starter diet is also significant, but is only present in 400 lines of data, and if all of the 100 missing age data lines do not coincide with the lysine data lines, then there could be only 300 lines of data where FCR, age and lysine are reported. Such data reduction is very real and hampers analysis considerably, especially if many independents are considered in a model. Rejection of some independents and restriction of the number of independent variables in total is often the only way sufficient data remain to test models. In a recent example of the author's, a literature review of 113 papers, yielding approximately 1000 rows, was reduced to fewer than 50 rows of useable data for these reasons. As a result, independent variables that may be highly significant from a biological viewpoint could well be overlooked due to the infrequency of their reporting. As mentioned before, this is due to poor reporting in the original papers and highlights the need for minimum standards in publication (Hooijmans *et al.*, 2010; Kilkenny *et al.*, 2014), not only so that the work can be repeated, but also to facilitate post hoc analysis.

7.2.5 Outputs and interpretation

The great value of holo-analysis is the quantification of the response due to changing the dose or level of the significant input variables. For example, understanding the relationship between carcass yield of a broiler and the level of lysine in the diet enables the user to determine the optimum economic return if the costs of lysine and value of carcass are known. Even greater value is evident if the model has identified other contributory factors to the response; for example, the energy level of the diet, which may alter the relationship between lysine level and carcass yield. If several high-cost variables are captured in such models (e.g. levels of phosphorus, other amino acids, inclusion of additives), it provides even more value as the costs of each of these inputs can and do change independently. As a result, the levels of each of these inputs, lysine included, needed to optimize the output of interest, will change with time even though the model itself does not. The optimum may not always be intuitive, particularly if there are several interactions between independents in the model. Ideally, a good model would be constructed from all the highest-cost nutrients and husbandry inputs so that the

maximum efficiency and greatest savings could be obtained. The problem is that, first, these inputs, despite their commercial relevance, are not always recorded in the literature and, second, they are not always found to be significant in the models (which in itself challenges the use of high levels of such inputs). Future work should ensure that there is alignment between industry and academia to ensure that commercially relevant and costly inputs are always captured in academic research.

Whereas the focus of this chapter has been on practical application of holo-analysis of data from the literature, these methodologies are equally applicable to data collected in commercial situations. Many large commercial animal and poultry production companies collect data at breeder farms, broiler and layer farms and feed mills, as well as at the time when feed is formulated. Often, these data are captured in isolation, i.e. in separate datasets. If, however, these data can be captured and aligned so that the performance of the animals at the farm can be related to breeder stock, mill and feed formulation, then the process of holo-analysis can be applied to the extended dataset generated. If there are significant effects of the breeder, feed formulation and manufacture, and husbandry processes on final carcass yield, such extended datasets are clearly essential to tease these out.

Two distinct advantages accrue from such an activity. The first is that the data collected relate to the commercial company's own situation and, as a result, any models generated apply to that company and, probably to some degree, are of a bespoke nature. It is unlikely, for example, that any other company would have the same set of raw materials, formulations, feed milling processes, husbandry conditions and costs. Thus, profit optimization or cost minimization would likely settle on a set of circumstances that suit the company concerned and probably no other. In short, profit maximization would far more likely be achieved using data from the company concerned rather than literature or generic performance data. The second is that, should the data be collected on a real-time basis, the robustness of any models generated could be tested on a relatively frequent basis and newer, better estimates generated as more data are added with time. Given the volume of data produced by medium-sized to large companies, it would not take long to have at hand a dataset that has greater width and depth than is available from all of the literature. For example, commercial feed mills have the capacity to record feed formulation, throughput, energy consumed per tonne, conditioning time and temperature, pellet press temperature and temperature rise and cooling conditions amongst many other variables, very few of which are ever recorded in scientific papers. If any of these conditions affect final profitability, either through influencing the nutritional value of the diet, or the cost of diet manufacture, such a relationship should be quickly established and the conditions of feed manufacture set to optimize overall profitability. Furthermore, the more complete and organized the data, the more streamlined the above processes become, making such analysis relatively straightforward for even the largest of outfits and may easily become regular practice, allowing almost minute-by-minute optimization.

Optimization of animal production over such a broad set of inputs, i.e. from ingredient selection, through feed manufacture to husbandry, is possible today using commercial data but has yet to be considered in academic research projects, as it is simply too large a question to consider. There is an argument, therefore, that the application of holo-analysis may move away from the academic literature and towards large-scale datasets generated by commercial companies. Perhaps it will be from this forum that topics for research will be generated as a result of interesting associations discovered from empirical data generated in the field. Regardless of where holo-analysis is implemented, it will be far more successful if adherence to the tenet 'the quality of the data determines the quality of the output' is upheld.

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8 Presentation and Publication of Your Data

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8.1 Publication Is Not the End of Your Research

This chapter on presentation and publication of your data may be the last in this book, but presentation and publication should be among the first things you consider when designing experiments. Too often, researchers begin to think about publication only after they have completed their experiment. As a result, they find themselves in unnecessary difficulty in presenting their results convincingly or explaining them clearly. In fact, it can be argued that the only reason for doing experiments is to write them up so that other people, scientists or non-scientists, can read them and be influenced by them. That is because the written word is the only possible medium by which researchers can reach all but a tiny portion of the people who may potentially be interested in their findings and reasoning.

