VARIATION IN THE DIGESTION OF ENERGY BY BROILER CHICKENS

By

Robert James Hughes

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Faculty of the Sciences

The University of Adelaide

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Abstract

Feed is the largest single cost factor (60%) in production of chicken meat with cost of energy being a major consideration given that birds eat to satisfy an energy requirement. The Australian chicken meat industry is highly dependent on supply of energy from cereals such as wheat and barley that are known to vary widely in apparent metabolisable energy (AME). In contrast, sorghum is a relatively consistent source of energy.

Diets for broiler chickens are comprised mainly of cereal grains, legumes and protein-rich meals of plant and animal origin. The diets are formulated to provide essential nutrients for maintenance and rapid growth of the flock as a whole. However, some dietary ingredients may also have chemical and physical properties that can be detrimental to the processes of ingestion, digestion, absorption, transport and utilisation of nutrients. Soluble non-starch polysaccharides (NSP) in cereal grains such as wheat and barley can depress digestion of energy by broiler chickens.

This thesis examined the general hypothesis that the effects of soluble NSP in cereal grains on gut structure and function, digesta transit time, and gut microflora differ substantially between individual chickens within a flock, thus contributing to variation in the digestion of energy by the flock as a whole. A major goal of the research was to determine what characteristics of the gastrointestinal tract of broiler chickens were the key determinants of digestion of energy. Twelve experiments were conducted during this study. Breath tests involving measurements of carbon dioxide, hydrogen and methane were developed as non-invasive indicators of digestive function, and were used in conjunction with conventional methods for measuring energy digestion in commercial strains of chickens.

Sex of the chicken had a significant effect ($P<0.05$) on AME values obtained for a diet based on wheat with a high soluble NSP content. Females were superior to males (14.6 vs 14.0 MJ/kg DM), but strain of chicken had no effect on AME. Villus height and crypt depth in the intestinal epithelium were measured to determine if any relationships between gut morphology and AME could explain why males and females differed. Males had significantly greater ileal villus height than females ($P<0.05$). In one of the two strains of chickens studied, villus heights in the duodenum and jejunum tended to be greater in males than females ($0.05<P<0.10$). In the second strain of chicken, villus heights in the duodenum and ileum were lower ($P<0.05$) than those in the first strain, with little

differences observed between males and females. Crypt depth was unaffected by strain or by sex of the chickens. Thus, individual measurements of gut morphology were poor indicators of AME. Furthermore, only 33% of the total variation in apparent metabolisable energy (AME) could be accounted for by combinations of measurements of villus height and crypt depth in the duodenal, jejunal and ileal sections of the small intestine. It was concluded that other determinants of digestive capacity were collectively more important than gut morphology.

Energy excretion by male chickens was observed to rise in an exponential manner relative to energy intake, whereas the increase in females was linear. It was reasoned that increased energy excretion by males could be due to increased endogenous energy losses from the distal part of the intestinal tract, reduced production of volatile fatty acids (VFA) by microbial fermentation in the caeca, or reduced absorption of VFA by caeca. These possibilities pointed to the need for a closer examination of the role of gut microbiota on the digestive function of chickens. It was also clear that further studies should differentiate between digestion of energy in the small intestine (by measurement of ileal digestible energy) and whole of tract digestion (by measurement of AME).

Ileal digestible energy (DE) values for wheat and barley were unaffected by sex of chickens, whereas AME values were lower in male chickens compared with females. These results suggested that the sex-specific effects of microbiota occurred mainly in the hindgut. Furthermore, the influence of the gut microbiota on between-bird variation in AME was partially dependent on the type of cereal grain used in the diet, as indicated by the observation that the differential between males and females in expiration of hydrogen and methane in the breath changed according to the type of grain consumed. That is, the metabolic activity of the gut microbiota was influenced both by the sex of the chicken and by the properties of the diet.

The results of these studies provided evidence that microbial colonisation of the gut is a key determinant of the digestive function of chickens. Further work is needed to determine why microbial colonisation of the gut is variable and why it differs substantially between male and female chickens. Then it may be possible to control the initial colonisation of newly hatched chicks and to maintain a health-promoting profile throughout the life of chickens in order to enhance efficient production, and product quality and safety.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signature

R. J. Hughes

Discipline of Animal Science

The University of Adelaide

Adelaide, South Australia 5000

Australia

Dedication

This thesis is dedicated to the memory of the late Dr R.B. Cumming (formerly of the University of New England, Armidale, New South Wales, Australia) who inspired me to ponder the differences between individual birds within the flock, and to explore the consequences of that variation through basic and applied experiments with chickens.

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With the continuing support of you all, I would like to think that my best is yet to come.

"If you are scared, don't do it: if you do it, don't be scared -- Old Mongolian proverb.

Publications associated with the thesis

Some of the work described in this thesis was presented at various meetings in Australia and New Zealand and published in proceedings of conferences and scientific journals.

Carter, R.R. and Hughes, R.J. (2002). Age dependent responses of chickens to enzymes in wheat and barley diets. A*ustralian Poultry Science Symposium* **14,** 101.

Hughes, R.J. (2000). Nutritional management of broilers - what awaits us? In 'Proceedings of the New Zealand Poultry Industry Conference, Palmerston North': pp.103-115.

Hughes, R.J. (2001). Variation in the digestive capacity of the broiler chicken. *Recent Advances in Animal Nutrition in Australia* **13,** 153-161.

Hughes, R.J. (2003). Sex and the single chicken. A*ustralian Poultry Science Symposium* **15,** 172-176.

Hughes, R.J. and Choct, M. (1999). Chemical and physical characteristics of grains that are related to variability in energy and amino acid availability in poultry. *Australian Journal of Agricultural Research* **50,** 689-701.

Hughes, R.J. and Choct, M. (2000). Factors influencing the energy content of cereal grains in broiler diets. In 'Proceedings of the New Zealand Poultry Industry Conference, Palmerston North': pp.134-145.

Hughes, R.J., Choct, M. and van Barneveld, R.J. (2001). Factors influencing the energy values of Australian cereal grains fed to broilers. *Australian Poultry Science Symposium* **13,** 30-38.

Hughes, R.J., Symonds, E.L., Tivey, D.R. and Butler, R.N. (2000). $^{13}CO_2$ breath tests for evaluating gastro-intestinal function in broiler chickens. *Australian Poultry Science Symposium* **12,** 170-173.

Hughes, R.J., Tivey, D.R. and Butler, R.N. (2001). Hydrogen and methane breath tests for assessing metabolic activity of gut flora in broiler chickens. *Australian Poultry Science Symposium* **13,** 168-171.

Hughes, R.J., Tivey, D.R. and Butler, R.N. (2002). Breath tests for estimating digesta transit time in chickens. *Australian Poultry Science Symposium* **14,** 108-111.

Hughes, R.J., Tivey, D.R. and Choct, M. (2000). Gastro-intestinal tract structure and energy metabolism in broilers. *Australian Poultry Science Symposium* **12,** 166-169.

Abbreviations

AME	apparent metabolisable energy
ANOVA	analysis of variance
AU	Adelaide University
CE	competitive exclusion
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CV	coefficient of variation
DE	digestible energy
DM	dry matter
DMD	dry matter digestibility
EE	energy excreted
EEL	endogenous energy losses
FCR	feed conversion ratio
GE	gross energy
GEI	gross energy intake
GIT	gastrointestinal tract
GLM	general linear model
GRDC	Grains Research and Development Corporation
LSMEANS	least squares means
MOS	mannan oligosacharide
NSP	non-starch polysaccharide(s)
OCTT	oro-caecal transit time for digesta
PPPI	Pig and Poultry Production Institute
RIRDC	Rural Industries Research and Development Corporation
SARDI	South Australian Research & Development Institute
SAS	Statistical Analysis System®
SD/SE	standard deviation/standard error
TME	true metabolisable energy
UNE	University of New England
VFA	volatile fatty acids
WCH	Women's and Children's Hospital
WTTT	whole tract transit time for digesta

Chapter 1. Literature Review

1.1 Introduction

Feed is the largest single cost factor (60%) in production of chicken meat, with cost of energy being a major consideration given that birds eat to satisfy an energy requirement (Miller 1974). Over- or under-estimation of other nutrient levels relative to energy results in less efficient production, variable growth rate, and considerable wastage *via* excreta, all of which lead to increased cost of production. Absolute and relative shortages of feedstuffs for poultry can be expected in the near future if current trends in usage of grain for livestock continue in Australia (Meyers Strategy Group 1995). During periods of shortage or high costs, it is likely that lower value grains and by-products with higher levels of anti-nutritive components will be used. Wider variations in flock performance and dressed carcass weight and composition can be expected to follow.

Benefit/cost analyses of poultry research conducted in Australia (Black 1995) point to highly significant returns on investment in nutrition research that leads to an increase in efficient production of lean chicken meat. In Australia, annual cost savings in excess of AUD$6M can be made for each 1% improvement in feed conversion. Basic studies are required to elucidate the modes of action of factors contributing to specific problems in commercial flocks in order that these are diagnosed quickly and effectively. Also, development of computer models for improving production and processing efficiency will require highly specific information on fundamental aspects of nutrition and physiology of broilers, particularly in the area of energy metabolism.

A fundamental mathematical assumption that must be made for least cost feed formulation by linear programming methods is that nutritive values of feed ingredients are additive. For example, the value assigned to apparent metabolisable energy (AME) for one ingredient is assumed independent of the dietary inclusion rate of that ingredient and is not influenced by the presence of any other ingredient or component of the diet (Miller 1974). Cost-effective use of feed ingredients for poultry requires detailed knowledge of the nutrient composition of the ingredients and the likely effects of any anti-nutritive properties of those ingredients.

There is a rapidly growing body of evidence that this assumption of additivity of nutrient values does not always hold true in commercial practice. A prime example is the effect of soluble non-starch polysaccharides (NSP) on digesta viscosity, starch digestibility and AME (Annison 1993a), and the ameliorating effect of exogenous glycanases (Choct *et al.* 1996*a,b*) that in the last decade have become a common component in broiler feeds (Bedford and Morgan 1996). Small increments in dietary concentration of soluble NSP such as arabinoxylan from wheat or β-glucan from barley can result in non-linear increases in viscosity of digesta with a resulting loss of performance by interference with digestion and absorption of nutrients (Annison 1993a).

The Australian chicken meat industry is highly dependent on supply of energy from wheat and barley. Both cereals are known to vary widely in AME due to the presence of NSP such as arabinoxylan and β-glucan (Hughes and Choct 1999). Surveys by Mollah *et al.* (1983) and Rogel *et al.* (1987) indicated a range of 10-16 MJ/kg in AME values for wheat. Starch contents of wheats ranged from 588-719 g/kg dry matter (Mollah *et al.* 1983) and 504 to 596 g/kg (as fed basis) (Rogel *et al.* 1987). When low-AME wheats were fed to chickens, considerable amount of starch was detected in the excreta, indicating incomplete starch digestion in the small intestine. Because starch constitutes up to 70% of the total energy of wheats, variation in its contents may be reflected in the AME values. The ileal digestibility for starch, but not the starch content of the grain, was highly correlated (R^2=0.886) with the AME (Mollah *et al.* 1983; Rogel *et al.* 1987). The physical structure of the grain and the chemical properties of the starch granules were also excluded as causative factors for the low-AME wheat phenomenon because AME was unaffected by grain hardness, and starch isolated from low-AME wheat was completely digested by chicken pancreatic amylase (Rogel *et al.* 1987). Age of the birds significantly affected the starch digestibility of wheats. The ileal digestibility of starch ranged from 21.9 to 98.3% when the chickens were 3 weeks of age, but it was highly digestible and much less variable when the chickens were 6 weeks of age (Rogel *et al.* 1987). This indicated that starch *per se* is not a factor in the low-AME wheat phenomenon (Choct *et al.* 1999).

Recent studies by R.J. Hughes *et al.* (unpublished data) on probable causes of intestinal dysfunction in commercial flocks point strongly to a highly significant bird component to the problem. These observations led to the development of the hypothesis that wide between-bird variation in gut function persists in commercial strains of broiler chickens

despite heavy selection for economically important traits such as lean tissue growth and feed efficiency, and despite usage of feed enzymes in broiler diets. To date, the importance of bird-related factors in resolution of nutritional problems in commercial practice has received relatively little attention.

In the following sections of this review, I discuss (1) the digestive physiology of chickens, with particular reference to energy metabolism, and (2) those properties of feed ingredients that affect gastro-intestinal function.

1.2 Factors influencing energy metabolism in chickens

Tivey and Butler (1999) described the digestive capacity of an animal as the integration of residence time of digesta, enzyme secretion, absorptive mechanisms, microbial activity, surface area, and barrier function. This concept of digestive capacity in relation to the individual chicken was developed as a framework for investigation of the dynamic relationships between bird-related and feed related factors which can interact to influence energy metabolism in chickens (Figure 1).

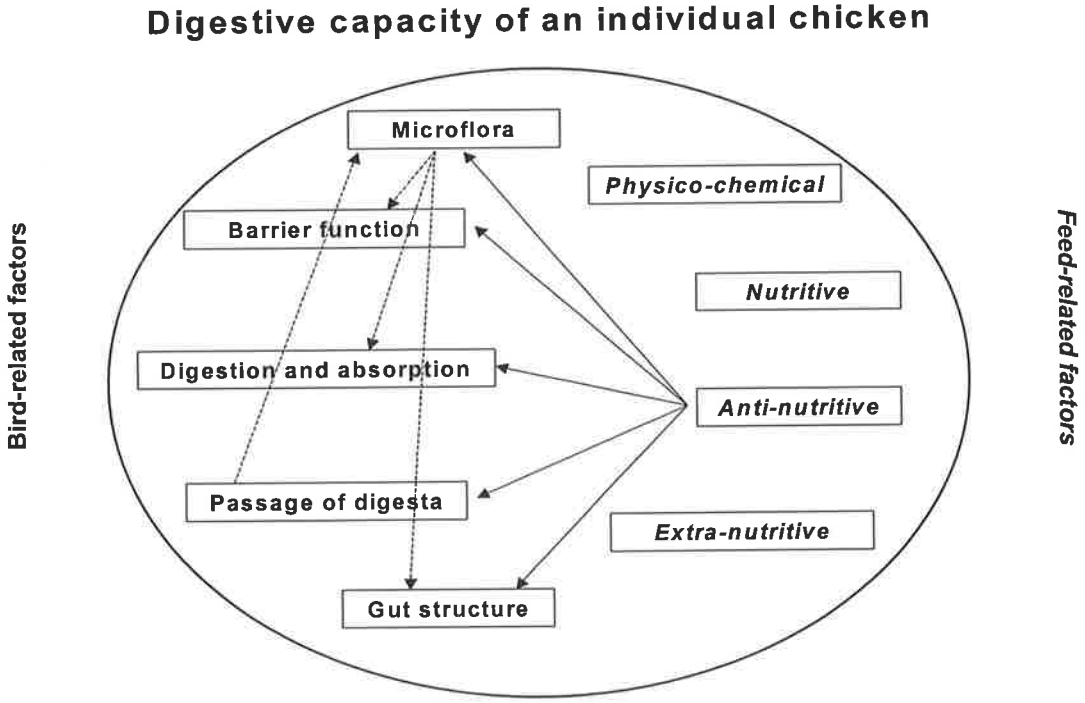


Figure 1. A schematic representation to demonstrate that the digestive capacity of the chicken is an integration of bird-related and feed-related factors. Adapted from Hughes (2001*a*).

The particular example shown in Figure 1 represents anti-nutritive effects of soluble NSP (feed-related factor) acting on all bird-related factors. It also shows a cascading effect of metabolic activity of microflora on three other bird-related factors, as well as an effect of changed rate of passage (due to increased viscosity associated with soluble NSP) on microbial proliferation in the small intestine (Choct *et al.* 1996*a,b*). Besides NSP, other components of the feed can interact with bird-related factors to influence digestive capacity.

The importance of each of these major determinants of digestion and assimilation of nutrients (as described by Tivey and Butler 1999 in relation to the Kyberplus system for diagnosis of gastrointestinal diseases) is discussed in the following sections of this review, as well as the known interactions between the determinants, as outlined in Figure 1.

1.2.1 Gut structure

Net utilisation of energy by the chicken will be influenced by requirements of the gut for growth and maintenance (Choct 1999), and by its overall gut surface area. The latter will be determined by gross morphological features such as length and cross-sectional area of the duodenal, jejunal and ileal segments, and by finer morphological features such as villus height and surface area of the epithelium in each of those segments (Jin *et al.* 1998; Iji 1999).

The embryonic development of the gastro-intestinal tract of the chicken was reviewed by Uni *et al.* (1996) and Kannan *et al.* (1993). Vieira and Moran (1999) noted that while the gastro-intestinal tract was structurally complete at hatching the lengthening of the villi and associated enzymic activity took up to two weeks to reach a mature stage. Iji *et al.* (2001) confirmed these findings and added that the rates of development in gross size and epithelial function were more rapid in the duodenum than in lower regions of the small intestine, particularly in the 7-day post hatch period.

