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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