Therefore, given the importance of the written word to the research process, it makes sense when planning experiments for researchers to ask themselves questions like, 'How can I present these data as forcefully as possible?' and 'What is the most compelling way I can present the implications of my results?'. The answers to these questions can often influence the experimental procedure, the treatments applied and the variables studied.

Of course, at the planning stage, you don't have any results; so how can you plan so carefully what you intend to do with results that don't exist? Here is where well-planned experiments and well-planned writing mutually lead to good research outcomes. Sure, you don't have results when setting up the experiment, but if you have a well-reasoned and justified hypothesis (as proposed in Chapter 1) you certainly have a plausible expectation of the

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results you are likely to get. These expected results are the ones you consider when planning your presentation and explanation. Then, if you see potential difficulties that might arise, you can adjust your plans for the research to cope with these. This will lessen the 'if only I had realized . . .' and 'why didn't I consider . . .?' reproaches that writers of scientific papers so often have to deal with.

But that is only the start. We will see later that the hypothesis plays an even greater role in the structuring of your article and making it easy to write and read.

8.2 Scientific Style – a Myth Laid Bare

There is no doubt that English is the *de facto* language of science; about 97% of all the nearly 10,000 journals used to calculate citation indexes are in English. The very first scientific articles written many centuries ago were probably written in Latin (which was already a 'dead' language), or those written in English were full of complex and uncommon words derived from Latin. This ensured that everyone who read them was aware that the writers came from a select and erudite class that was intellectually superior to the general public. Elements of this archaic idea persist to this day and many people believe (and, worse still, are often taught) that good scientific English is different from and more complex than the simpler English of Anglo-Saxon origin used in everyday communication. However, a large and increasing proportion of the world's scientists do not have English as their mother tongue. So, for them, the laborious task of reading so-called 'erudite text' is bad enough, and the thought of being obliged to write that way is almost overwhelming. Indeed, even many native English-speaking scientists admit that this is why they become faint-hearted at the prospect of having to publish the outcome of their work and so they leave their findings to rot in notebooks and filing cabinets.

Look at the following two statements:

> A combination of probiotics and naturally occurring components such as prebiotics, nonspecific substrates, plant extracts, and microbial metabolites that act synergistically to improve host health would be appealing and may yield a new dimension in using probiotics in the sphere of safe food practices.

> Combining probiotics that would act synergistically with naturally occurring components such as prebiotics, nonspecific substrates, plant extracts, and microbial metabolites may be a new and appealing way of using probiotics to make food safer and healthier for animals.

They both attempt to say the same thing but the final part of the first is needlessly flowery by adding concepts of dimensions and spheres that have little or nothing to do with the subject. Without these, the second sentence is more direct and easier to read.

The best scientific style is plain, simple English; the plainer and simpler, the better. This is the sort of language you would use in explaining your

work in a conversation with a friend. After all, when you write you should always be thinking of your readers, and it is nice to think of them as friends. This is OK, but are there rules that must be obeyed when writing about research?

Yes, there are: three of them. First, what you write must always be *precise*. If you are imprecise you are not being scientific. Second, it must be *clear*. Statements that are ambiguous or difficult to follow can lead to readers interpreting them wrongly and that is certainly not good science. Third, it must be *brief*. All words that are not needed add to the risk that the reader will become confused. In the example above we have shortened the sentence by six potentially confusing words and so improved it. The welcome news for prospective scientific writers is that, apart from precision, clarity and brevity, there are no other rules. When most writers become conscious of this a huge weight of uncertainty is lifted from their shoulders and they can feel liberated to write more freely than they first thought was possible – just like talking to a friend.

A word of caution. Every field of science has its exact and specific terminology that may be unfamiliar to scientists in other fields or to non-scientists. The example above has several such terms. These are the precise words for that field and to use other words that are more familiar but imprecise is therefore totally unacceptable and not good scientific writing. However, the words that bind these terms together into a sensible statement are those that need to be chosen for their simplicity and inability to confuse.

8.3 Telling a Scientific Story

The only reason for writing anything is to have someone read it, and that applies to all forms of literature, including scientific literature. There can, of course, be lots of secondary reasons that compel scientists to write, such as bolstering their CV or their reputation, pleasing the administration, getting new results or a point of view 'out there', or vanity, or pride. But the primary purpose for writing is to have as many people as possible read what you have written, understand it and be persuaded by it. That means you are writing for a reader and not for yourself. So the key to being able to write good scientific articles is to understand what induces a scientific reader to want to read what you have written. It goes without saying that the subject matter should be good science and the rules about precision, clarity and brevity must be adhered to. But for your article to stand out among the hundreds of thousands, or millions, that are written each year it has to go beyond that. It has to tell a scientific story. Most of the rest of this chapter is about how to do this successfully.

Broadly, a scientific article presents data and discusses their implications for advancing knowledge either in a specific field or in a wider context.

Many articles do no more than this. Yet, those same data and their implications, if properly handled, could be the foundation for an absorbing story ensuring that a reader, at least one in the same field, would feel compelled to

read it. The key to turning mere data into a compelling scientific story is to deliberately create anticipation in the reader's mind. That is, to have readers wanting to read the next passage of text because they expect that it will contain something that is going to be interesting to them. Gopen and Swan (1990) first raised the concept of expectation in scientific writing in an article entitled 'The science of scientific writing'. In it, they outlined how to use 'reader expectation' to make sentences follow one another fluently and in a way that does not tire the reader. Simply, they recommended that sentences never begin with new material, but that sentences begin with words already made familiar in the previous sentence. This enables readers to expect what is coming in the body of the sentence and relate it to what they have already learned.