Uni *et al.* (1995; 1996) reported significant differences in morphology and functionality of the small intestine in two strains of broiler chickens on hatching. The differences in villus volume and enterocyte density had disappeared within 14 days of hatch by which time the chickens had acquired similar rates of digestion of starch. Presumably then, variations in energy uptake in chickens from 22 to 29 days of age as reported by Hughes and Choct

(1997) were not due to delayed or variable rate of development of the gastro-intestinal tract, which could otherwise influence digestive function (Iji 1998).

Baranyiova and Holman (1976) concluded that the availability of food soon after hatching stimulated the growth of the small intestine, in particular, the absorptive surface area. On the other hand, they noted that starvation for up to 5-days post-hatch resulted in no changes to villus height or diameter during this period. Yamauchi *et al.* (1990) conducted a comparative study of the small intestinal segments of male White Leghorn (egg-type strain), broiler chicken, Pekin duck and wild duck. Their measurements of gross morphology included weight, length and cross-sectional area of the duodenum, jejunum and ileum, all adjusted for body weight. They concluded that a larger intestinal surface area was closely associated with the faster growth rate in heavier type of birds and that within each type of fowl, body weight of domestic fowls increased with an increase in surface area.

Clarke (1967) noted earlier that post-embryonic growth in the epithelial surface area of the duodenum occurred through increase in the size of villi but that the number remained constant for the entire life of the fowl. On the other hand, the duodenal crypts increased greatly in number and depth but changed little in diameter (Clarke 1967). Forrester (1972) and Clarke (1972) demonstrated that the number of villi in the intestine of the rat also appeared to be constant. On the basis of these findings, Wright and Alison (1984) concluded that the villus was a fixed unit of mucosal architecture and that any adaptive changes were mediated through variation in the size of villi, and not by variation in the numbers and/or epithelial function.

Ferrer *et al.* (1995) used transmission electron microscopy to measure the length, diameter and density of microvilli and cell apical diameter in tip-villous enterocytes in newly hatched chickens, and at two and six weeks thereafter. They suggested that microvillus length and density best explained the changes observed in apical surface occurring during development

Smits (1996) used carboxymethylcelluloses (CMC) with low and high viscosity to study the physiological effects of soluble NSP on the morphology of the intestinal epithelium of broiler chickens. In one experiment, high viscosity CMC given to chickens from 21 to 35 days of age (a) reduced growth rate, feed efficiency, and faecal digestibility of fat,

(b) raised the viscosity of digesta and the ATP concentration (an indication of bacterial proliferation) in the upper and lower sections of the small intestine, and (c) caused an increase in weight and length of the total intestinal tract. In a separate experiment with younger chickens (7 to 18 days of age), fat digestibility was depressed, and jejunal villus height and number of goblet cells per unit length of villus were raised. There were no effects on proliferation rate of enterocytes in the jejunum or ileum, crypt depth, villus to crypt ratio or composition of mucin. Smits (1996) concluded that the anti-nutritive effect of soluble NSP was not primarily associated with mucosal damage, although an increased goblet cell population may tend to indicate otherwise, as might an increase in mucus, which was not measured. However, fat digestibility was more severely affected in the first experiment (34 versus 64%) than the second (68 versus 76%), despite the chickens being older, and there were no direct or indirect measures of bacterial activity reported for the second experiment to indicate the relative size of the microflora population. Hence, it remains possible that a heavy bacterial overgrowth of small intestinal digesta (such as that reported by Choct *et al.* 1996*a*) could have a detrimental effect on the epithelial structure or function. Another point of concern is that Smits (1996) used female chickens only in these two studies. The possibility that energy uptake is partially dependent on sex of the chicken is discussed in later sections.

Finally, Silva and Smithard (1996) reported that exogenous enzymes in broiler diets significantly reduced crypt cell proliferation rate. It would be worth determining whether this effect occurred as a direct result of enzymes attaching to the epithelial surface or was an indirect effect resulting from reduced opportunity for bacterial overgrowth following depolymerisation of NSP by enzymes. Either way, the mechanism(s) involved require elucidation if this is a repeatable response to feed enzymes. It should be noted that in a later study, Silva and Smithard (2002) observed no change in crypt cell proliferation rate in the small intestine from male broiler chickens given a rye-based diet supplemented with a commercial enzyme product with mainly xylanase activity and some protease activity. However, they were able to detect the presence of exogenous enzyme in the small intestine, and there was a reduction in digest viscosity associated with an improvement in growth.

In conclusion, dynamics of epithelial cell populations in the mammalian small intestine are well studied (Wright and Alison 1984*a,b*). Maintenance of a healthy, functional gastrointestinal mucosa requires a finely controlled balance between cell proliferation and

apoptosis. Some bird- and feed-related factors (discussed in subsequent sections) may disturb this balance.

1.2.2 Passage of digesta

Rate of gastric emptying of solids and liquids, and transit time in the small intestine are known to influence the nutritional status of human subjects (Tivey and Butler 1999). It follows that the extent of opportunities for contact between ingested food, digestive enzymes and bile salts, and the time available for contact between digested particles and absorptive surfaces will influence uptake of energy and other nutrients by chickens (van der Klis and van Voorst 1993; Uni *et al.* 1995).

Duke (1986, 1992) reviewed the literature on gastric motility in avian species. In the latter review, Duke (1992) concluded that while the characteristics of gastroduodenal motility were well defined in turkeys, regulation of motility was not. He indicated that regulation involved complex interrelationships between neural and hormonal activities in the intestinal tract. The presence of digesta in the duodenum resulted in release of the hormones, pancreatic peptide and cholecystokinin that acted to slow gastric emptying. Moreover, passage of digesta was influenced by age and health of chicken, consistency and hardness of the feed, dietary concentrations of fat, fibre and protein, antibiotics, environmental temperature, and fasting and overeating (Duke 1986).

Clench and Mathias (1992) observed a reversal of the flow of digesta in response to fasting in adult cockerels. They described the phenomenon as a rhythmic oscillating complex that could be an adaptive mechanism for the return of undigested food in the caeca to the small intestine during a period of inadequate food intake, in birds only. Godwin and Russell (1997) noted that the reverse peristalsis was highly effective in fasted birds but appeared to have little effect in the fed animal. The reversal of flow of digesta could result in the entry of uric acid, other potentially toxic waste products and harmful microorganisms into the small intestine.

The reflux of uric acid could have a detrimental effect on gut epithelial function under these circumstances. Langar *et al.* (1993) reported a reduction in villus height in the small intestine with an associated decline in nutrient absorption in poultry given diets containing 1 to 1.75% uric acid. On the other hand, reflux of volatile fatty acids with bacteriostatic

action (Corrier *et al.* 1990) may be beneficial in inhibiting microbial proliferation in the small intestine. Furthermore, Choct and Kocher (2000) reported that the caecal flora of the broiler chicken produce some xylanase and β-glucanase activitities, which may be refluxed into the small intestine where the action of these enzymes could add to variation in energy digestion and absorption through the effects of reduced digesta viscosity (as discussed in more detail in section 1.3.4 below). Clearly, the nutritional implications of bacterial enzyme activity in the gut warrant investigation.

The effects of the gut microflora are discussed further in sections 1.2.4 and 1.2.5 below).

1.2.3 Digestion and absorption

Capacity to digest and absorb carbohydrates develops during incubation, providing the newly hatched chick with a relatively mature system for utilisation of starch, the main carbohydrate in the diet of poultry, according to Moran (1985). On the other hand, the capacity to utilise fat can take 10 days or so to develop in broiler chickens due to a lag in lipase secretion by the pancreas (Jin *et al.* 1998). They concluded that total digestive enzyme activity tended to increase during the early post-hatch period because of rapid increase in the weight of the pancreas and intestines. Similarly, Jin *et al.* (1998) noted that utilisation of some NSP, oligosaccharides and protein was less efficient during this early period than later in the life of chickens and turkeys.

Uni *et al.* (1995) reported differences in ability to digest starch in the period 0 to 4 days post-hatch between two strains of broiler chicken. However, by day 14, starch digestion was greater than 90% in both strains, which compares favourably with that seen in broiler chickens 28-days of age (Choct *et al.* 1995; 1996*a*) and 35-days of age (Choct *et al.* 1999). These changes are consistent with those noted by Vieira and Moran (1999) who concluded that the full capacity of the small intestine to retrieve nutrients took up to two weeks to develop. They attributed this in part to the initial orientation of enterocytes towards luminal absorption of immunoglobulin from the remaining contents of the yolk sac, rather than towards nutrient retrieval. Uni *et al.* (1996) earlier concluded that nutrient supply from the yolk was less crucial than a lack of feed in the first 36 hours post-hatching, which subsequently delayed normal intestinal development for several days.

Vasquez *et al.* (1997) examined the age-related changes in density of Na-dependent D-glucose transporter in jejunal brush-border membrane vesicles of chickens. Transporter site density was highest at one week then declined in the second week and remained constant thereafter. They concluded that changes in the density of Na+-dependent D-glucose transporter as well as in lipid content and fluidity were involved in the age-related changes observed in D-glucose uptake.

Uni *et al.* (1998) studied changes in the structure and function of the duodenum, jejunum and ileum in broilers from hatch to 14 days of age. They noted that development of the small intestine was rapid from day 2 after hatch but that the rates of development differed between the segments of the small intestine. Villus volume in the duodenum reached a plateau after 7 days but continued to increase in the jejunum and ileum. Indices of tissue activity, ribosomal capacity, and cell size decreased with age, but at differing rates at the three intestinal sites. Sucrase-maltase activity (expressed as micromoles of substrate hydrolysed per g of intestinal tissue per hour) was low in the duodenum, and highest in the jejunum and ileum at hatch. Maximum activity in the jejunum occurred two days post-hatch then decreased. Density of the enterocytes changed little from 0 to 14 days post-hatch.

As with the physical changes in gut structure observed in the two-week post-hatch period (section 1.2.1 above), major biochemical changes in the development of the gut also seem to have stabilised within a relatively short time after hatch. Nevertheless, it is possible that the subtle differences in gut structure and function evident within groups of individual chickens of the same strain are sufficient to effect the uptake of energy in the highly variable manner observed by Hughes and Choct (1997).

Kocher (2001) used NMR spectrometry to demonstrate structural changes in soluble NSP isolated from digesta in chickens given diets containing sorghum, lupins and commercial enzyme products with xylanase, glucanase and pectinase activities. The enzymes did not affect AME values or growth characteristics of the chickens, but did cause a reduction in viscosity of the digesta that is otherwise thought to be associated with the anti-nutritive properties of NSP (as discussed further in section 1.3.2). On the other hand, Iji (1998) showed that addition of a commercial enzyme product with xylanase, glucanase and pectinase activities to the diets failed to affect visceral organ weights, or morphometry of

the jejunal and ileal mucosa. Iji (1998) also showed that the specific activity of maltase in the jejunum was raised in chickens given wheat or barley supplemented with commercial enzyme product, but was reduced in chickens given maize or sorghum with supplementary enzymes. Activities of sucrase, aminopeptidase N and alkaline phosphatase were unaffected by these exogenous enzymes.

This raises an interesting question as to whether degradation products from exogenous enzyme activity can influence gene expression in mucosal tissue, or in the metabolic activity of commensal bacteria, as suggested by the work of Choct and Kocher (2000) mentioned in section 1.2.2 above. It also seems plausible that some of the variation in AME remaining after treatment by exogenous enzymes to effectively eliminate the deleterious effects of highly viscous digesta (section 1.3.2 below) could be the result of small polymer fragments attaching to the gut epithelium, thereby interfering with absorption of nutrients.

The effect of the sex of the individual animal on its functional capacity to digest and absorb nutrients has received little attention by researchers. Indeed, much of the recently acquired knowledge about nutrient utilisation by commercial broiler chickens has been gained by study of males only (Hughes 2001*a*). This is, perhaps, surprising given that the importance of sex effect on energy metabolism was noted long ago by Guirguis (1975, 1976).

There are tantalising hints in the scientific literature that males and females differ in other unexpected ways of a fundamental nature. Recent studies with chickens support the hypothesis that sex of the animal can influence digestive capacity through at least two different mechanisms. Firstly, Iji *et al.* (2001) observed a greater *in situ* expression of α-glucosidase in jejunal mucosa in female chickens compared with males, irrespective of whether the diet contained a commercial enzyme product with xylanase, glucanase and pectinase activities. Secondly, Yaghobfar (2001) observed that layer and meat strain females utilised energy from maize more efficiently than males when fed dry mash, but the reverse was true when feed was offered as wet mash. The difference was attributed to significantly greater endogenous energy losses (EEL) in males. Other examples of sex-related differences include the slower rate of growth in male piglets immediately after

weaning reported by Dunshea *et al.* (1998), and the observation of Chicurel (2000) that sex and environment can affect the expression of genes.

Finally, Hughes (2001*b*) noted that there were many observations of sex differences in responsiveness to drugs such as scopolamine and caffeine. These could be accounted for by differences between males and females in the extent of their ability to absorb, store, metabolise and excrete them, rather than to differences in mechanisms at the cellular or receptor level. He pointed out that there were examples of sex differences in a variety of behavioural and physiological processes in rats and other animals. Female rats were able to familiarise themselves with a novel environment more rapidly than males. This ability appeared to be dependent more on visual than olfactory cues (Hughes and Beveridge 1990) that Hughes (2001*b*) attributed to sex differences in organisation of the rat's visual cortex as observed by Reid and Juraska (1995).

1.2.4 Microflora

Dietary factors which lead to increased activity of gut microflora can have detrimental effects such as depressed starch digestion (Choct *et al.* 1996*a*), reduced apparent protein digestibility (Smits *et al.* 1997), and reduced availability of amino acids (Steenfeldt *et al.* 1995). On the other hand, endogenous enzymes produced by gut microflora may have beneficial effects. For example, Choct and Kocher (2000) detected xylanase and β-glucanase activities of gut microbial origin in caecal contents but not in ileal digesta from chickens given a low-ME wheat diet. If these observations are indicative of changes in the profiles of bacterial populations in these chickens then it follows that variation in production of microbial enzymes could contribute to the variability in energy uptake by birds through three different mechanisms. These involve the effects of digesta viscosity on digestion and absorption of nutrients (Smits *et al.* 1997; Williams 1995), use of nutrients from digesta to support microbial proliferation (Bedford and Apajalahti 2001), and effects on gut motility and rate of passage of digesta through the gut (Tivey and Butler 1999).

Klasing (1996) described the homeorhetic responses that take place when a chicken is faced with a bacterial challenge. These include decreased appetite, the partitioning of dietary nutrients away from growth, skeletal muscle accretion in favour of metabolic processes that support the immune response and disease resistance, and alteration of

nutrient requirements during and after the infectious challenge. During the challenge, the requirements for amino acids and most trace minerals are decreased whereas these are increased later to repair damaged tissue and to accelerate growth. Klasing (1996) recommended an increase in dietary carbohydrate to compensate for the decrease in appetite in order to assist the recovery and lift the performance of immune-stressed animals.

Abrams *et al.* (1963) concluded that the gut microflora reduced the lifespan of ileal epithelial cells in mice by accelerating the rate of loss of cells from the villi. They also noted that the microbial flora exerted a profound influence upon cell population dynamics, not only in the epithelium but also in the lamina propria and to the defensive functions of the lymphoid tissue in the mucosa. Finally, Abrams *et al.* (1963) emphasised that during its migration from the crypt to the tip of the villus, the epithelial cell underwent significant changes to enzyme systems, chemical constituents, ultrastructural organisation, and probably functional capacity. Recent studies have confirmed these findings in poultry (Iji 1998, 1999) and pigs (Kelly and King 2001).

Inclusion of an antibiotic in the diet of pigs can result in a reduction in the weight and a change in the morphology of the small intestine (Parker and Armstrong 1987). These changes included elongation of villi and a higher villus: crypt ratio, which was indicative of a slower rate of enterocyte-cell migration from the crypt to the villus. It was suggested that reduced microbial activity in digesta or microbial activity at the level of the brush border would reduce both the damage to enterocytes and the need for cell renewal in the gut. On the other hand, gut bacteria may assist in the normal development of the gastrointestinal tract, at least in pigs (Kelly and King 2001).

Osborne and Seidel (1989) induced increased mucosal DNA, RNA, and protein contents in the distal ileum of rats by infusion of exogenous putrescine into the ileal lumen. They noted that polyamines such as putrescine, spermidine, and spermine were implicated as regulatory compounds in the growth of the intestinal mucosa during mucosal maturation and in repair of damage to the intestinal mucosa. The results of Osborne and Seidel (1989) are consistent with earlier reports that polyamines produced by fermentation by gut bacteria could influence colonic mucosal structure in rats. One would expect that endogenous polyamines could contribute to variation in nutrient digestibility in chickens, as would

biogenic amines in poorly produced fish meals and other fermented protein meals (as discussed in section 1.3.5).

Williams (1995) has pointed out that gut microflora can significantly influence metabolism of avian gut tissue which in turn will affect absorption of amino acids. Protein supplements with poor digestibility will undergo more microbial fermentation than highly digestible material. For example, differences between ileal and faecal digestibilities in intact compared with caecectomised cockerels were minor for cereals and oilseeds, but were large for some animal meals. Nevertheless, relatively small differences between ileal and faecal digestibilities in grains observed by Williams (1995) could become significantly more important when comparing differences between different samples of grain.