If we expand Gopen and Swan's concept at the level of the sentence to the whole article, we stop readers of scientific articles wondering what on earth they might find in the next passage and have them expecting to find something. Then the article becomes a scientific story and the reader becomes a seeker of knowledge rather than attempting to be an absorber of information. These are the articles that readers follow easily, understand clearly and quote most confidently – all of the features that you want to encourage. So, as you are writing each section of your article you should have two objectives. The first is to provide the information that is relevant to that section and the second is to give the reader something to expect in the sections that will follow. Readers, so groomed, will pick up the new information more quickly and logically than if they have to integrate fresh concepts without being prepared.

8.4 Structuring the Scientific Story

The physical structure of most scientific articles follows the 'IMRAD' format:

- Title
- Introduction
- Materials and Methods
- Results (and)
- Discussion
- Bibliography.

A checklist called ARRIVE (animal research: reporting *in vivo* experiments) includes 20 recommendations on what each of these sections should contain when describing experiments with animals (Kilkenny *et al*., 2010). It was put together by a group of eminent scientists called the CONSORT Group (Schulz *et al*., 2010) in response to a perceived need to improve the quality of research papers by encouraging authors not to omit information vital to the integrity of the experiment when they report it. This is a handy checklist to use, as you do not want to have a paper rejected due to unintended omissions. But it does nothing to ensure that your data, though complete, make a compelling scientific story.

So, let us look at how you can write under each of these IMRAD headings, concentrating on the two aspects: what they should contain; and how they can prepare the reader for the sections that follow. You will see that this approach will simplify the writing by ensuring that you do not clutter your article (and, even more importantly, the mind of the reader) with irrelevant material and that your article will be fluent.

8.4.1 The Title

What it should contain

Many thousands of people will probably read your title. Inevitably, many thousands fewer are likely to read any of the rest of the article. So the *Title* has an important job to do and the first part of that job is to ensure that the reader has accurate information regarding what the article is about.

Imagine a title 'A new approach to feeding free-range chickens'. It could refer to a new feeding regime, or a specific supplement, or a new component of the feed, or a new design for a feeder – or almost anything. Not very helpful to a busy reader who may be a specialist in one or other of these possible areas but with little interest in the others. Forcing that reader to seek out the body of the article just to find whether it is of interest may be just enough to entice them to ignore the article altogether. To avoid this, think carefully about the keywords in the article and ensure that all the important ones appear in your title. In fact, without all the important keywords, no title can claim to describe the work accurately. The title above contains the words *approach*, *feeding* and *free-range chickens* – hardly inspirational. By contrast, by including the important keywords you have a base for a good title. But that is only the base. You need to go further to entice the reader to take the trouble to start reading.

Preparing the reader

Let us assume that you have designed a feeder that opens automatically at certain times of day and encourages free-range chickens to eat more and grow faster. There is certainly a good scientific story to be told here. Make sure that no potential reader misses it by having a dull title like the one above. You may believe that the most important information in your article could be the design of the feeder (in other words, your news is about the *Materials and Methods*) or the fact that it increases rate of growth by 10% (*Results*) or that, by using this new gadget, rearing of chickens in a free-range system can be economically more attractive (*Discussion*). It is up to you as the researcher to decide which but, having done so, it is up to you as the author to make sure that the reader is stimulated by a title that specifically divulges the good news.

The important key words would probably be similar whatever the emphasis you choose. They would probably include: free-range chickens, timed-access feeder, rate of growth, intake of feed, economics of free-range

systems and supplementary feeding. However, you can emphasize the aspect that you have chosen simply by adjusting the order in which you use these key words. In an isolated statement like a title, a reader will automatically assume that words at the beginning of the statement are more important that those in the middle or at the end.

For example:

- To emphasize the feeding system: '*Automated timed-access feeders increase intake of supplement and growth rate of free-range chickens*'.
- To emphasize the growth: '*Increasing intake of feed and growth rate of free-range chickens by using automated timed-access feeders*'.
- To emphasize the economic possibilities: '*Improving the economics of free-range rearing of chickens by using automated timed-access feeders*'.

These are all 'definitive titles' (Siso, 2009) that endeavour, in a few carefully chosen words, to give a mini summary of the article, including its most important message, rather than 'indicative titles' that merely nominate the field in which the research was carried out. They are much more likely to attract the busy reader than our original bland and non-specific title, or even something stereotypic like '*The effect of automated timed-access feeders on intake and growth rate of chickens*'.

8.4.2 The Introduction

What it should contain

Books on scientific writing suggest a wide variety of things that ought to be in an *Introduction*. They include vague proposals like:

- Define the scope of the study.
- Define the problem.
- Identify the gaps.
- State the objective.
- Summarize the background.
- State the question to be asked.
- Provide a context for the work.
- Explain the theory behind the work.

Of course you should cover all of these, but to what extent? If you systematically set out to deal with each in turn you would quickly run to a dozen or more pages. To avoid such wordiness you have to be more focused. This is where you can use the hypothesis effectively to connect the thinking behind the experimentation to the writing. Put forward your hypothesis and justify it as thoroughly as the literature and the information available allow. This literature and information are the focus of your *Introduction*. You can now decide very positively whether to include or omit information or references based simply on whether or not they contribute to the justification of your hypothesis. Your *Introduction* will be shorter than it might be otherwise

but that is not a bad thing. Brevity is always appreciated by editors trying to save precious space in their journals and by busy readers wanting to get the message from your article in the shortest possible time. And you, too, with a single focus will find the writing easier than if you are constantly worried about how far to go in describing things or citing references.

Preparing the reader

Giving a reader some background information is a waste of time unless, at the same time, the reader is being made aware of what this information is the background to. So the *Introduction* has to give an idea of what you expected to find at the same time as providing the setting for your experiment. For almost all experimental situations, that expectation is your hypothesis. It is what you logically predicted would happen given the information available from all sources before you carried out your experiment. In other words, the *Introduction* is putting into words exactly the same thinking that you did or ought to have done when the experiment was designed. So you can consider an *Introduction* as a formalization and recording of your reasoning before you began the work. You should be familiar with it when you start writing and have little need to introduce material that is new to you. In practice, this is sometimes not as simple as it sounds because the discipline of committing your thoughts carefully and precisely to paper often draws attention to gaps or flaws in the logic of your original reasoning. This is why writing is fittingly a part of the experimental process and not just an add-on after the experimental work is done.

As a result, readers who discover from your *Introduction* what you expected and, even more importantly, why you expected it will be armed with a series of questions for which they will be expecting answers later in the article. Questions like, 'How would the authors design an experiment to test whether this expectation is supported or not?', 'When I read the results will they support or disprove this hypothesis?', 'How will the authors explain it?', 'What will be the consequences if it is supported or not?', will automatically ensure that readers seek information. Not only that, but they will be viewing the information from the same point of view as you because they will have shared the same reasoning. As a result, it ensures that the rest of the article – methods, results and discussion – is straightforward for you to write well and for the reader to understand.

The concept of creating expectation to make stories flow and motivate readers is not confined to scientific writing and expectation can be stimulated by means other than using a hypothesis. But good researchers in animal nutrition telling their scientific stories are fortunate that they already have their hypothesis as the cornerstone of their experimental method. With it, they have at their disposal the ideal means of introducing the description and explanation of their work without looking further. The justification of the hypothesis not only offers an immediate and clear means of showing the reader why the work was done and the necessary background that inspired it; it also does so in the most logical and scientific way possible.

Let us look at an example. Hesselman and Åman (1986) studied the use of β-glucanase in chicken diets based on barley. An abridged summary of their introduction could have been (but wasn't) like this if they had followed a popular idea that introductions are only for presenting a broad context for the research:

- Barley is a major grain used throughout the world in diets for broiler chickens.
- In Sweden, over 3 million tons are used annually (the work was done in Sweden).
- It supplies energy through its high content of starch.
- The digestion of starch in the broiler chicken is not well documented.
- This is one of the first studies into digestion of starch in broiler chickens.
- Our aim was to measure the degradation of starch from high- and low-viscosity barley in different sections of the gastrointestinal tract after supplementation or not with β-glucanase.

This one has no hypothesis but an aim preceded by a series of loosely related pieces of information that gives very little to expect or look for in the remainder of the article. That barley is an important grain and that Sweden produces 3 million tons of it may be interesting but we can find that out anywhere and it is highly unlikely that it will help us follow the scientific story. Neither is the fact that no one seems to have looked at this before. An introduction like this would be largely irrelevant yet one often finds examples like this in scientific literature.

In fact, a summary of the *Introduction* that they did use was more like this:

- β-Glucanase improves the feeding value of barley.
- β-Glucanase breaks down the cell wall of the endosperm of barley.
- This makes starch more available for digestion in the gastrointestinal tract.
- Adding β-glucanase to barley-based diets in broilers improves growth and feed conversion.
- Starch in non-barley, synthetic diets (with no cell walls) is readily digested in the anterior small intestine.
- We hypothesized that the known increase in performance when β-glucanase is added is due to better absorption of starch in the anterior small intestine.

All the information it gives is relevant. As readers, we are told what the writers plausibly expected to find and so we, too, know what to expect. Later, when we come to the appropriate section we can assess how they went about it, what they found and how they explained it against the expectation that this type of *Introduction* has deliberately created for us.

And what about an aim? There is nothing wrong with an aim, so we could add the almost cryptic one from the first version:

- We tested this by measuring the degradation of starch from high- and low-viscosity barley in different sections of the gastrointestinal tract after supplementation or not with β-glucanase.

This aim is no longer cryptic. It is a logical consequence of the first five bullet points summarized by the hypothesis in the sixth.

8.4.3 The Materials and Methods

What it should contain

A good *Materials and Methods* is one that should give readers who are competent researchers in the field enough information to repeat the experiment if they so wish. This means describing, simply and accurately, what you did, how you did it and how you analysed it, chemically, statistically or in other ways.

Bear in mind, however, that most readers, when searching through a paper for what it has to offer, do not read this section very thoroughly. Some journals in the medical sciences have recognized this by moving the *Materials and Methods* to the end of the article and printing it in smaller font than the rest of the article as if it were just an appendix. When someone first reads an article, *Results* and *Discussion* are far more appealing. But if readers find something of interest there, they often come back to the *Materials and Methods* and read it very carefully either to verify that the work was done appropriately or to acquaint themselves with methods that may be new to them. For this reason, you can improve the reader's first impression by carefully providing meaningful headings to describe the detailed elements of your *Materials and Methods*. By reading only those headings, they can get a quick and broad picture of the experimental procedure and main resources used. Later, they can get the details by reading the text beneath the headings

For example, in an experiment into environmental factors that change the nutritional value of wheat, Choct *et al*. (1999) had these headings in the *Materials and Methods*.