Smits (1996) provided unequivocal evidence that the mechanism by which soluble NSP depresses fat digestibility in chickens hinges on the reduction of bile salts following bacterial overgrowth of the small intestine. He demonstrated that reduction in fat digestibility was particularly severe in the case of animal fats that contained a high proportion of long chain fatty acids as these are dependent on bile for absorption. It seems likely that a reduction in absorption of fat soluble vitamins and withdrawal of other essential nutrients by microbial proliferation would immediately compromise the growth performance and feed efficiency of the animal, and lead to possible health problems through general inflammation of the gut and invasion of tissue by pathogenic organisms.

The concept of competitive exclusion (CE), as proposed originally by Nurmi and Rantala (1973), involves the establishment and maintenance of a normal population of gut microflora to afford protection from colonisation by organisms pathogenic to chickens (e.g., Salmonella spp.) or to humans (e.g., Campylobacter spp.), or both. The topic of CE has been reviewed extensively (Snoeyenbos *et al.* 1979; Stavric *et al.* 1987; Beery *et al.* 1988; Stern and Meinersmann 1989; Schoeni and Wong 1994; Cox and Chung 2000). The original concept of CE has widened to include non-living entities such as fructo- and mannan-oligosaccharides. Iji and Tivey (1998) reviewed the role of oligosaccharides in the regulation of gut microflora. They suggested that the regulatory mechanisms involving oligosaccharides could include:-

a. Provision of alternative binding sites for pathogens, thus preventing invasion of the gut tissue

b. Direct stimulation of the blood immune system after crossing the intestinal mucosal barrier
c. Preservation of the systemic immune system by blocking translocation of pathogens
d. Fermentation of carbohydrates to produce short-chain volatile fatty acids with bacteriostatic properties
e. Direct stimulation of the villus-crypt axis
f. Induction of intestinal microflora to produce glycolytic enzymes.

1.2.5 Barrier function

The gastrointestinal tract performs a selective barrier function between the tissue of the host animal and its lumenal environment. On the one hand, the mucosa must allow efficient transport of water and nutrients (section 1.2.3), but on the other, it must resist the passage of potentially harmful microorganisms, and toxins produced either by gut microorganisms (section 1.2.4) or ingested in the feed (section 1.3.5). The integrity of the barrier can be disrupted when microorganisms and toxins damage cells lining the lumen, or alter tight junction integrity.

The barrier comprises physical and chemical components. Mucus coating the villi, tight junctions between enterocytes on the villi and commensal bacteria attached to the mucosa block the approach and entry of potentially harmful agents such as other bacteria, viruses, fungi, parasites, toxins, incompletely digested feed and antigens in feed, and, of course, digestive enzymes. Mucins secreted by goblet cells in the intestinal villi in mammals (Wright and Alison 1984*b*) and birds (Langhout 1998) also provide a chemical defence by binding to bacteria. The causative agent of gastric ulcerations in humans, *Helicobacter pylori*, secretes a number of highly reactive compounds (Guslandi 1999), one of which is urease that is associated with degradation of the protective mucus barrier in the stomach. Savage (1983) pointed out that a capacity to hydrolyse urea and polysaccharides in mucinous glycoproteins, and an ability to utilise the products of hydrolysis as sources of energy, carbon and nitrogen, were advantageous to microorganisms colonising mucous gels in the gastrointestinal tract of birds and mammals. Savage (1972) also noted that some microbes seemed able to suppress alkaline phosphatase activity in the duodenum of the mouse, possibly by increasing the rate of migration of enterocytes from crypt to villus tip,

thus reducing the time available for maturation of the cells. Savage (1972) suggested that this sort of physiological interaction between microflora and host tissue could have consequences for the health and well-being of the host animal by altering its capacity to digest and absorb essential nutrients.

The intestine is also an immunologic organ of considerable significance; capable of mounting innate and specific challenges to antigens associated with microorganisms and ingested feed. Mucosal immunity in mammals in general (Bienenstock and Befus 1980), pigs and rats (Kelly and King 2001) and chickens (Befus *et al.* 1980; Boyd and Siatkas 1996; Muir 1999; Lowenthal *et al.* 2000) has been reviewed extensively. Lillehoj (1997) described the avian immune response by gut-associated lymphoid tissues to microbial pathogens as a complex interaction of soluble factors, leukocytes, epithelial cells and other physiological mechanisms. Over-stimulation of the mucosal immune system through stresses associated with diet, environment and management can severely impair efficient growth and feed conversion (Klasing *et al.* 1987; Klasing *et al.* 1999; Husband 1996; Klasing 1996; Cook 2002). Benson *et al.* (1993) reported that dietary energy level and the sources of dietary energy influenced the chicken's response to immunologic stress. Koutsos and Klasing (2002) discussed the partitioning of nutrients away from lean tissue growth to support increased demands for nutrients as the immune system mounted a defence against infection. They recommended that diets be re-formulated to allow for reduced feed intake altered nutrient requirements during the infection, and afterwards to repair tissue damage. Klasing (1988) also pointed out that the nutritional status of the chicken influenced cytokine release and regulation of the immune response. For example, protein deficiency can lead to decreased release of interleukin-1, which acts in concert with interleukin-6 and tumour necrosis factor α to decrease feed intake and to increase resting energy expenditure, along with a host of other effects on intermediary metabolism (Klasing 1988).

1.2.6 Summary

It is clear from the preceding sections that the intestinal microflora play an integral part in the functioning of the digestive system of chickens (Bedford and Apajalahti 2001). The complex interrelationships between gut bacteria and the host tissue involves "crosstalk" (Kelly and King 2001) through chemical signals produced by both participants in the

conversation. Bedford and Apajalahti (2001) and Kelly and King (2001) drew similar conclusions in that the life-long health and performance of chickens and pigs requires an orchestrated balance between the host and its resident bacteria. The following sections discuss the physical and chemical properties of feed ingredients in poultry diets that could upset the equilibrium between the host and its gut microflora.

1.3 Properties of feed that affect energy metabolism in poultry

Grains and products of grains comprise 80% or more of most diet formulations for poultry in Australia. Cereal grains such as wheat and barley, combined with legumes and oilseed meals, provide not only the bulk of the energy and other essential nutrients for commercial poultry production, but are also the prime source of anti-nutritive components which are likely to have significant bearing on how effectively all dietary components are utilised by poultry.

Sources of variation in the physical and chemical characteristics of plant material used in poultry diets include variety, seasonal effects and growth sites, crop treatment and grain fumigants, and post-harvest storage conditions and period of storage. The available energy, protein and amino acid contents of grains fed to poultry, which best represent nutritive value, are extremely wide. In addition, variation in the availability of energy and protein in plant material can be attributed to a wide range of anti-nutritive factors such as non-starch polysaccharides (NSP), enzyme activity, tannins, alkyl resorcinols, protease inhibitors, α-amylase inhibitors, phyto-haemagglutinins, alkaloids, saponins, and lathyrogens. The relative importance of such factors will also differ according to the type of grain in question. All of the above aspects were reviewed by Hughes and Choct (1999).

1.3.1 Anti-nutritive properties of non-starch polysaccharides (NSP)

Of the known causes of variation in energy value of grains, soluble NSP stand out as major determinants of the availability of energy and other nutrients for poultry according to Smits (1996), Langhout (1998), and Hughes and Choct (1999).

The physical and chemical properties of the major carbohydrate components of dietary fibre were discussed in detail by Annison (1993b), Oakenfull (1993), and Evers *et al.* (1999). Other anti-nutritive effects of NSP have also received a great deal of attention in

recent years (see reviews by Chesson 1993; Annison and Choct 1991; Annison 1993a; Drochner *et al.* 1993; Smits and Annison 1996; Langhout 1998). Sharma and Schumacher (1995) showed that dietary fibre altered the amount and composition of mucins in the small intestine of rats. More recently, Langhout *et al.* (1999) and Iji (1999) examined the effects of NSP on microbial activity, gut structure and function, and nutrient digestion, absorption and transport. Iji (1999) also suggested that the molecular basis for the changes in intestinal structure and function induced by NSP could involve regulation of thyroid and parathyroid hormones by glucose and other end products of digestion. Uni *et al.* (2001) showed that exposure to heat stress at 3-days of age led to an immediate reduction in feed intake, lowered triiodothyronine (T_3) level in blood plasma, depressed enterocyte proliferation, and reduced expression and activities of sucrase, aminopeptidase and alkaline phosphatase in jejunal tissue. They concluded that exposure of chickens to heat stress at an early age influenced T_3 concentrations, which in turn altered the intestinal capacity to proliferate, grow and digest nutrients. However, their experiments were not able to differentiate between the effects of reduced feed intake and heat stress *per se*.

1.3.2 NSP in cereal grains

The 'low-ME' wheat phenomenon in broilers, first described by Mollah *et al.* (1983) and Rogel *et al.* (1987), is caused by soluble NSP (mainly arabinoxylan and some β-glucan) in cell walls (Annison 1993a). Evidence for the anti-nutritive effect of soluble NSP includes:-

a. AME and soluble NSP are negatively correlated.
b. various sources of purified NSP depress AME.
c. *in situ* degradation of cell wall NSP by glycanases increases AME.
d. NSP isolated from wheat depress AME in a dose-dependent manner.

Recent studies (Choct *et al.* 1995; Choct *et al.* 1996*a*; Hughes and Choct 1997) provide further evidence of the occurrence of low-ME wheats. The reduction in AME is directly attributable to NSP. However, dissenting views were expressed by Nicol *et al.* (1993) who found no correlation between soluble NSP and AME, and by McNab (1996) who suggested that low AME values were artefacts generated by faulty methods. In addition, Wootton *et al.* (1995) observed a positive correlation ($r = 0.63$; $n = 19$) between pentosan content of wheat and AME. This suggests that the higher the fibre content in diets, the better their nutritive value for poultry, a view which contrasts with the conventional understanding of

monogastric nutrition. This is not surprising given the narrow range of AME values (only three samples falling below 14 and none below 13 MJ/kg DM) and the methodology used to determine the pentosan levels.

The anti-nutritive effect of soluble NSP on AME is manifested through inhibition of digestion of starch, lipid and protein in the foregut (Choct and Annison 1992). The possibility that gut microflora have a role in depression of starch digestion and AME (Annison 1993a) was confirmed by Choct *et al.* (1996*a*) and Smits *et al.* (1997). The mechanism of action of soluble NSP is thought to involve increased viscosity of digesta which limits contact between digestive enzymes and substrates, and for contact between nutrients and absorption sites on the intestinal mucosa (Annison 1993*a*; Bedford and Morgan 1996; Smits *et al.* 1997). The possibility that NSP in chicken diets can have direct effects on gut structure and function as has been shown in rats (Johnson and Gee 1986; Brunsgaard and Eggum 1995; Brunsgaard *et al.* 1995) should not be overlooked.

Satchithanandam *et al.* (1996) reported increased mucin levels in the stomach and colons of rats given diets high in soluble NSP. They suggested that polysaccharides of host origin (mucin) or in the diet (NSP) could contribute to diversification of bacterial species in the gastrointestinal tract. These observations point to dietary NSP having a beneficial effect (by stimulating an increase intestinal barrier function; section 1.2.5) and a detrimental effect (by fuelling bacterial proliferation in the small intestine; section 1.2.4).

1.3.3 NSP in legumes and oilseeds

Drochner *et al.* (1993) concluded that the pectin content of legumes was a limiting factor in nutritive value of mixed poultry feeds. Digestibility of pectin ranged from 33 to 90% and the presence of pectin significantly reduced apparent digestibility of crude fat and crude protein. Mosenthin *et al.* (1994) showed that, in pigs, dietary pectin increased endogenous protein and amino acid secretion into the small intestine, and stimulated nitrogen assimilation by bacteria in the large intestine. They also showed that pectin depressed secretion of α-amylase but had no effect on other pancreatic secretions.

Brenes *et al.* (1993) claimed that the seed coat of low alkaloid *L. albus* contained an anti-nutritive factor that was deactivated by α-galactosidase. In contrast, Hughes *et al.* (1998) observed that incorporation of seed coat material from *L. angustifolius* cv Gungurru and *L.*

albus cv Kiev mutant in a highly digestible diet based on sorghum and casein only had an energy dilution effect with no evidence of any anti-nutritive activity. This supports the finding that insoluble NSP are not anti-nutritive in poultry (Angkanaporn *et al.* 1994).

1.3.4 Exogenous enzymes for degrading NSP

The roles of NSP-degrading enzymes in improving the nutritive value of grains and in reducing the problems associated with wet, sticky droppings were reviewed extensively by Bedford and Morgan (1996), Chesson (1993), Classen (1996), and Williams (1997).

Enzymes are thought to act on two fronts. The first action is to reduce viscosity of digesta by partial depolymerisation of main and side chains in complex carbohydrates such as arabinoxylan and β-glucan in cereals, and pectic polysaccharides in legumes. This is likely to influence the digestive capacity of the chicken through effects discussed in section 1.2. The second action involves disruption of cell walls to expose substrates such as starch to digestive enzymes. Chesson (1993) pointed out that reduction of digesta viscosity by exogenous enzymes did not fully account for production responses in broilers fed barley-based diets. He considered that release of nutrients following enzyme degradation of cell walls was an important factor. In the case of wheat-based diets, Bedford and Morgan (1996) concluded that digesta viscosity was the major factor limiting performance of broilers. These two modes of action of enzymes require further elucidation for both cereals and legumes, particularly the latter. The greater degree of complexity of side-chain structures and cross-linking in NSP in legumes could be a contributing factor to the relative lack of success in application of specific enzymes for legume NSP compared with cereal NSP achieved to date (Kocher 2001).

Bedford and Apajalahti (2001) proposed that exogenous enzymes improved animal performance largely through interaction with the gut microflora. They described this interaction as a "two-way negotiated process between host and intestinal microflora", with enzymes tipping the equilibrium in favour of the host for efficient digestion and absorption of nutrients in the small intestine, which also results in a reduced flow of undigested nutrients into the hindgut to fuel fermentation by anaerobic bacteria. Bedford and Apajalahti (2001) pointed out that alteration of the microflora profiles in the small and

large intestines would influence the effects of microflora on structure and function of the intestines, and mucosal and systemic immune functions (section 1.2 above).

With the possible exception of vaccines, rarely has the Australian poultry industry witnessed such a rapid and widespread uptake of a successful technology as that involving addition of NSP-degrading enzymes to poultry feed, particularly for broiler chickens. Adoption of technology usually follows a different path, one of slow, gradual uptake as one problem after another is solved by adequately funded research until producers have sufficient confidence in the innovation to try it for themselves. In the case of enzymes, the benefits were seen to be immediate and large in commercial practice. As a result, our understanding of the true function and role of enzymes on the processes of digestion and absorption of nutrients remains incomplete because industry is reluctant to invest in basic research now that there is a practical solution to problems associated with NSP. However, there remain many unanswered questions of a fundamental nature concerning the structure and composition of NSP in grains, such as why and how different species and varieties of grain vary in their structure and composition of NSP, and whether these differences influence the digestion and absorption of nutrients in livestock, and gut health.

Hence, feed enzyme technology provides a practical solution to the problems associated with NSP such as arabinoxylan and β-glucan by simultaneously lifting the availability of energy and improving flock uniformity (Bedford and Morgan 1996; Hughes *et al.* 1996). However, feed enzymes did not eliminate variability in AME of wheat examined in these studies, nor on variability in AME of barley as shown by Kocher *et al.* (1997). It seems plausible that while exogenous enzymes can depolymerise NSP to the extent that these no longer contribute to digesta viscosity, the shortened fragments of NSP retain the power to directly effect intestinal structure and function in chickens as was noted for rats (section 1.3.2 above). Furthermore, a change in gut microflora appears to be a significant factor related to the large variation between individual chickens given low-ME wheat or diets supplemented with arabinoxylan isolated from wheat (Choct *et al.* 1996*a,b*).

1.3.5 Anti-nutritive properties of feed other than NSP

As pointed out in section 1.2.4 above, anti-nutritive activities of feed components are often manifested either through change in the microflora population in the gut or by direct effect

on gut tissue. Some key examples of anti-nutritive factors beside NSP in feed ingredients, and associated effects on the key determinants of digestive capacity of poultry (Figure 1) are outlined in the following sections.

Mycotoxins

van Barneveld (1999) reviewed the many and varied effects of mycotoxins from grains on the health and welfare of agricultural animal species. Hoerr (2001) discussed the consequences of damage to the intestinal mucosa of chickens inflicted by mycotoxins. For example, trichothecene mycotoxins damaged the tips of villi, and disrupted cellular proliferation in the crypts. Hoerr (2001) also noted that aflatoxin impaired digestion by decreasing bile secretion from the liver, and bicarbonate secretion from the pancreas. Hence, mycotoxins are likely to impair the digestive capacity of chickens through the damage caused to structural integrity and function of the intestinal tissue of chickens fed mouldy grains.

Tannins

The effects of tannins on nutritive value of sorghum for poultry were reviewed by Gualtieri and Rapaccini (1990) and Nyachoti *et al.* (1997). Gualtieri and Rapaccini (1990) concluded that low tannin sorghum was comparable to maize in nutritive value, whereas Nyachoti *et al.* (1997) concluded that there remained a great deal of variation in nutritive values of different varieties of sorghum. This variation which may have wrongly been attributed to tannins in previous studies, a view supported by observations that poor correlations exist between tannin content of sorghum and the performance of poultry (Elkin *et al.* 1996). Tannin content is not regarded as a limiting factor in the use of Australian varieties of sorghum (Kondos and Foale 1986).

Wiseman and Blanch (1994) observed similar discrepancies between tannins and performance of poultry given diets containing some cultivars of faba bean. In contrast, Flores *et al.* (1994) reported that tannin extracted from field beans depressed digestibility of semi-purified starch from field beans fed to young and adult birds, whereas digestibility of starch from triticale was depressed in chicks but not in cockerels. Marquardt *et al.* (1977) extracted and purified condensed tannins from faba beans and observed that these has similar chemical characteristics to the tannins present in sorghum. Hence, it seems

possible that tannins in sorghum could have similar adverse effects if present in sufficient quantities.

Marquardt (1979) noted that a thermo-labile component in testa of broad beans suppressed appetite and availability of nutrients in chickens. The anti-nutritive factor was condensed tannin that was not associated with inhibition of trypsin and did not affect pancreas size. Davidson *et al.* (1981) detected a heat-stable anti-nutritive factor in forage pea that caused weight loss and reduced the rate of lay in hens. On the other hand, Yu *et al.* (1996) concluded that condensed tannins were not responsible for depressed apparent and true ileal digestibility of N and amino acids in rats and that other components of cottonseed hulls were implicated.

Contradictory evidence concerning the antinutritive properties of tannins in commonly used feed ingredients probably arises as a result of the wide variation in structure and composition of the polyphenolic compounds, and from artefacts created by a variety of extraction methods.

In addition to the deleterious effects of tannins on nutritive value of feed ingredients, many polyphenolic compounds in plant material are known to have antimicrobial properties (Cowan 1999), which could alter the dynamics of the normal gut microflora when fed to chickens. Cowan (1999) pointed out that a number of physiological activities in human have been assigned to tannins. These include stimulation of phagocytic cells, host-mediated tumour activity, and a wide range of anti-infective actions through several different modes of action. Of these, abilities to inactivate microbial adhesins, enzymes and cell envelop transport proteins, may provide ways of protecting the gut of newly hatched chickens from pathogenic bacterial species such as clostridium and salmonella, while allowing beneficial species to proliferate.

Protease inhibitors

Trypsin inhibitors are widespread in plant seeds (Liener 1990). They are found in legumes as well as most cereal grains. Their nutritional and physiological significance in the latter has always been questionable due to these much lower levels and activities compared with their concentrations in legumes, particularly raw soybeans. Ikeda and Kusano (1983) showed that the inhibition of trypsin activity *in vitro* resulted from binding with pectin and

xylan substrates. Birk (1985, 1987) demonstrated the long- and short-term effects of soybean trypsin inhibitors on growth rate, and pancreatic enlargement in rats and chickens. Although trypsin inhibitors can depress bird performance if raw soybean meals are included in the diet, it is now common practice in the feed industry in Australia to steam process diets at or above 85°C, which will effectively deactivate protein inhibitors (Liener 1990).

α-Amylase inhibitors

Inhibitors of α-amylase occur widely in plants (Kneen and Sandstedt 1946). Indeed, plant materials rich in α-amylase inhibitors or starch blockers have been used to treat obesity and diabetes mellitus in humans (Pusztai *et al.* 1995). These workers warned of the potentially deleterious effects of reduction in starch digestion and microbial overgrowth of digesta in the intestine and large bowel if livestock were given feed containing naturally-enriched or transgenic plants with high levels of inhibitor gene expression. On the other hand, α-amylase inhibitors are moderately heat stable but are inactivated by cooking (Pusztai *et al.* 1995). However, Ernest *et al.* (1992) reported that inactivation of α-amylase inhibitors in wheat by steam treatment improved protein and fat digestibility in broilers by 6.5 and 4.0%, respectively, but did not affect starch digestibility.

Urease

Urease activity in poorly prepared soybean meals may contribute to variability in digestion of energy and other nutrients in chickens by degradation of the protective mucus layer in the small intestine (section 1.2.5), as can occur with urease produced by gut bacteria (Guslandi 1999; Savage 1983).

Phyto-haemagglutinins

Williams and Burgess (1974) noted that feeding raw navy beans to quail resulted in growth depression and death. The toxicity was attributed to high concentrations of phyto-haemagglutinins, which impaired body defence mechanisms and allowed tissue invasion by normally harmless intestinal microflora.

Bioactive proteins, peptides and amino acids

The antigenic properties of proteins in legumes were reviewed extensively by Gatel (1993). Dietary allergy appears to be more important in young animals rather than adults. Prior exposure to small doses of antigen can predispose an animal to digestive disorders at a later age but which may not be manifested in clear clinical signs such as reduced growth and morbidity (Gatel 1993). Chickens housed in metabolism cages often experience a period of reduced growth and feed efficiency when novel ingredients such as lupins and cottonseed meal are first introduced into the diet (R. J. Hughes *et al.*, unpublished data). However, it is not clear from these observations whether this response was associated with immune stress or more related to adaptation of the digestive tract in terms of its digestive and absorptive capacity.

Cadaverine and putrescine are biogenic amines derived from enzymic decarboxylation of amino acids during fermentation of plant meals such as silage (Hughes 1970) and animal protein meals such as fish meal (Bakker 1994; Bryden *et al.* 1996). The latter product is used widely in feed for broiler chickens (Dudley-Cash 1993). These amines are known to have pharmacological activities including alteration of gastric motility (Bakker 1994) and remodelling of the villus-crypt axis in chickens (Shinki *et al.* 1991). Kelly and King (2001) noted that polyamines produced by bacteria were potent maturation factors implicated in increased expression of α1,2 fucosylation of mucins in the rat gut at time of weaning and that bacterial sources of polyamines may affect maturation of the post-weaned intestine in pigs. Presumably, exogenous polyamines could have similar effects in both pigs and poultry.

However, it seems unlikely that biogenic amines could have contributed to the wide variation in energy uptake reported by Hughes and Choct (1997) as their experimental diet was free of protein meals prone to fermentation prior to processing. However, their diet contained 134 g/kg casein, a high protein food-grade product derived from milk, which is not normally used in commercial diets due to its very high cost. Casein is frequently used in experimental diets to determine apparent metabolisable energy values of cereal and legumes (Annison *et al.* 1996), as it is a highly digestible protein source. The possibility that constituents of casein could affect energy uptake or growth performance of chickens by affecting barrier function and endocrine regulation, as shown in guinea pigs by

Malikova *et al.* (1991), or gut structure, as reported in pigs by Pluske *et al.* (1996), has not been considered previously.

In contrast to the deleterious effects of plant proteins as discussed by Gatel (1993), Revell (1998) outlined the effects of growth factors which provide extra-nutritional benefits such as reduction in the risk of disease and stimulation of cellular proliferation in the gut. Other feed components having potential therapeutic effects include lectins, NSP and biogenic amines (Cowan 1999), all of which are regarded as anti-nutritive factors in other circumstances. Revell (1998) and (Adams 2000) recommended that further research was required on the functionality of foods which they described as "nutraceuticals" and "nutracines", respectively.

The possibility that bioactive substances ingested by the animal or produced by gut microflora can contribute to variation in energy uptake in healthy chickens is worthy of further study.

1.4 Summary

The diet of broiler chickens provides not only the nutrients essential for maintenance and rapid growth of the flock as a whole, but the feed itself can also have other chemical and physical properties which are detrimental to the processes of ingestion, digestion, absorption, transport and utilisation of nutrients. To date, most of the research conducted on broiler chickens has been directed at improving the overall health and performance of the flock, with little attention being paid to the individuality of chickens within the flock.

The general hypothesis examined in this project was "effects of soluble NSP in cereal grains on gut structure and function, digesta transit time, and gut microflora differ substantially between individual chickens within a flock, thus contributing to variation in the digestion of energy by the flock as a whole". A major goal of the research was to determine what characteristics of the gastrointestinal tract of broiler chickens were the key determinants of digestion of energy. During the course of my studies I developed the concept of digestive capacity of the individual chicken as a frame-work for investigation of the relationships between bird-related and feed related factors which influence energy metabolism in chickens, as outlined in Figure 1.

Chapter 2. Influence of gut structure on digestion of energy by broiler chickens

2.1 General introduction

Newly hatched chickens undergo a rapid transition from utilisation of lipids in yolk to usage of carbohydrate from cereal grains as the major source of energy for maintenance and growth. Sklan (2001) described the first few days after hatching as a period of intense morphological and functional development of the small intestine. The preferential growth of the small intestine relative to the live weight of the chicken is at its peak during this early period (Sklan 2001). In addition, the relative increase in weight of the duodenum is greater than that of either the jejunum or ileum (Uni *et al.* 1999). Iji (1998) studied the rapid changes in visceral organ weights, and intestinal structure and function in relation to body mass of rapidly growing broiler chickens, and concluded that uneven growth and productivity among individual chickens could be traced to variable development and functionality of the small intestine. Iji (1998) did not differentiate between male and female chickens in his studies, nor did he measure the AME of the feed.

Chickens reared from hatch in the presence of older chickens can exhibit wide variation in live weight without showing any signs of clinical disease (R.J. Hughes and D.G. Schultz, unpublished data), with variation among male chickens usually being much higher than in female chickens. As the number of prior batches of chickens reared in the shed without thorough cleaning between batches increased, live weight gain of both sexes decreased, and the variation between individuals increased. These symptoms are consistent with those of chickens faced with a sub-clinical challenge which can suppress appetite and result in the partitioning of nutrients away from growth to support an immune response (Klasing 1996). Iji and Tivey (1998) concluded that chickens exposed to some classes of pathogenic bacteria could benefit from addition of synthetic mannan oligosaccharide (MOS) to the diet. MOS is understood to act mainly through its capacity to provide alternative binding sites for bacteria with type-1 fimbriae (Spring 1997), thus preventing invasion of the gut tissue.

Two experiments were conducted to determine whether energy digestion was influenced by the structure of the gastrointestinal tract, and whether any relationships between gut morphology and AME were dependent on the sex of the chicken. Both experiments involved the rearing of chickens in clean and dirty environments to increase the extent of between-bird variation in live weight, and any corresponding effects on gastrointestinal structure and energy digestion. MOS was added to the feed to see whether it was possible to reverse any deleterious effects of sub-standard rearing conditions on gut structure and energy digestion.

2.2 Effects of gross structure of the gastrointestinal tract on AME of commercial broiler feed

2.2.1 Introduction

Cumming (1992) proposed that chickens needs an active gizzard for effective grinding and acidification of chyme as a prelude to efficient digestion, and as part of its natural resistance against coccidiosis which can impair intestinal function. He showed that the relative weight of the gizzard (expressed as a proportion of live weight) was inversely correlated with the number of Eimeria oocysts in excreta. Cumming (1992) attributed the reduction in oocyst to their mechanical disruption caused by the greater grinding ability of larger gizzards.

Plavnik *et al.* (2002) observed an increase in gizzard weight, and improvements in weight gain and feed efficiency in broilers given a diet containing 200 g/kg whole wheat rather than milled wheat. In an earlier study, Svihus *et al.* (1997) showed that the relative weights of the proventriculus and gizzard were increased in male broilers given a diet containing whole barley (553 g/kg) rather than ground barley. However, the relative weights of the crop, small intestine, pancreas, and hindgut were unaffected by milling of the grain. Svihus and Hetland (2001) showed that starch digestibility in male broilers was higher when they were given a diet containing whole wheat (385 g/kg) rather than milled wheat. They attributed the improvement in starch digestibility to increased gizzard weight.

Hence, there is reason to believe that gizzard size may be related to between-bird variation in digestion of energy, and that the importance of gizzard size differs between the sexes.

An experiment conducted at PPPI in 1997 (R.J Hughes, A. Kocher, R.B. Cumming and M. Choct, unpublished data) showed poor statistical relationships between AME of a wheat-based diet (mean value 13.2 MJ/kg DM) and weights of freshly dissected gizzard and other gut sections, despite high replication (n=32) and wide variation in AME values of the diet (range 8.4 to 15.8 MJ/kg DM). A possible reason for the failure to detect a relationship between AME and gizzard size was unwanted variation introduced by differing degrees of drying of tissue between collection and weighing. A further possibility is that the high standard of hygiene used during the rearing of chickens for that study may have led to only minor challenge from bacteria and coccidial oocysts.

For the purposes of the following study, it was reasoned that measurements of dissected sections of the gastrointestinal tract emptied of digesta then dried to constant weight might better reflect the capacity of that GIT section to perform its respective function in the digestion of energy. In addition, sub-standard conditions of hygiene were used during rearing with the intention of increasing the variation in AME and performance of chickens by exposure to coccidial oocysts and bacteria. The inclusion or not of MOS in the diet was included as an experimental treatment to see whether it was possible to alleviate any effects of pathogenic gut bacteria.

This experiment tested the hypotheses (a) that AME of commercial broiler feed was significantly correlated with dried weights of sections of the GIT, and (b) that the relationships differed according to sex of the chicken.

2.2.2 Materials and methods

Birds, housing and management

Broiler chickens (Ross strain) obtained from the Bartter Steggles hatchery, Cavan SA on Thursday 22 April 1999 were raised on starter crumbles (Ridley Agriproducts, diet code #503540) from hatch to 19 or 20 days of age in two sets of experimental rearing pens (Appendix 1). One set of pens was housed in a clean environment and the other in a dirty environment. The clean rearing environment involved isolation of chickens in a room cleaned to current industry standards. The dirty environment involved exposure of chickens to air-borne debris from an older flock of healthy chickens kept in the room at the same time.

At 19 days of age, chickens were transferred in pairs to 48 single-bird metabolism cages (Appendix 2) located in a controlled-temperature room set at 25 - 27°C initially, and given commercial finisher pellets (Ridley Agriproducts, diet code #504540). The following day, one chicken was removed from each cage. The reason for initially placing two chickens per cage was that prior experience has shown that chickens adapted more quickly to the cages when placed with a mate.

The two experimental dietary treatments were 0 and 5 g/kg MOS (Bio-mos™). MOS was mixed with coarsely milled finisher pellets (Ridley Agriproducts, diet code #504540), which were then cold-pressed into pellets approximately 6 mm long and 4 mm in diameter. The control diet was finisher pellets treated in the same manner but without MOS.

All of the above procedures were repeated commencing at 20 days of age with chickens from the same rearing groups. These chickens were placed in a second set of 48 metabolism cages located in the same controlled-temperature room as the first set of 48 cages. It was necessary to conduct the experiment in two phases in this manner due to limited availability of staff experienced in these procedures

The temperature setting in the room was reduced daily until it was 22°C at the end of the experiment.

AME measurement and collection of intestinal tissues

AME value of diets in this and other experiments described in this thesis were determined in a conventional energy balance study involving measurements of total feed intake and total excreta output and subsequent measurement of gross energy (GE) values of feed and excreta by isoperibol bomb calorimetry (Parr Instrument Company, models 1261 and 1281).

The first three days enabled the chickens to adapt to the feed. Feed intake was measured during this period. A sample of pelleted feed (approximately 70 g) was collected on day 3 for subsequent measurement of dry matter and gross energy. During the following four days, feed intake was measured and all excreta were collected in stainless steel trays beneath the cages. All excreta was collected daily and transferred to aluminium trays for drying overnight at 90°C in fan-forced ovens. All of the pooled excreta from each pen were milled (2mm screen) in a stainless steel hammer mill, and then a sub-sample

(approximately 70 g) was retained for the measurement of gross energy. Moisture content of fresh excreta collected on day 5 of the metabolism study was measured by overnight drying at 90°C in fan-forced ovens. Dry matter contents of sub-samples of pelleted feed and milled feed (1mm screen) were determined by drying overnight at 105°C in a fan-forced oven for the calculation of AME as shown in the equations in Appendix 3.

On completion of the 7-day AME study, each chicken was killed by intravenous injection of pentobarbitone into the brachial vein, and then weighed. The body cavity was opened and the gastro-intestinal tract (GIT) from the proventriculus to the ileo-caecal junction was dissected. The empty proventriculus, gizzard, duodenum, pancreas, jejunum and ileum were rinsed with water, dried by blotting, then weighed. The GIT sections were dried at 40°C to constant weight.

Serology

Chickens not required for the 7-day metabolism experiment were transferred at 20 days of age from the rearing pens in the clean and dirty environments to a shared floor pen in the dirty environment. They were given finisher pellets without MOS. At seven weeks of age, blood sera samples were collected from the brachial vein of 20 chickens. The sera were tested by ELISA methodology for the presence of antibodies against Marek's Disease Virus (MDV), Infectious Bursal Disease (IBDV) and Chick Anaemia Virus (CAV), courtesy of Dr Tom Grimes, Steggles Pty Ltd.