- Apparent Metabolisable Energy determination
- Calculation of dry matter, gross energy, and AME
- Soluble and insoluble non-starch polysaccharides
- Starch
- Nitrogen
- Statistical analysis
- Ethical considerations

A quick scan of these subtitles gives an impatient reader a broad outline in a second or two of the most important factors that were measured. However, it is not apparent from these headings where the wheat samples came from or that they were tested in birds. Even though this information was in the detail, do you think a couple more headings would have improved its value for the skim reader?

- Experimental design and birds
- Source of wheat samples
- Calculation of dry matter, gross energy, and AME
- Soluble and insoluble non-starch polysaccharides
- Starch
- Nitrogen
- Statistical analysis
- Ethical considerations

We now know from just these few words that this was an experiment in birds that tested wheat from a range of sources for its composition, energy value measured in two ways, its starch and non-starch components and its nitrogen. And all of this was done ethically and analysed statistically. A succinct broad picture.

You don't have to spell everything out to describe your research. Usually some of the methods, analyses and even materials have been used and described by other authors. If this is the case, you only need to refer to the article where the work was first described. If yours is similar to but not identical with the earlier work, then refer to that work and say how yours differs, e.g. 'a modification of the technique of Bloggs (2013) in which we did . . . instead of . . .'. Leaving a 'paper trail' like this for people to read if they wish to repeat the experiment can save you many needless lines or even pages of text.

Preparing the reader

Preparing the text for *Material and Methods* does not require as much of the logic and reasoning that are essential in sections like the *Introduction* and *Discussion*. It is mainly factual description and is usually disjointed, which is why headings are useful to re-orient readers as they pass from one subsection to another. Most authors therefore find *Material and Methods* relatively easy to write and often build up their confidence by writing it first. However, there are a few minor considerations.

First, get the order right. Start with the methods you used, particularly the experimental design, and the animals and the venue rather than with the materials. Readers need a broad picture of the way experiments were done before they can appreciate where chemicals, analyses and pieces of equipment fit into the picture.

Second, you may have had to develop a method especially for your experiment. You have two options. If the main aim of writing your article was to describe and validate the method, describe it in the *Materials and Methods* section and present the results of the validation later, in the *Results* section. If, on the other hand, the method was devised to produce results for another aim, describe both the method and the results of the validation in the *Materials and Methods* section and don't confuse your reader by cluttering up your *Results* with data that are not integral to your scientific story. If you are in any doubt about which of these two options best fits your method, look at

your hypothesis. If the results for the validation merely show that it is an appropriate method but don't provide data to confirm or refute the hypothesis, present them in the section on methods.

8.4.4 The Results

What it should contain

Consider this section as an exclusive box into which only results, and only *your* results, have right of place. That means, no *Discussion*, no added *Introduction* or *Materials and Methods* and nobody else's results – just yours. It also means that your results should be presented objectively and without bias or comment. There is no place for bias in a scientific paper and the place for comment is the *Discussion* section.

However, objectivity does not automatically mean dullness. As far as your story is concerned, not all results are equal. Some will be crucial to the story and some will not. Some may even be a distraction that would interrupt the theme of your story. Before you write the *Results* you may not be certain which of your results are the really crucial ones. But, by the time you have finished, both you and the reader need to be very clear because, if you aren't, your *Results* are going to be very dull indeed.

You therefore need an effective way to confirm which of your results are the important ones and which are not. This is where the hypothesis comes into play once more. In the *Introduction* the hypothesis told readers what you expected to find, so when they begin to read your results for the first time, readers will begin to match what they see against that expectation. Without question then, the most important of your results are those that provide evidence that allows you to state as clearly as possible that your hypothesis was supported or rejected. Results that don't have anything to do with the hypothesis that you carefully articulated and justified are those that you have to query whether to include at all.

Be wary here, though. This does not mean excluding inconvenient results that may conflict with others supporting your hypothesis – often referred to as 'cherry picking' your results. All results that have anything to do with the hypothesis must be part of the *Results* and your eventual story will have to account for any that cause you problems in making an unequivocal conclusion. If, on the other hand, you measured some variables not associated with the hypothesis because you had the opportunity to do so but they don't show anything new or out of the ordinary, consider not including them. They can only clutter up the important results and confuse the reader. On the other hand, such peripheral results *will* occasionally be new and interesting although they are not part of the main story. In this case you would be letting yourself down if you didn't present them and eventually discuss them. The important thing is to take care to present and discuss them so that they do not get in the way of the main story that the reader expects to read.

Preparing the reader

The most potent place to present your most important results is at the beginning – immediately under the heading, *Results*. By putting them there you are doing two essential things. You are saying to readers, 'Look, this is what I found, isn't it great?' and you are ensuring that readers are at once able to satisfy the curiosity that you deliberately cultivated in their minds in the *Introduction*. Don't ignore this opportunity as so many authors do when they start *Results* with explanations of relative trivia like abnormalities that may have cropped up in the experiment, or missing plots or weather details, or measurements to show that the animals were normal or other relatively minor information. That, if it is relevant at all, should come later.