Statistics

Base SAS® software (SAS Institute) was used in this and other studies described in this thesis. Wilcoxon's test was used to determine whether data were normally distributed. Analysis of variance (by GLM procedure) was used to examine the effects of rearing environment, diet and sex, and all possible 2-way interactions and the 3-way interaction. Duncan's Multiple Range Test was used to separate means of main effects (rearing environment, diet and sex) and *T*-tests (by LSMEANS procedure) were used to separate means for significant ($P<0.05$) interactions in analysis of variance. Stepwise regression (by REG procedure with maximum R^2 selection) was used to develop prediction equations for AME and dry matter digestibility (DMD) of the diet from weights of dried gut sections.

2.2.3 Results

Means, standard deviations, numbers of observations, coefficients of variation, and ranges of values for dietary AME and dry matter digestibility are shown in Table 1. These included data from one chicken with values of 12.30 MJ/kg DM for AME and 0.588 for dry matter digestibility coefficient which were considered real and therefore included in all statistical calculations. The next lowest values were 13.20 MJ/kg DM for AME and 0.616 for digestibility coefficient.

Table 1. Means, standard deviations (SD), numbers of observations, coefficients of variation (CV) and ranges of values for dietary AME and dry matter digestibility.

	Mean	SD	n	CV	Range
AME of diet DM (MJ/kg)	13.70	0.29	96	2.1	12.30-14.36
Dry matter digestibility (g/g)	0.645	0.014	96	2.2	0.588-0.678

AME of the diet (13.7 MJ/kg DM) and dry matter digestibility (0.645 g/g) were unaffected ($P>0.05$) by cleanliness of the rearing environment, sex of chicken, or addition of MOS to the diet from 22-29 days of age (Table 2).

Live-weights of chickens from the clean environment were greater at the start ($P<0.001$) and end ($P<0.05$) of the 7-day metabolism study in comparison with those from the dirty environment (Table 2), but the rate of gain in live-weight during the study was greater ($P<0.01$) for chickens from the dirty environment. Similarly, feed conversion was significantly improved ($P<0.01$) in chickens from the dirty environment (Table 2). Male chickens were heavier ($P<0.001$) than females at the start and end of the 7-day metabolism study (Figure 2), and ate more feed ($P<0.001$), but there was no difference ($P>0.05$) between sexes in the rate of gain.

There was a significant interaction ($P<0.05$) between rearing environment and diet on live weight at the end of the 7-day metabolism study (Figure 2) which resulted in chickens from the clean environment given MOS being heavier than controls. In contrast, there was no difference due to MOS in chickens from the dirty environment.

Table 2. Summary of significant main effects of rearing environment, diet and sex. Means with the same letter within a main effect are not significantly different (P>0.05)

	Rearing		Diet		Sex		Pooled
Variable	Clean	Dirty	Control	MOS	Female	Male	SEM
Live weight at start (g/bird)	896 a	855 b	876	875	806 b	939 a	6.9
Live weight at end (g/bird)	1305 a	1276 b	1289	1290	1191 b	1382 a	9.2
Growth (g/bird)	413	421	418	416	386 b	446 a	5.8
Rate of growth (g/kg) [1]	464	495	481	478	481	478	7.6
Feed intake (g/bird/day)	114	112	113	113	105 b	121 a	1.2
Feed conversion ratio	1.94 a	1.87 b	1.90	1.90	1.90	1.91	0.015
AME of diet DM (MJ/kg)	13.68	13.72	13.73	13.67	13.71	13.69	0.043
Dry matter digestibility (g/g)	0.644	0.646	0.646	0.644	0.644	0.645	0.0020
Gizzard (g/bird)	4.09	3.99	3.95	4.13	3.91	4.17	0.092
Proventriculus (g/bird)	1.15	1.05	1.09	1.11	1.02 b	1.18 a	0.037
Duodenum (g/bird)	2.51	2.39	2.45	2.45	2.30 b	2.59 a	0.056
Pancreas (g/bird)	0.93	0.94	0.94	0.94	0.93	0.94	0.029
Jejunum (g/bird)	5.04	4.79	4.81	5.03	4.67 b	5.15 a	0.090
Ileum (g/bird)	3.65	3.63	3.51	3.78	3.44 b	3.83 a	0.138
Gizzard [2] (g/kg MBW [3])	4.11	4.04	4.00	4.14	4.17	3.98	0.096
Proventriculus (g/kg MBW)	1.15	1.06	1.09	1.11	1.08	1.12	0.036
Duodenum (g/kg MBW)	2.50	2.43	2.47	2.45	2.45	2.48	0.054
Pancreas (g/kg MBW)	0.93	0.96	0.95	0.95	0.99 a	0.90 b	0.031
Jejunum (g/kg MBW)	4.97	4.83	4.78 b	5.02 a	4.94	4.86	0.079
Ileum (g/kg MBW)	3.64	3.67	3.53	3.77	3.67	3.64	0.136

[1] Rate of growth=growth×1000÷live weight at start

[2] Adjusted dry weight=dry weight×(live weight at end)$^{0.75}$ ÷ (mean live weight at end)$^{0.75}$

[3] Metabolic body weight (MBW)

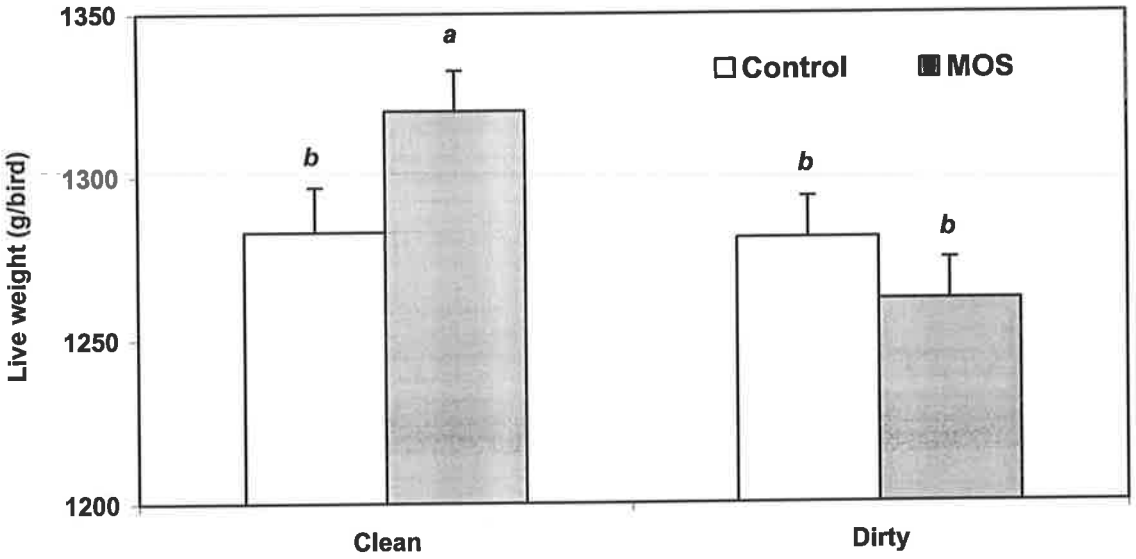


Figure 2. Effects of cleanliness of the rearing environment and diet on live weight of chickens at the end of the 7-day metabolism study (means ± SE; n = 24). Means with a common letter are not significantly different (P>0.05).

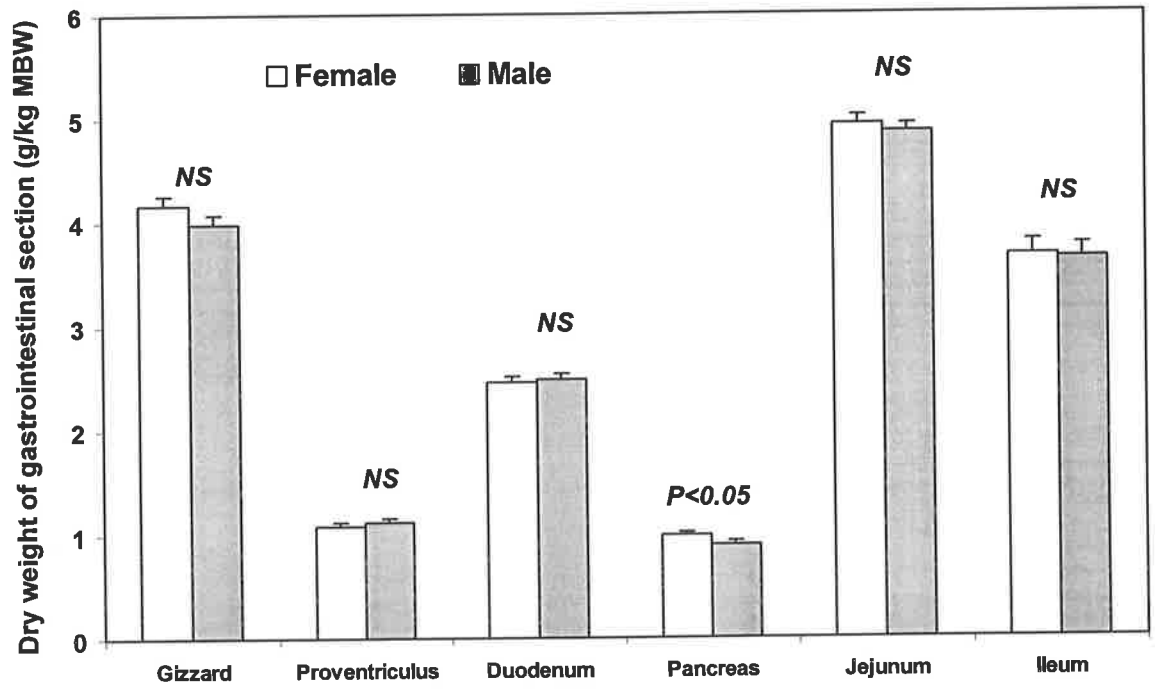


Figure 3. Effects of sex of chicken on dry weights (in g/kg metabolic body weight MBW) of gastrointestinal tract sections (means ± SE; n = 48). *NS* indicates not significant (P>0.05).

With the exception of the pancreas (Table 2), dry weights of gastrointestinal sections were heavier in males than in females. When dry weights of gut sections were adjusted for metabolic body weight (MBW, weight$^{0.75}$), the pancreas was significantly heavier (P<0.05) for females compared with males (Figure 3). The adjusted dry weights of other sections did not differ (P>0.05) between the sexes. The dry weight of the proventriculus tended to be heavier (P=0.06) for chickens reared in the clean environment (Table 2), and the trend (P=0.09) was still evident when the data were adjusted to constant metabolic body weight.

There was a significant interaction (P<0.05) between rearing environment and sex for the adjusted dry weight of the jejunum (Figure 4). In the clean environment, adjusted dry weight was greater (P<0.05) for females than males, but there was no difference (P>0.05) between sexes in the dirty environment.

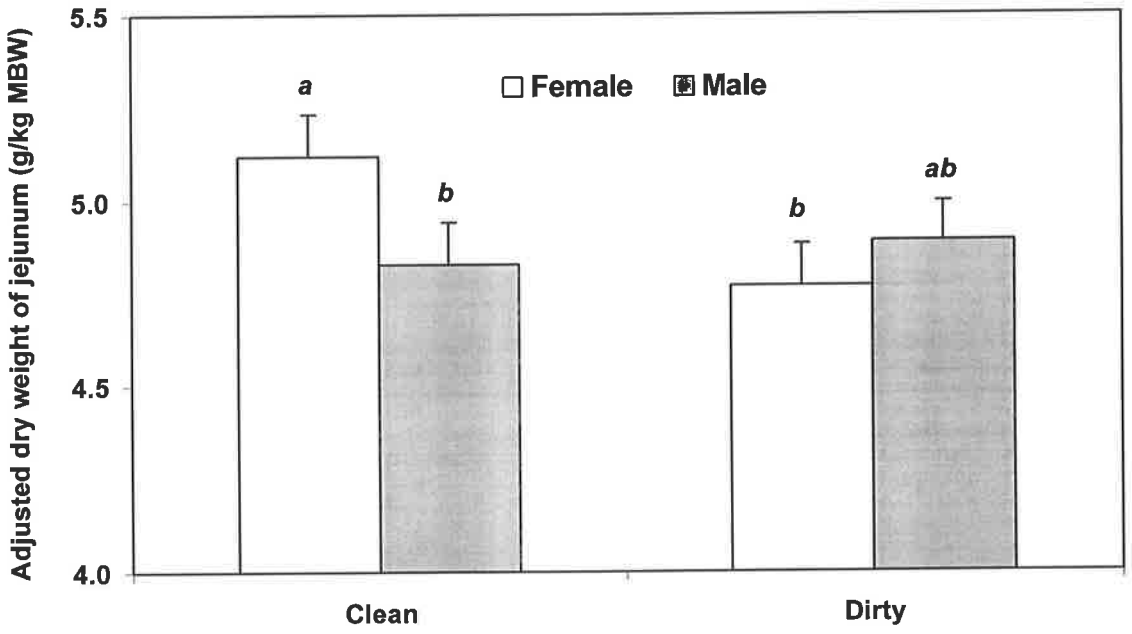


Figure 4. Effects of cleanliness of the rearing environment and sex of chicken on the adjusted dry weight (g/kg metabolic body weight) of the jejunum (means ± SE; n = 24). Means with a common letter are not significantly different (P>0.05).

Step-wise regression analysis was used to develop a prediction equation for AME of the diet and dry matter digestibility (DMD) from adjusted weights of gut sections. In both cases, dry weight of the gizzard had the strongest association (P<0.05) of any variable with AME and DMD. Gizzard weight accounted for 13% and 20% of the variation in AME and

DMD, respectively. Only 17% of the variation in AME and 24% of the variation in DMD could be accounted for by a combination of measurements of all of the six different gut sections.

ELISA tests showed an absence of challenge from immuno-suppressive agents such as MDV and IBDV. Three out of 20 chickens tested positive to CAV.

2.2.4 Discussion

There were indications that chickens reared in the dirty environment showed compensatory growth when moved to the metabolism cages, which were located in a relatively clean environment. Chickens from the dirty environment had superior performance in terms of rate of gain in live weight and feed conversion ratio.

The lack of effects of MOS on energy metabolism and growth of chickens observed in this study is in contrast to the work of Iji (1998; 1999) who obtained improvements in health and performance of chickens with the same dietary concentration of MOS. The failure to detect a difference between males and females in the estimation of AME values of the diets is at odds with previous studies at PPPI (R.J. Hughes and D.G. Schultz, unpublished data). Perhaps the reason for this is that the challenge from bacterial and viral organisms carried on air-borne dust and dander was too mild, as implied by the ELISA results which showed an absence of responses by most chickens to three common viral pathogens.

The significant although weak statistical associations between dry weight of the gizzard and AME and DMD are consistent with the view of Cumming (1992) that larger gizzards more effectively grind ingested feed with resulting benefits in increased surface area for attack by digestive enzymes and mechanical disruption of coccidial oocyts.

The results of this study also tend to suggest that any advantage that females might have over males in terms of energy digestion could be associated with them having a larger pancreas in relation to metabolic body weight. Recently, Engberg *et al.* (2002) reported increased gizzard and pancreas weights relative to live weight, and increased activities of pancreatic enzymes in chickens given a mash feed compared with those given pellets. Growth and feed conversion were poorer in the mash-fed chickens, however. Engberg *et al.* (2002) suggested that the feeding of coarsely ground mash may have stimulated

secretion of hydrochloric acid by the proventriculus, and increased retention time of feed in the proventriculus and gizzard. Similarly, Ikegami *et al.* (1990) noted that rats fed dietary fibre had an enlarged pancreas with increased contents of enzymes and nucleic acids, but there were no changes in the activities of the pancreatic enzymes when expressed per milligram of tissue. They concluded that the enlargement of the pancreas and increased secretion of digestive juices compensated for the decrease in diffusion of substrates and enzymes in the digesta, and reduced contact with the mucosal surface.

Iskander and Pym (1987) reported that male chickens had a relatively smaller proventriculus and gizzard weight (expressed in g/kg live weight) than females. In contrast, there were no differences in these organs due to sex of the chicken in the current study. However, there was an indication that the proventriculus and jejunum might be reduced in size when chickens were reared in a dirty environment. Nevertheless, none of the gross characteristics of the gastrointestinal tract were firmly linked with any change in the digestion of energy metabolism by either sex of chicken, despite there being relatively wide ranges of normally distributed AME and dry matter digestibility values.

2.2.5 Conclusions

Females appear to have more potential ability than males for digestion and absorption of carbohydrate and fat as a result of them having a relatively larger pancreas and jejunum than male chickens. However, gross measurements of dry weights of different sections of the upper part of the gastrointestinal tract explained only a small proportion of the variation in AME values across the flock.

2.3 Effects of morphology of the small intestinal epithelium on AME of commercial broiler feed

2.3.1 Introduction

In the previous experiment (reported in section 2.2), chickens were 21 or 22 days of age before receiving MOS in the diet. This could have been too late to confer any benefits from reduction in the numbers of bacteria attaching to the intestinal mucosa and invading the gut tissue, and that earlier use of MOS may be necessary. Also, it was evident that gross morphology of the gastrointestinal tract was an insensitive indicator of variation in

AME values, and that differential responses by males and females may be masked if the nutrient specifications of the diet were too high. In the following experiment, changes in villus/crypt structure of the small intestinal epithelium were measured to determine whether these were more sensitive indicators of digestive function, as suggested by Iji (1998).

The specific objectives of this experiment were to quantify the variation in gut structure and energy metabolism associated with cleanliness of the rearing environment and the sex of chicken, but with MOS added to the diet of half of the birds from day of hatch. The chickens were reared in clean and dirty environments to 15 days of age, and then given a wheat-based experimental diet prior to measurement of AME of a commercial diet with the same nutrient specifications as that used in the previous experiment. The reasoning behind this approach was to nutritionally stress chickens by exposure to an experimental diet comprised mainly of wheat in the expectation that the chickens would subsequently express a greater degree of variation in digestive capacity than seen in the previous experiment.

This experiment tested the hypotheses (a) that AME of commercial broiler feed was significantly correlated with measurements of villus-crypt architecture of the small intestinal mucosa, and (b) that the relationships differed according to sex of the chicken.

2.3.2 Materials and methods

Birds, housing and management

Sexed broiler chickens (Ross strain) obtained from the Bartter Steggles hatchery, Cavan, SA on Thursday 10 June 1999 were raised from hatch to 15 days of age in experimental rearing pens (Appendix 1) in clean and dirty environments as described previously. Birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540) containing MOS (Bio-mos™) at 5 g/kg, or a control diet of starter crumbles without MOS.

At 15 days of age, the chickens were transferred in pairs to 96 single-bird metabolism cages (Appendix 2) located in a controlled-temperature room set at 25 - 27°C initially, and given wheat-based experimental diets containing MOS at 0 or 5 g/kg. Each kg of diet contained 800g wheat, 152 g casein, 20 g dicalcium phosphate, 11 g limestone, 7 g DL-methionine, 5 g minerals and vitamins, 3 g salt, and 2 g choline chloride (60%). At 18

days of age, one chicken was removed from each cage and AME values of the wheat-based diets with or without MOS were measured over the following 4-day period. At 22 days of age, chickens were given one of two commercial finisher diets again with or without MOS. AME values of these diets were measured over the following 7-day period. Throughout the period 15 to 29 days of age, chickens were given MOS at 0 or 5 g/kg as per the level in the diet they received during rearing. The temperature setting in the room was reduced daily until it was 22°C at the end of the experiment.

Collection of intestinal tissue and histology

A total of 24 chickens were selected on the basis of AME values obtained on the wheat-based diet. Chickens with lowest, highest or average AME value within each combination of rearing treatment, sex of chicken and dietary addition of MOS were killed by intravenous injection of pentobarbitone, then weighed. Two 1 cm long sections from the midpoints of the duodenum, jejunum and ileum were flushed with ice-cold buffered phosphate saline at pH 7.4. One section was cut longitudinally. Both sections were fixed in neutral buffered formalin for 24 hours then retained in 70% ethanol prior to further processing. Tissue slices 1-2mm thick were enclosed in a plastic tissue cassette then processed over a 16.5-hour period in an automatic tissue processor. Processing involved serial dehydration with ethanol, clearance with histolene, then impregnation with wax. The tissue was imbedded in paraffin wax for sectioning by microtome. Separate 7 μm sections were placed on a glass slide for staining with haematoxylin and counter-staining with eosin, then mounted in a xylene-based medium. Villus height and crypt depth were viewed at x40 and x100 magnifications, respectively, on a compound microscope fitted with a trinocular head with x10 eyepieces and a colour video camera. Images were analysed with Video Pro software (Leading Edge Pty Ltd, Adelaide, South Australia). At least 15 villi and 15 crypts were measured in each type of tissue from each chicken.

Serology

Chickens not required for the metabolism experiment were transferred at 15 days of age from the rearing pens in the clean and dirty environments to a shared floor pen in the dirty environment and given finisher pellets without MOS. At seven weeks of age, blood sera

samples were collected from the brachial vein of 48 chickens, and tested for the presence of antibodies against MDV, IBDV and CAV, as in the previous experiment (section 2.2.2).

Statistics

Analysis of variance (by GLM procedure) was used to examine the effects of rearing environment, diet and sex as described in section 2.2.2. Stepwise regression was used to develop prediction equations for AME of the diet and dry matter digestibility (DMD) from measurements of villus height and crypt depth in the duodenum, jejunum and ileum.

2.3.3 Results

Addition of MOS significantly improved ($P<0.05$) the live weight of chickens at 18 days of age (Table 3), irrespective of the cleanliness of the rearing environment.

Table 3. Summary of significant main effects of rearing environment, diet and sex in the period 22 to 29 days of age. Means with the same letter within a main effect are not significantly different ($P>0.05$).

	Rearing		Diet		Sex		Pooled
Variable	Clean	Dirty	Control	MOS	Female	Male	SEM
Live weight 18 d (g/bird)	627 a	592 b	603 b	616 a	584 b	636 a	4.9
Live weight 22 d (g/bird)	835 a	795 b	808 b	822 a	773 b	859 a	6.4
Live weight 29 d (g/bird)	1250 a	1212 b	1225	1237	1162 b	1303 a	9.7
Growth (g/bird)	415	417	417	415	389 b	444 a	6.1
Rate of growth (g/kg) [1]	498 b	524 a	517	506	505	518	7.9
Feed intake (g/bird/day)	115	113	113	115	107 b	122 a	1.2
Feed conversion ratio	1.96	1.92	1.92	1.95	1.94	1.93	0.019
AME of diet DM (MJ/kg)	13.47	13.59	13.60	13.46	13.58	13.48	0.060
Dry matter digestibility (g/g)	0.622	0.629	0.626	0.625	0.627	0.624	0.0027
Duodenal villus height (µm)	1440 a	1299 b	1334	1405	1367	1372	42
Jejunal villus height (µm)	1046	995	1026	1015	1019	1022	35
Ileal villus height (µm)	543	518	535	526	533	528	21
Duodenal crypt depth (µm)	237	230	241	226	248	222	12
Jejunal crypt depth (µm)	210	198	211	197	212	197	8
Ileal crypt depth (µm)	190	183	190	183	192	182	4

[1] Rate of growth=growth×1000÷live weight at start

MOS had no effect ($P>0.05$) on the AME of the wheat diet (mean 15.3 MJ/kg DM) measured in the period 18 to 22 days of age. There was a significant interaction ($P<0.05$) between cleanliness of the rearing environment and sex of the chicken on AME. Males reared in the dirty environment showed a significant depression ($P<0.05$) in AME relative to females reared in the same environment, but no significant difference between sexes existed in the clean environment (Figure 5).

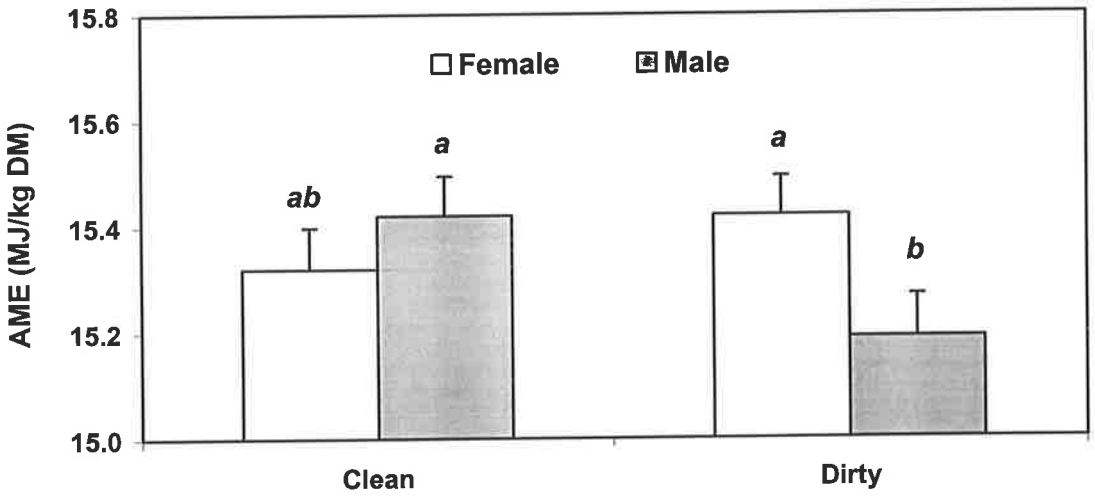


Figure 5. Effects of rearing environment and sex on AME of a wheat-based diet (mean ± SE; $n = 24$) in the period 18 to 22 days of age. Means with a common letter are not significantly different ($P>0.05$).

In the 7-day period (22 to 29 days of age), AME of the commercial finisher diet (13.53 MJ/kg DM) and dry matter digestibility (0.626 g/g) were unaffected ($P>0.05$) by the cleanliness of the rearing environment, sex of chicken, or addition of MOS to the diet (Table 3). Means, standard deviations, numbers of observations, coefficients of variation, and ranges of values for dietary AME and dry matter digestibility are shown in Table 4.

Table 4. Means, standard deviations (SD), numbers of observations, coefficients of variation (CV) and ranges of values for dietary AME and dry matter digestibility of the commercial diet fed in the period 22 to 29 days of age.

	Mean	SD	n	CV	Range
AME of diet DM (MJ/kg)	13.53	0.41	96	3.0	12.06-14.82
Dry matter digestibility (g/g)	0.626	0.018	96	2.9	0.569-0.682

Live-weights of chickens from the clean environment were greater ($P<0.001$) at 18 and 22 days of age (Table 3). However, the interaction between rearing environment and diet approached significance ($P=0.06$) for the 22-day weights, and significantly affected ($P<0.01$) live weight at 29 days of age, and feed intake and growth in the period 22 to 29 days of age when chickens were fed the commercial finisher diet (Table 5).

Table 5. Effects of rearing environment and diet on live weight (g/bird) at 18, 22 and 29 days of age, and on feed intake (g/bird) and growth (g/bird) in the period 22 to 29 days of age. Means with a common letter in the same row are not significantly different ($P>0.05$) according to pair-wise *t*-tests.

	Clean		Dirty		Pooled
Variable	Control	MOS	Control	MOS	SEM
Live weight 18 days	622 ab	629 a	582 c	606 b	7
Live weight 22 days	832 a	833 a	781 b	816 a	9
Live weight 29 days	1258 a	1237 a	1187 b	1249 a	14
Growth 22 to 29 days	426 ab	403 b	406 b	432 a	9
Feed intake 22 to 29 days	116 a	114 ab	110 b	118 a	2

Effects of cleanliness of the rearing environment on villus height and crypt depth in duodenal, jejunal and ileal sections are shown in Figure 6. Villus height in duodenal mucosa was significantly ($P<0.05$) reduced in chickens reared in the dirty environment (Table 3). Other morphological features were unaffected ($P<0.05$) by rearing conditions, dietary addition of MOS, and sex of chickens.

There was a significant ($P<0.05$) but weak association ($R^2=0.17$) between DMD and crypt depth in ileal tissue (Figure 7). However, AME was not related to crypt depth (Figure 7) or any other single measurement or any combination of measurements of morphological features of the small intestine. AME and DMD values for individual chickens ranged from 12.7 to 14.3 MJ/kg DM and 0.59 to 0.66, respectively (Figure 7).

ELISA tests showed an absence of challenge from immuno-suppressive agents such as MDV and IBDV. Eight out of 48 chickens tested positive to CAV.

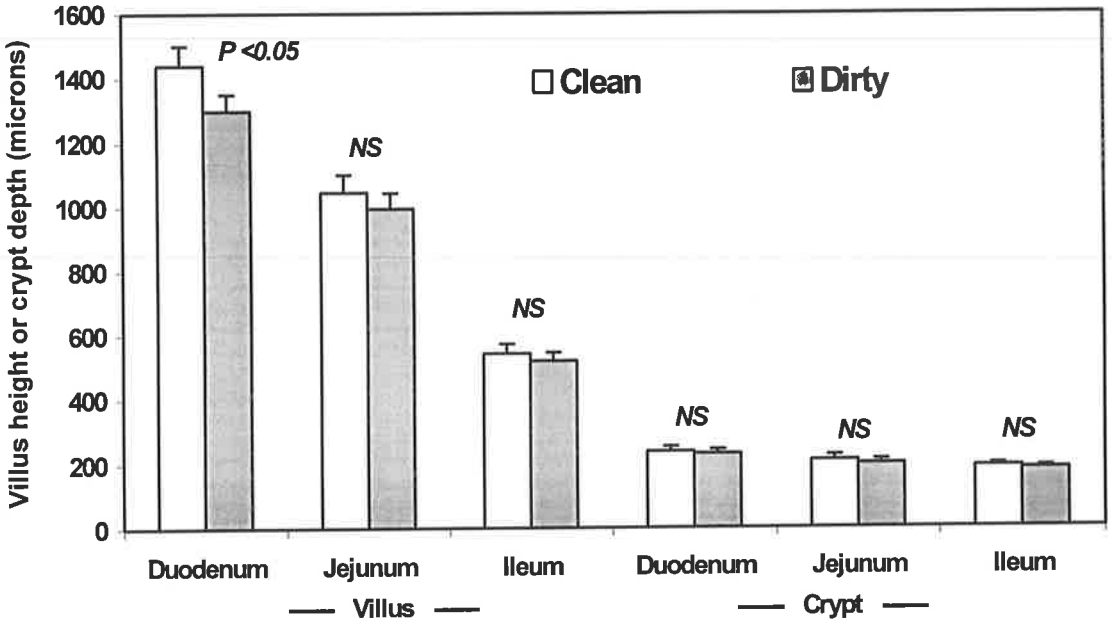


Figure 6. Effects of cleanliness of the rearing environment on villus height (μm) and crypt depth (μm) in duodenum, jejunum and ileum (mean ± SE; n = 48). *NS* indicates not significant (P>0.05).

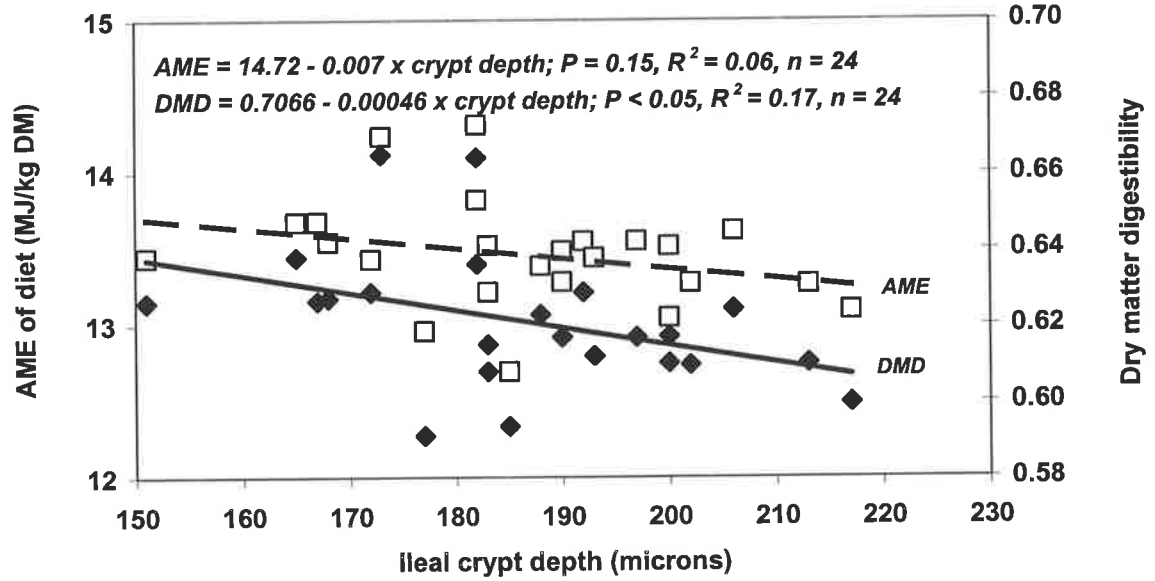


Figure 7. Relationships between crypt depth in ileal mucosa and AME (□) and DMD (◆) values for a commercial finisher diet.

2.3.4 Discussion

Addition of MOS (5 g/kg) to the rearing diet improved the live weights at 18, 22 and 29 days of age for chickens reared in the dirty environment. The benefit of adding MOS to the diet did not extend into changes in gut structure. This lack of response to dietary MOS on intestinal morphology is not consistent with the results of Iji (1998) who observed a 25% increase in villus height of the jejunal mucosa (from 1246 to 1554 μm) when MOS was added at the rate of 5 g/kg to a sorghum based diet. Villus height and crypt depth in duodenal, jejunal and ileal mucosa were all unaffected by MOS in the current work.