Then, as far as possible, present all your data relating to your hypothesis in descending order of importance, followed if appropriate by results unconnected to the hypothesis that you picked up fortuitously and feel are important enough to interest the reader. By doing this, you will convey to readers the relative importance, as you see it, of the evidence you have gathered from your experiment. Your *Results* will have light and shade instead of appearing to be a homogeneous mass of facts and numbers likely to intimidate the most dedicated reader.

You can help the reader even further by making your tables or graphs work together with the text. All results must be presented in words and most scientific papers have tables or figures. The golden rule is that both text and tables should be self-explanatory; that is, they should not need the reader to read both before being able to grasp what either one is trying to convey. We use tables and figures because they make numbers far easier to read than if they are in a line of text. But, having presented the numbers in this convenient way, how do we best handle the text that describes those same numbers? Take Table 8.1 below with results for clutch size and weight of eggs from a hypothetical experiment comparing four breeds of native chickens.

This table presents precise numbers and is comprehensive enough to be read on its own without resorting to the text. The caption describes the table, the row and column headings are clear and include the units of measurement where needed, and the footnote summarizes the main statistical information. Now, what do we need to put in the text? We could repeat the numbers but that would not only be needless duplication, it would also present the numbers in lines of text which are very awkward to read (that is why we put

Table 8.1. Mean and SD of size of clutch and weight of eggs from four breeds of native chickens.

	Breed			
	Red Runner	Blue Peter	White Pecker	Brown Clucker
Mean clutch size (days)	10.4 ± 1.6^{a}	9.6 ± 1.9^{a}	9.2 ± 1.0^{a}	10.7 ± 2.3^{a}
Mean egg weight (g)	38.2 ± 4.1^{a}	37.6 ± 3.7^{a}	51.3 ± 3.2^{b}	38.9 ± 2.9^{a}

Note: numbers in rows with different superscripts are significantly different ($P < 0.01$).

numbers in tables in the first place). Our obligation as scientific writers is always to be precise but having a table with precise numbers fulfils that obligation and frees us to meet our second obligation, clarity, in the text. We can now make it clear what we want the reader to draw from these numbers. So, a suitable text might be:

> The mean clutch size did not differ between the four breeds but the White Pecker breed produced eggs that were about 25% larger than those of any of the other three breeds (Table 8.1: $P < 0.01$).

In fact, the number, 25%, is not precise at all, but is close enough and the whole statement encapsulates precisely what we want the reader to have in mind when we talk about these results in the *Discussion*. Making the text and tables complement one another in this way, without losing any scientific integrity, encourages readers to follow your story instead of trying to work out why they are looking at large blocks of numbers.

8.4.5 The Discussion

What it should contain

One dictionary's definition of discussion is 'a conversation or debate about a certain topic'. Many authors seem to take this definition at face value and compose their discussions as a general and unfocused discourse that is disjointed and often too long. To produce a good *Discussion* in a scientific article, that definition has to be refined in at least four ways.

First, it is the completion of your scientific story, so it must be a discussion of *your* results and not other people's. You must place your results front and centre; in other words, don't say that your work agrees or disagrees with that of Smirch *et al*. Use Smirch *et al*.'s work to support or disagree with yours – yours is the focus, not theirs.

Second, like the *Results*, you will consider some points you want to make to be more important than others. And, like the *Results*, the important points are those that develop arguments relevant to the hypothesis, which remains your focal point; the more relevant, the more important. If you are considering making discussion points that are dissociated from the hypothesis, reflect on whether they are worth presenting. In terms of your story, they will be a distraction because they stray from the main theme, so they had better be good to help compensate for the diversion they will cause. It is imperative that this priority within your arguments comes clearly across to the reader and a simple way of doing this is to construct your *Discussion* with arguments in descending rank order.

Third, to raise an argument in a scientific *Discussion* without drawing a conclusion is a certain way to turn the reader off. Every discussion point must have your concluding statement. Statements citing extensive literature along with a selection of your results without answering the all-important

question 'So what?' are of no value in scientific articles. They just leave the reader confused and often angry and dissatisfied. Sometimes you won't be able to draw a satisfactory conclusion and, if this is because you still don't have enough evidence, then say so, but help the reader by concluding what evidence is still needed and possibly how an experiment might be devised to find it. It may not be earth-shattering, but it can help readers understand the true state of the field at the time you were writing and may germinate ideas for future work. If so, it is a very worthwhile conclusion.

Fourth, the single most common complaint by editors about *Discussions* is that they are too long. There are two good ways to ensure that yours is not in that category. First, resolve never to discuss any point unless you can draw a useful conclusion at the end of it, as we have just seen. Second, cut down on the material that adds little to the scientific debate. For example, telling everyone that you are the first to show something may boost your ego but is it important to the scientific proposition in question? You are discussing your results so there is no need to say constantly throughout the *Discussion*, 'Our results show that . . .' or 'Analysis of our results reveal that . . .'. Take it that the reader has already read your *Results*. Don't repeat them, or only repeat just what is necessary to introduce a new topic for discussion. Certainly don't repeat methods, or tracts from your *Introduction*.

Preparing the reader

Words are precious, so don't waste them. Help the reader by disciplining yourself to write the *Discussion* using paragraphing. The form of the paragraph was not invented for scientific writing but it might well have been, so well does it fit the goals that scientific writers seek to achieve. It is a wonderful and relatively easy way to accomplish the dual aims of writing compactly and ensuring that readers can follow your reasoning to the end of your article.

A conventional paragraph consists of three sections. The opening or topic sentence tells the reader the issue that the paragraph, or block of writing, is going to be discussing. The heart of the paragraph is a sentence or, more usually, a group of sentences, that develops the argument and subject matter, and the final sentence is the concluding sentence which, as its name implies, summarizes the reason for writing the paragraph in the first place. These three sections are exactly the structure needed for presenting well-reasoned scientific arguments. Use them well and you can never be rightly accused of talking nonsense.

To illustrate: look no further than the paragraph above. Its first sentence says that it is about paragraphs. The last sentence says the 'So what?'; the conclusion that you can use paragraphs to write *Discussions* that are clear and concise, and the sentences in the middle tell you why I was able to draw that conclusion.

So, before sitting in front of a keyboard to write your *Discussion* as a single piece of text, do some planning on paper or on a whiteboard. Think very carefully about what topics you feel you should discuss. Then, think

even more carefully about what the specific conclusion is going to be for each topic. If you feel that you don't have a conclusion, consider not bringing up that topic at all. The world will not be deprived if you leave it out and your *Discussion* in general will be less vague and your other, more definite topics in particular will be sharper for its absence. Then decide on the order of the topics based principally on their relative relevance to the theme of your whole article, the hypothesis, only varying that order where logic dictates that it is sensible to do so.

Now you can sit in front of the keyboard much more confidently with a blueprint that has a *Discussion* with a known number of paragraphs set out in a rational order, each with a first and last sentence already composed. Armed with this blueprint you will find writing a complete and compact *Discussion* a much easier and rapid task. Everything that you intend to write and every reference that you cite in each paragraph can be judged on two simple criteria: 'Is it directly related to the topic in the first sentence?' and 'Does it lead to the conclusion in the last sentence?'. If what you wish to say doesn't meet both of these criteria, leave it out or, maybe, save it for another paragraph where it will. If it does meet these criteria, you can go ahead and write with confidence knowing that your writing is direct and focused and will be appreciated for its logic by your readers.

8.4.6 The Summary

A good *Summary* tells the reader four things:

1. Why you did the experiment.
2. How you did the experiment.
3. Your main results.
4. Your main conclusions.

Fortunately, it should be easy to write because you will have already reflected on all of this information very carefully and even have most of the words already written.

1. The *Why* was to test your hypothesis, so just re-quote your hypothesis, which is, after all, the conclusion to your *Introduction*.
2. The *How* is a broad statement of your *Methods*, leaving out the details. To illustrate from an earlier example, 'We tested this by measuring the degradation of starch from high- and low-viscosity barley in different sections of the gastrointestinal tract after supplementation or not with β-glucanase'.
3. The *Main Results* are only those that concern the hypothesis and no other; that is, those to which you drew particular attention in the *Results* section.
4. The *Main Conclusions* are also only those that concern your hypothesis. In most cases, they can be a verbatim duplicate of the concluding sentences of the first paragraph or two of your *Discussion* – the most important ones.

It can't be much simpler than that!

8.5 Scientific and Political Correctness

An ever-present problem in writing scientific articles is to make sure that what you wrote was what you thought you wrote and that it is precise, clear, brief and scientifically correct. Your main problem here is that you become over-familiar with your work and, having spent some time on your first draft, you may also be over-familiar with your writing. Of course, you should carefully scrutinize the draft for typographical errors, errors of fact, transcription and calculation but this is not enough. You, as the author of this work, have probably lived with it for many weeks or even months. So you can sometimes assume that readers know as much as you do and leave out what may seem to you to be trivial details without which they may misunderstand you. The only way to avoid this is to have other people, ideally two or more people, read your draft critically. You need at least one person who is familiar with your work, or at least your field of work, to check that your writing is scientifically correct and credible. But if you can also find a person outside your immediate field to question your text and make suggestions, you could potentially make your article accessible to a wider group of readers. The important thing is that these 'friendly' reviewers be as thorough as you expect referees and editors will be, so that you avert as many problems as possible later at the publishing stage.

Publishers and research institutions require that certain statutory conditions be reported in scientific articles. In *Animal Nutrition*, the most common of these is the details of approval by official animal ethics committees, but human ethics committees could also be involved (for example, in experiments involving taste panels) and from time to time new regulations for other studies like recombinant DNA may legally require reporting. For most readers these statutory requirements add little to the scientific story being told, so it is sensible to report compliance where it will least interrupt the flow of information – for instance towards the end rather than at the beginning of the *Materials and Methods* where, strangely, many authors choose to put them.

Another, more concerning problem is that of plagiarism and breach of copyright – the practice of taking someone else's words and passing them off as one's own. Plagiarism is made easier nowadays by having masses of text readily available in electronic form on the Internet, which is simple to copy. Paradoxically, the power of the Internet also makes it possible to detect possible plagiarism by comparing new text with millions of published works for common sequences of words. Editors of journals do this routinely and there are also many freely available anti-plagiarism programs that authors can use to ensure that they have not inadvertently left themselves vulnerable to embarrassment. For example, authors have occasionally been stunned to have been accused of plagiarizing their own material only to realize that they had signed away copyright of the original material to a previous publisher. None of this means that you cannot use other people's words or ideas. After all, if they have expressed an idea or described something well and it matches

what you wish to say better than you can, why not? All that is necessary is to cite the original author correctly and, if it is a verbatim extract, use quotation marks to show that you are not claiming the words as your own.