The significant reduction in villus height in the duodenal mucosa from chickens reared in the dirty environment (Figure 7) is consistent with a general inflammatory response arising from accumulation of inhaled or ingested non-pathogenic microbes, dust and dander in the dirty environment (Klasing *et al.* 1999). However, while there was a general tendency for reductions in villus height in jejunal and ileal mucosa, neither of these was significant. Similarly, crypt depth was numerically lower (but not significantly so) in all intestinal segments in chickens from the dirty environment. The lack of change in the villus/crypt architecture tends to suggest that, while this particular rearing environment was dusty, it was not biologically dirty in the sense of promoting overt responses to bacteria, coccidia or viruses. The ELISA results from this and the previous experiment imply an absence of responses in most chickens to three common poultry viruses. The level of exposure to oocysts and bacteria would have been limited to viable organisms carried on air-borne particles settling in the rearing pens which were otherwise clean at the start. In retrospect, a better approach may have been to use re-cycled litter from previous batches of chickens to induce a response by chickens to a dirty environment.

As in the first experiment (described in section 2.2), no single measurement or combination of measurements of morphology of the gastrointestinal mucosa were firmly linked with any change in the energy metabolism by either sex of chicken, despite there being wide ranges of values for AME and dry matter digestibility.

Relatively high AME values for the experimental wheat diet and the commercial diet indicate a low level of nutritional stress experienced by the chickens used in these two experiments. This may explain why the variation in gut structure was less than expected,

and that poorer quality feedstuffs are required in experiments in order to determine whether gut morphology of the intestinal mucosa becomes a limiting factor in the digestive capacity of chickens.

2.3.5 Conclusion

Villus height and crypt depth of different sections of the small intestinal mucosa are poor indicators of the digestive capacity of broiler chickens given commercial broiler feeds.

2.4 General discussion

The relative weight of the pancreas of female chickens was greater than that in males, irrespective of dietary addition of MOS or cleanliness of the rearing environment. The relative weight of the jejunum in female chickens was greater than that in males, but only when chickens were reared in a clean environment. Other morphological features of the intestine did not differ between the sexes.

Relationships between AME and DMD of the diet and gut structure were examined in two experiments intended to reveal the extent of potential variation in energy digestion by application of treatments involving cleanliness of the rearing environment, sex of chickens and addition of MOS to the diet. Less than 20% of the variation in AME or DMD was associated with gross structural characteristics such as the dry weights of duodenum, jejunum or ileum relative to metabolic body weight, or with finer morphological measurement of villus height and crypt depth in these sections of the small intestine, despite there being relatively wide ranges of normally distributed AME and DMD values in both experiments.

There were no indications that the statistically significant although weak assocations between AME or DMD and some morphological features of the small intestine differed according to sex of the chicken.

2.5 Conclusions

The relative weights of the pancreas and jejunum of female chickens were greater than those of the males, but no other morphological features of the intestine differed between the sexes. The possibility that variation in AME is associated with differences in gut

morphology warrants further investigation with experimental diets known to contain relatively high levels of anti-nutritive factors such as soluble NSP in order to quantify the extent that the morphology of the intestinal tract and its mucosa can affect digestion of energy in chickens.

Chapter 3. Variable responses in digestion of energy from a wheat-based diet are associated with sex and strain of chicken and morphology of the small intestinal mucosa

3.1 Introduction

Results of the experiments described in Chapter 2 to examine relationships between gut structure and energy digestion were inconclusive, probably because the diets contained insufficient levels of anti-nutritive factors to affect AME. A main conclusion was that further investigation was required with poorer quality feedstuffs to determine whether soluble NSP affected the digestion of energy by altering the morphology of the small intestine.

Hughes and Choct (1997) demonstrated wide variation in AME (8.8 to 14.9 MJ/kg DM) in a single experiment involving a sample of wheat containing a high level of soluble arabinoxylan given to broilers hatched and reared under identical conditions. They concluded that the "low-ME" wheat phenomenon was not entirely dependent on the physico-chemical nature of wheat but it was a multi-faceted problem associated with characteristics of digestive physiology of individual broiler chickens. More recently, Hughes *et al.* (2001) reported AME values ranging from 12.3 to 13.5 MJ/kg DM for a single barley sample. That is, individual birds demonstrated quite different responses in energy metabolism when fed the same diet.

The experimental diets used in the studies reported by Hughes and Choct (1997) and Hughes *et al.* (2001) were comprised of grain, casein, limestone, dicalcium phosphate, salt, and a mineral and vitamin supplement. Carbohydrate was the main source of energy in these diets, with fat making a relatively small contribution. Hence, large variation in AME was probably associated with similarly large variation between individual birds in starch digestion (as noted by Mollah *et al.* 1983 and Rogel *et al.* 1987) and absorption of sugars, rather than by reduced lipid absorption due to the removal of bile salts from digesta by microflora in the small intestine as described by Smits (1996).

A likely explanation for the reduced and variable digestion of starch is the anti-nutritive effect of soluble NSP in the foregut (Choct and Annison 1992). The mechanism of action of soluble NSP is thought to involve increased viscosity of digesta which limits contact between digestive enzymes and substrates, and contact between nutrients and absorption sites on the intestinal mucosa (Annison 1993; Bedford and Morgan 1996; Smits *et al.* 1997).

The effects of sex of the chicken on AME values and gut morphology described in Chapter 2 point to the possibility that responses to soluble NSP may also differ between male and female chickens. To date, no one has quantified differences between individual birds in the way that they respond to soluble NSP. If large differences exist, it would help explain the wide between-bird variability in AME observed by Hughes and Choct (1997). ten Doeschate *et al.* (1993) reported a genotype by sex interaction on energy digestion; hence it seems reasonable to assume that not only the sex of the bird but also its strain may be an important factor in how individual chickens respond to anti-nutritive components such as soluble NSP.

The following experiment tested the hypotheses (a) that individual birds would respond differently in digestion of energy from a diet based on wheat with soluble NSP content 11.3 g/kg DM, (b) that the responses would differ according to strain and sex of chicken, and (c) that morphology of the small intestinal mucosa was associated with digestion of energy.

3.2 Material and methods

3.2.1 Birds, housing and management

Cobb (Strain #1) and Ross (Strain #2) broiler chickens were obtained from the Bartter Steggles hatchery, Cavan SA and the Baiada hatchery, Willaston SA, respectively, on Thursday 4 November 1999. Chickens were raised from hatch in four floor pens in a controlled temperature room. Male and female chickens of each strain were reared separately. All birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540).

At 18 days of age, chickens were transferred in pairs to 48 single-bird metabolism cages (dimensions shown in Appendix 2) located in a controlled-temperature room set at 25-27°C initially, and given commercial starter crumbles (Ridley Agriproducts, diet code #503540).

The temperature setting in the room was reduced daily until it was 22°C at the end of the experiment.

Previous experience showed that chickens quickly adjusted to the cages when accompanied by at least one other chicken. One bird was removed from each cage just prior to commencement of the experiment.

The 7-day experiment commenced at 21 days of age for chickens in the first set of 24 cages, and on the following day for chickens in the second set of 24 cages. All chickens were given free access to a wheat-based diet (Table 6) and water throughout the experiment. Chickens were weighed at the beginning and end of the experiment.

Table 6. Composition of the experimental diet

Ingredient	g/kg
Wheat	700
Meat and bone meal	76
Soybean meal	170
Sunflower oil	40
Sodium chloride	2.5
L-lysine HCl	2.5
DL-methionine	3
Vitamin and mineral premix with millrun diluent	5
Choline chloride (60%)	0.8

Dr Mingan Choct (UNE) supplied the wheat soon after harvest in the 1996/97 growing season. A subsequent experiment in August 1997 involving 32 individually housed chickens at PPPI verified that it had a low AME value (13.1 MJ/kg DM). The wheat was kept in a cool room (4°C) between AME testing and its subsequent use in this experiment to minimise any post-harvest change in AME (Choct and Hughes 1997). The diet was pelleted (4 mm diameter and 6 mm length) in a cold-press to avoid selective feeding.

3.2.2 AME procedures, digesta collection and measurement of viscosity

The AME of the wheat-based experimental diet described in Table 6 was determined in a conventional energy balance study involving measurements of total feed intake and total

excreta output, and subsequent measurement of gross energy (GE) values of feed and excreta, as per the procedures described in section 2.2.2.

On completion of the 7-day AME study, each chicken was killed by intravenous injection of pentobarbitone, and then weighed. The gastro-intestinal tract (GIT) from the base of the gizzard down to the ileo-caecal junction was dissected. Sections of the duodenum, jejunum and ileum were placed in fixative for histology by procedures outlined in section 2.3.2.

The ileal digesta was removed by gentle squeezing, and kept on ice prior to centrifugation (12,000 g, 10 min, 20°C). Supernatant and pellet from each sample were frozen and stored at –20°C. Viscosity of thawed supernatants was measured with a Brookfield DVIII viscometer at 25°C with a CP40 cone and a shear rate range of 5-500 s^{-1}. The samples did not exhibit shear thinning at these shear rates. Previous studies (M. Choct, P. Zviedrans and R.J. Hughes, unpublished) indicated that initial differences in viscosity of fresh digesta were retained by freezing and thawing provided thawed supernatants were measured immediately.

King (1998) described the relationship between energy excreted (EE) and gross energy intake (GEI) as a linear model:-

$$EE = \alpha + \beta \times GEI$$

where α = energy voided at fasting and β = rate of increase in EE as GEI increases. This approach was used to examine whether sex and strain of chicken influenced energy balance.

3.2.3 Statistical analysis

Analysis of variance (by GLM procedure) was used to examine the effects of strain and sex, and the strain by sex interaction. Duncan's Multiple Range Test was used to separate means of main effects (block, strain and sex) and *T*-tests (by LSMEANS procedure) were used to separate means for the strain by sex interaction when significant effects ($P<0.05$) were evident in the analysis of variance. Stepwise regression (by REG procedure with maximum R^2 selection) was used to develop prediction equations for AME of the diet from measurements of villus height and crypt depth in the duodenum, jejunum and ileum. Analysis of covariance (by GLM procedure) was used to determine whether the linear

coefficients of regression for EE on GEI differed between the two strains and between males and females.

3.3 Results

Results of statistical analysis are summarised in Table 7. The strain by sex interaction was not significant (P>0.05) except for jejunal villus height.

Table 7. Summary of main effects in analysis of variance

Variable	Strain [3]		Sex [3]		Pooled
	1	2	Female	Male	SEM
Live weight at start (g/bird)	928 a	874 b	846 b	956 a	12.0
Growth (g/bird)	403 a	392 a	377 b	418 a	11.4
Rate of gain (g/kg) [1]	439 a	450 a	449 a	440 a	13.5
Feed intake (g/bird/day)	108 a	104 a	98 b	113 a	2.1
Feed conversion (g feed: g gain)	1.90 a	1.86 a	1.84 a	1.92 a	0.043
AME of diet DM (MJ/kg)	14.43 a	14.24 a	14.63 a	14.04 b	0.146
Dry matter digestibility (g/g)	0.699 a	0.693 a	0.708 a	0.685 b	0.0071
Ileal viscosity (cP) [2]	14.8 a	19.6 a	15.0 a	19.4 a	2.60
Duodenal villus height (µm)	1299 a	1199 b	1245 a	1253 a	26.7
Jejunal villus height (µm)	1027 a	1001 a	1017 a	1012 a	22.3
Ileal villus height (µm)	596 a	577 a	560 b	613 a	15.5
Duodenal crypt depth (µm)	221 a	229 a	223 a	228 a	6.1
Jejunal crypt depth (µm)	237 a	239 a	233 a	243 a	9.2
Ileal crypt depth (µm)	231 a	237 a	226 a	242 a	9.3

1 Rate of gain = Growth × 1000 ÷ Live weight at start of experiment

2 Data were $\log_e$ transformed to normalise the distribution prior to analysis

3 Means with the same letter within a main effect are not significantly different (P>0.05)

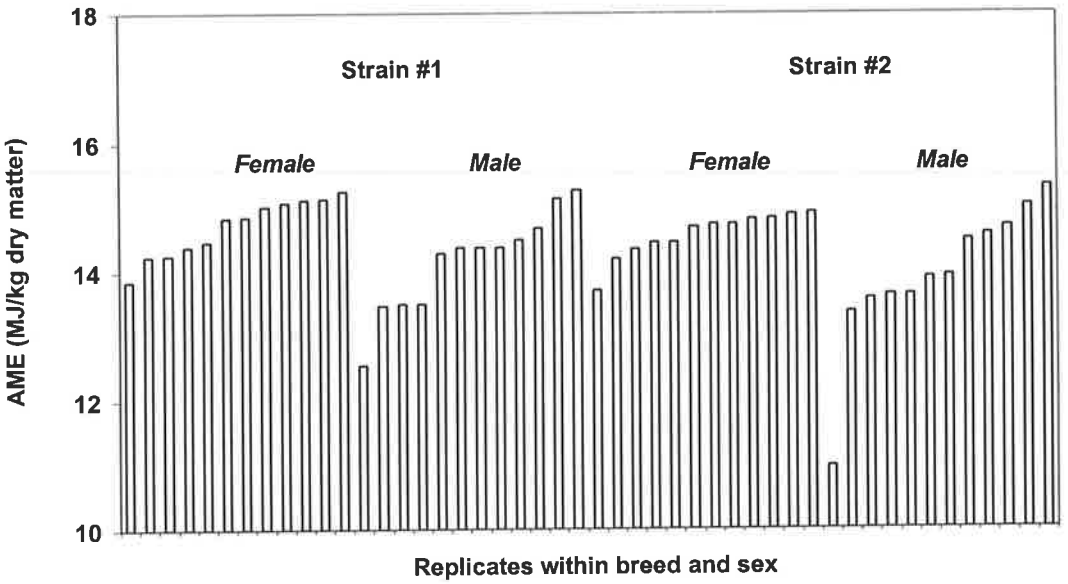


Figure 8. Variability in apparent metabolisable energy (AME) of a wheat diet given to male and female chickens of two commercial strains. AME values are sorted in ascending order within each combination of strain and sex.

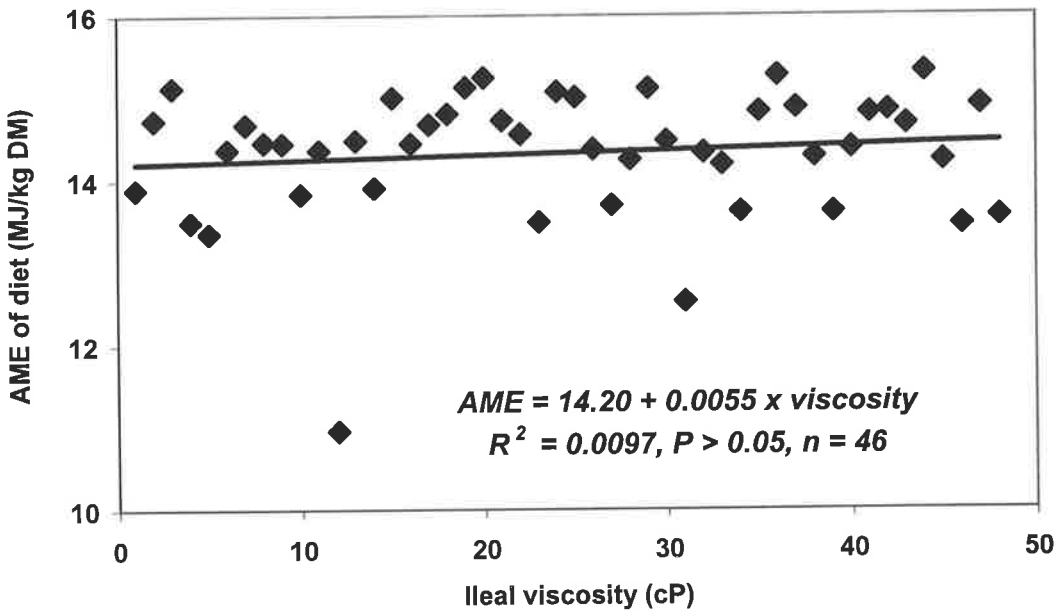


Figure 9. Lack of a relationship between AME and viscosity of ileal digesta from chickens given a diet based on wheat with soluble NSP content 11.3 g/kg DM.

Males weighed more than the females, and chickens of Strain #1 were heavier than Strain #2 at the start of the experiment (Table 7). Males ate more feed and gained more weight than the females during the 7-day experiment, however, the females were superior to males in AME and digestibility of dry matter (Table 7). AME values for individual male and female chickens of both strains are shown in Figure 8. The coefficients of variation for Strain #1 females and males were 3.1 and 5.5%, respectively, and those for Strain #2 were 2.4 and 8.0%, respectively.

There were no effects ($P>0.05$) of sex or strain on viscosity of ileal digesta, and the regression between AME and viscosity (Figure 9) was not significant ($P>0.05$). The coefficients of variation for viscosity and AME across sex and strain were 74% and 5.4%, respectively.

Significant differences ($P<0.05$) due to sex and strain were detected in villus height of the mucosa in different sections of the small intestine (Table 7). Males had significantly greater ileal villus height than females. Chickens from Strain #1 had significantly greater duodenal villus height than Strain #2 chickens. Jejunal villus height was significantly affected by the interaction between strain and sex (Figure 10) with male chickens of Strain #1 having significantly greater jejunal villus height than male chickens of Strain #2, but no difference due to strain was evident for females.