8.6 Which Journal Is Best for My Article?

Never lose sight of the primary aim of publishing your work – to have as many people as possible read, understand and be influenced by it. One might expect it to be simple to choose a journal that best meets this aim. It is a little more complex than this.

First, until you have written the article and developed, honed and committed your results and ideas to words, you may not be able to judge who your main readers are likely to be. The discipline of writing often brings fanciful notions and prejudices to earth and sometimes changes them radically. So choosing the journal should be done late in the writing process rather than, as some people advocate, before you start.

Second, the rising importance of so-called 'metrics', or as some cynics say, 'quantifying the unquantifiable' – putting numbers on everything – complicates the decision. One of these metrics, the 'impact factor', has become extremely important. It puts a number on the quality of a journal. In broad terms, that number is probably acceptable but, like many such numbers, it has quickly become accepted as an immutable truth about quality without considering the inputs from which it was generated in the first place (Taylor, 2015). It is defined by its originator, Thompson Reuters, as 'the average number of citations received per paper published in that journal during the two preceding years'. Clearly, it says nothing about individual papers and, in addition, would severely discount papers whose message was slow in being taken up (Mendel would have contributed nothing to the impact factor of the journal in which he published his ground-breaking work – little notice was taken of it until 50 years later!). But impact factors are large in the thinking of administrators and distributors of money for research because they provide simple numbers that can be included in funding formulae. Therefore, administrators encourage researchers to try to publish in journals with the highest impact factors, often regardless of the readership of those journals.

Third, some journals, especially those with high impact factors, are very popular and have extremely high rates of rejection of papers – some higher than 90%. So, sadly, rejection is an increasingly familiar fact of publishing scientific articles and is always a blow to an author's self-esteem.

The dilemma is that, from an author's viewpoint, a more meaningful and satisfying metric is the citation index or some variation of it, like the h-index, z-index and others, which indicates the number of times an individual paper is cited. If you have been cited in another paper, it is a reasonable indication that the reader of that paper has read, understood and been influenced by your work. Therefore a journal that simply meets your prime aim in writing the paper should be the one to target.

8.7 Scientific Publication in the Future

The arrival of the Internet and unprecedented computer power have encouraged big changes in the way the scientific world is thinking about publishing scientific works. Obvious advantages such as online submission, editing and processing of manuscripts have almost universally replaced the old postal communication systems that had been in place for the previous century or more. But there is a more complex and rapidly changing evolution aimed at improving the peer review system and making research more accessible than it is in traditional, printed scientific journals. Unfortunately, much of this evolution is experimental and, so far, there is no common structure in place as publishers strive to find the best system for them that also meets the requirements of sound scientific publishing.

So, we have a wide range of 'open access' journals, many of which have little in common. The concept of open access is appealing because it invokes the idea of making new research available to the widest audience possible, but the term 'open access' can mean many things and comes in many forms. In its worst form it has produced many new and highly questionable publishers that appear to use the journals as scams to get publication fees from desperate or naïve authors (Bohannon, 2013). In its most responsible form such as *PLOS One* and many others, it is putting good science in front of many more readers than the traditional journals without the high subscriptions that these journals charge. In addition, they are making available new and helpful tools. For example, many of the respectable 'open access' journals require original data to be lodged in an accessible electronic repository. Many encourage feedback to the authors and readership and thus extend the peer review process from pre-publication to both pre- and post-publication. In fact, some institutions now require their researchers to submit their findings to open access only. Maybe this could become the norm in the future but balancing the speed of publication with the quality of the article will continue to be a major issue.

8.8 Will New Forms of Publication Change the Way We Write?

As we saw earlier, the most appropriate place to publish your work is where as many people as possible will read, understand and be influenced by it and this may be measured, at least approximately, by the number of times it is cited. Massive computer power has made this a routine and freely available measure. The proliferation of online publishing now widens the choice of journal available to authors and readers and therefore should increase the probability of being cited. But it does not alter in any way the fundamentals of good scientific writing that all authors should follow. Wherever and however it is published, the writing must still be straightforward, precise, clear and brief. Ultimately, meeting the objective of gaining as high a citation index as possible is simple.

Choose an appropriate journal or medium but, most important of all, *write well*.

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Nutrition Experiments in Pigs and Poultry

A Practical Guide

Edited by **Michael R. Bedford, Mingan Choct** and **Helen Masey O'Neill**

This practical research text provides an invaluable resource for all animal and veterinary scientists designing, analysing and interpreting results from nutrition and feed experiments in pigs and poultry.

The emphasis throughout is on practical aspects of designing nutrition experiments. The book builds on the basics and proceeds to describe the limitations of experiment design involving different ingredients. It goes on to describe the characterization of experimental diets including ingredient selection, composition and the minimum proximate analysis required. The text details measurements and the tools available for understanding diverse data sets, data analysis and eventual publication of the research.

This fully balanced and extensively referenced, yet practical, text is an invaluable resource to all animal, veterinary and biomedical scientists involved in designing nutrition experiments in pigs and poultry.

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