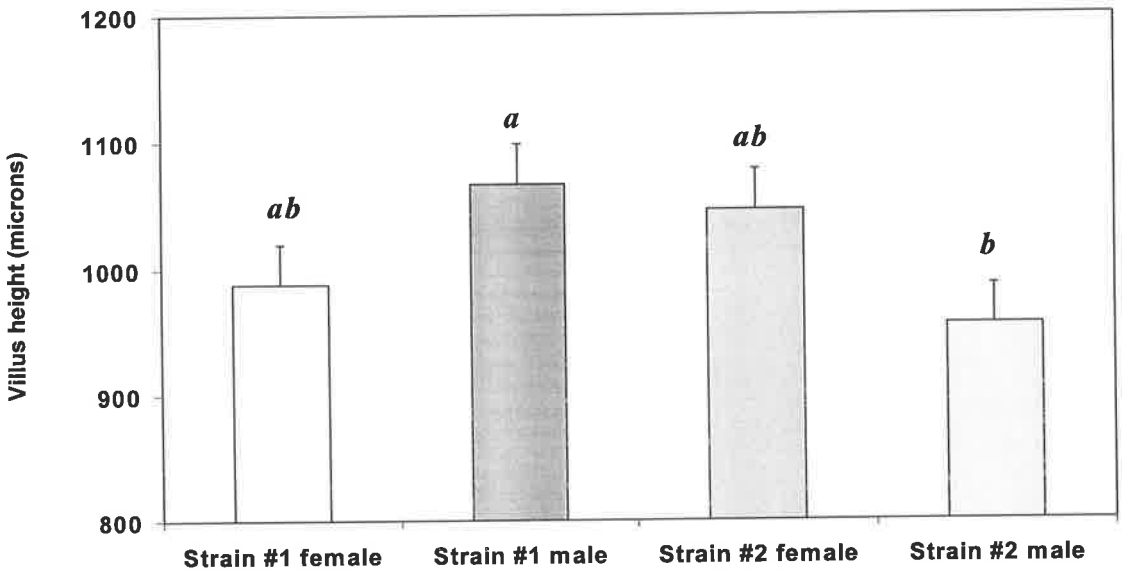


Figure 10. Effects of strain and sex of chicken on villus height in the jejunal mucosa (means ± SE; n = 12). Means with a common letter are not significantly different ($P>0.05$).

Best one-variable model:-

$$AME = 15.73 - 0.0059 \times \text{ileal crypt depth}$$

$$P < 0.01,\ R^2 = 0.179,\ r = -0.42,\ n = 48$$

Best two-variable model:-

$$AME = 14.37 - 0.0068 \times \text{ileal crypt depth} + 0.0013 \times \text{duodenal villus height}$$

$$P < 0.01,\ R^2 = 0.227,\ r = 0.48,\ n = 48$$

Best three-variable model:-

$$AME = 13.82 - 0.0047 \times \text{ileal villus height} + 0.0015 \times \text{duodenal villus height} - 0.54 \times \text{ileal villus to crypt ratio}$$

$$P < 0.01,\ R^2 = 0.290,\ r = 0.54,\ n = 48$$

Best four-variable model:-

$$AME = 13.14 - 0.0056 \times \text{ileal villus height} + 0.0015 \times \text{duodenal villus height} + 0.66 \times \text{ileal villus to crypt ratio} + 0.0036 \times \text{duodenal crypt depth}$$

$$P < 0.01,\ R^2 = 0.313,\ r = 0.56,\ n = 48$$

Best five-variable model:-

$$AME = 16.02 - 0.0052 \times \text{ileal villus height} + 0.0020 \times \text{duodenal villus height} + 0.60 \times \text{ileal villus to crypt ratio} - 0.0061 \times \text{jejunal crypt depth} - 0.27 \times \text{jejunal villus to crypt ratio}$$

$$P < 0.01,\ R^2 = 0.330,\ r = 0.57,\ n = 48$$

Figure 11. Relationships between dietary AME and morphological features of the small intestinal mucosa in chickens, as indicated by step-wise regression analysis.

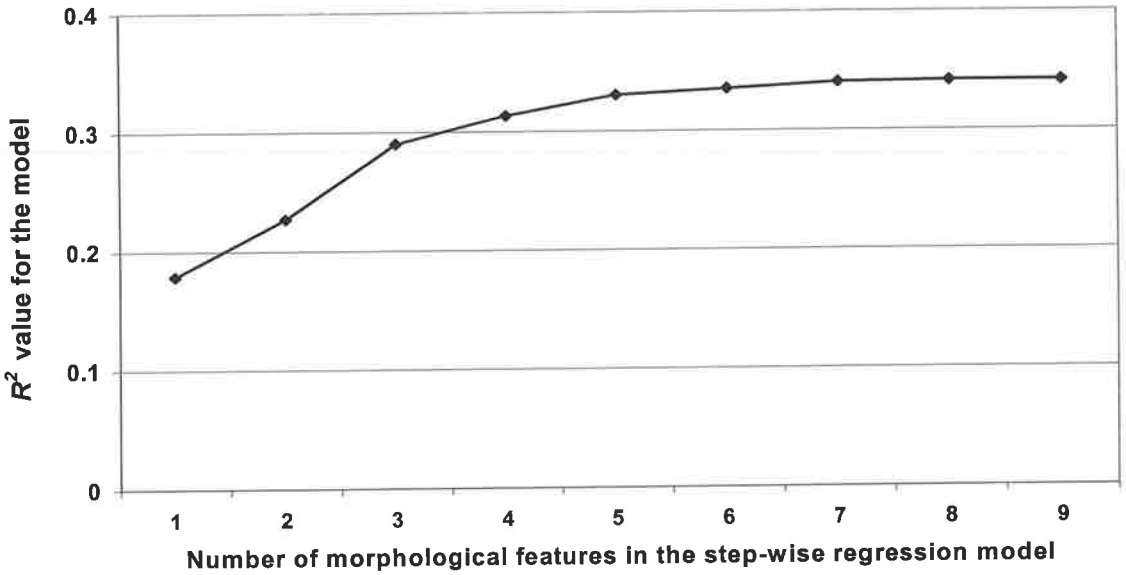


Figure 12. Relationships between dietary AME and morphological features of the small intestinal mucosa, as indicated by increasing R^2 values in step-wise regression analysis. The specific prediction equations are listed in Figure 11.

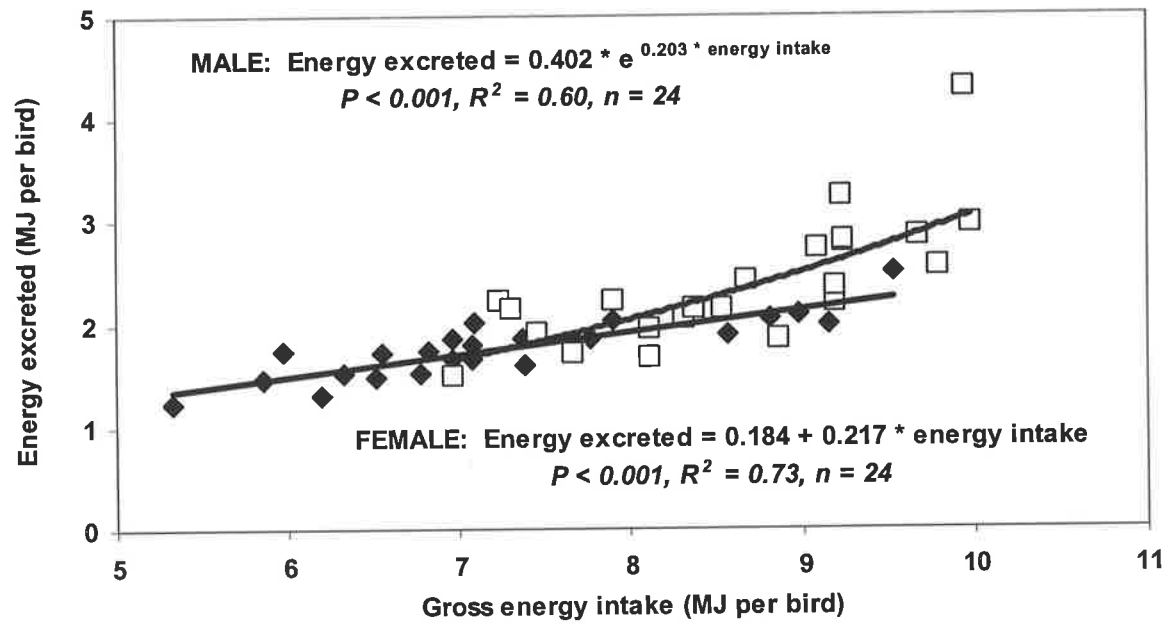


Figure 13. Relationships between energy excreted and gross energy intake for male (□) and female (◆) chickens.

Results of step-wise regression analysis to explore possible relationships between energy metabolism and villus/crypt architecture are summarised in Figure 11 and Figure 12.

Analysis of covariance of the effects of sex and strain of chicken on the relationship between energy intake and excretion (Figure 13) showed that strain was unimportant ($P>0.05$) but there was a significant effect ($P<0.05$) due to sex. Observation of the plot of data points for males (Figure 13) suggested that the relationship was curvilinear. Various curvilinear functions were fitted separately to the data from male and female chickens. An increase in R^2 value indicated a better fit for an exponential function ($R^2=0.60$) compared with a linear function ($R^2=0.55$). That is, there appeared to be an exponential increase in excreted energy with increase in gross energy intake (Figure 13). None of the curvilinear functions improved the R^2 value for females. The estimates for energy voided at fasting were 46 kJ/bird/day for females, and 101 kJ/bird/day for males. Even after correction for differences in live weight due to sex, the value for males was approximately 70% greater than that for females. Care is needed with the use of these estimates of endogenous energy loss as the models were extrapolated well beyond the range of measurements of energy intake.

3.4 Discussion

AME values for females were 4% higher than for males, with such a difference being highly significant in commercial terms. Furthermore, it is clear from the patterns of AME values obtained with individual chickens (Figure 8), and the corresponding coefficients of variation for strain by sex combinations, that females are more uniform than males. Some individual chickens, particularly males, responded poorly to the wheat diet compared with others. On the other hand, some males responded just as well as the best performing females. This is despite chickens being reared in the same room from hatch, and receiving the same rearing diet prior to the experiment. One would expect that all chickens would have been exposed to similar levels of challenge from pathogens in the environment and from effects of dust and dander in the air (Klasing *et al.* 1999), and any anti-nutritive components in the feed. Sex effects may be due to differences in energy costs to repair and maintain the gut (Wright and Alison 1984*b*), or in response to inflammatory effects of microflora (Koutsos and Klasing 2002). Whether effects of sex extend into the absorption and transport of nutrients remains to be determined.

Regression analysis showed no significant relationship ($P>0.05$) between AME and viscosity of ileal digesta. The lack of effects of sex and strain on viscosity of ileal digesta (Table 7), and the absence of a significant relationship between AME and viscosity (Figure 9) suggest that variation in AME values was not related to the gelling properties of NSP but that other factors were responsible.

Up to one third (33%) of the variation in AME was associated with physical features of the small intestinal mucosa (Figure 12). Ileal crypt depth was the single most important feature of the small intestinal mucosa associated with variation in AME (Figure 11). The strain and sex of chicken significantly affected villus heights of the mucosa in the jejunum and ileum, respectively. Villus/crypt architecture differed between strains for male chickens, but there were no differences observed in female chickens. These results imply that gut morphology may be an important determinant of digestion of energy but that other factors responsible for at least two thirds of the variation in AME are collectively more important.

Ileal crypt depth was the single most important predictor of AME (Figure 11) among the various measurements of small intestinal structure examined in this study. While ileal crypt depth *per se* accounted for only 18% of the variation in AME, it is possible that changes in the rates of proliferation of crypt cells in other sections of the small intestine, and in the maturation of cells during migration along the villus, may have occurred (Wright and Alison 1984*b*). Other possibilities include changes in the numbers of goblet cells on the villus surface (Langhout 1998), the amount and type of mucins secreted (Langhout *et al.* 1999), and the microbe-binding characteristics of the mucins (Kelly and King 2001). Changes such as these could affect the unstirred water layer (Johnson and Gee 1981) and, hence, limit the contact between nutrients and absorption sites on the mucosa (Smithson *et al.* 1981). Biochemical examination of mucosal tissue was planned in this project; however, this was prevented when tissue samples were lost during a power failure in the PPPI Nutrition Research Laboratory

Both Johnson (1987) and King (1998) pointed out that endogenous energy loss (EEL) could be a large source of error in measurements of AME and TME in assays involving the allocation of fixed amounts of test diet considerably less than *ad libitum* feed intake. The size of the EEL error relative to the AME value of the test diet diminishes as the amount of

feed allocated is increased and becomes trivial in fully fed birds. AME values obtained in all of the experiments described in this thesis were obtained with fully-fed birds not fasted at any stage. In a separate series of experiments (not reported in this thesis), I was unable to improve precision or accuracy of measurement of AME by incorporating periods of fasting at the start or end of the excreta collection period as advocated by Farrell (1999). Fasting did, however, significantly depress growth rate and feed efficiency, and greatly reduced the amount of digesta that could be collected for measurement of nutrient digestibility.

The curvilinear relationship between energy intake and energy excretion for male chickens (Figure 13) shows an apparent departure from linearity at higher intakes of energy. Explanations for this include a possible disturbance to a physiological process such as negative feedback control over flow rate of digesta, or increased proliferation of gut microflora. Svihus and Hetland (2001) concluded that overloading the digestive tract with starch can result in incomplete digestion of starch, large losses of energy due to microbial fermentation in the hindgut, and impaired feed conversion. They demonstrated that these losses could be avoided by inclusion of unground grain in the diet, presumably because whole grain enhanced gizzard size and function, which is a point that Cumming (1992) has advocated for many years.

It is worth pointing out that Svihus and Hetland (2001) used male broiler chickens only in their studies. Recent findings by Yaghobfar (2001) that males had higher endogenous energy losses than females, and the increased rate of energy excretion by males in comparison with females shown in Figure 13 indicate that key findings reported in recent publications ought to be verified with chickens of both sexes in order to maximise the value of this information for application in commercial industry practice.

3.5 Conclusions

These results are indicative of sex-related differences in gut morphology and digestion of energy, irrespective of the strain of the chicken. Possible reasons why female chickens have a 4% advantage over males in apparent digestion of energy are explored further in subsequent sections.

Chapter 4. Breath tests as non-invasive indicators of digestive function of broiler chickens and metabolic activity of gut microflora

4.1 General introduction

Previous attempts (described in Chapters 2 and 3) to identify key factors in the digestion of energy were hampered by the lack of variation between groups of chickens induced by the environmental treatments. Success or otherwise of experimental treatments was evident only after chickens were sacrificed for tissue samples, thus limiting the usefulness of the data and samples collected during that experiment. This indicated the need for a non-invasive tool or measurement that can be used to pre-select individual birds on the basis of their phenotypic traits, and for repeated application over time to track changes due to experimental treatments on gastro-intestinal functions such as gastric emptying, pancreatic enzyme activity, digesta transit time, and microbial proliferation in the small intestine.

Clinicians have known for many years that unusual breath odour is often an indication of gastrointestinal dysfunction in patients (Butler 1996). Analysis of expired breath for non-odorous gases is now a commonly used, non-invasive diagnostic method in human medicine (Amarri and Weaver 1995; Swart and van den Berg 1998). Tivey and Butler (1999) concluded that breath tests should prove to be powerful analytical tools for nutrition research and veterinary diagnostics in domestic and agricultural species.

Breath tests involving stable isotopes are safe alternatives to radio-scintigraphy, particularly for infants and pregnant women, and when multiple or frequent tests are required (Amarri and Weaver 1995; Swart and van den Berg 1998). The tests involve ingestion of a substrate enriched in a stable isotope such as ^{13}C, that is relevant to the particular rate-limiting intestinal process under investigation, followed by serial breath sampling. For example, ^{13}C-triglyceride is used to examine pancreatic lipase function (Vantrappen *et al.* 1989), and lactose ^{13}C-ureide is used for measuring small intestinal transit time (Heine *et al.* 1995). Isotope is released as $^{13}CO_2$ by a series of metabolic processes following digestion and absorption of labelled feedstuffs, then transported via the

blood stream to the lungs for excretion. The breath samples are analysed with an isotope-ratio mass spectrometer. The ratio of ^{13}C and ^{12}C isotopes in the breath is directly related to functionality of the gut in terms of release of digestive enzymes, epithelial function or digesta transit time, all of which are measured individually by this technology (Amarri and Weaver 1995; Swart and van den Berg 1998).

Other types of breath test used routinely in medical practice are based on expiration of hydrogen following microbial fermentation of non-labelled carbohydrates such as lactulose, a synthetic disaccharide, which is not absorbed by the small intestine (Robb and Davidson 1987; Wutzke *et al* 1997). Studies on humans and other species indicate that samples of breath can be taken with simple, inexpensive equipment and remain stable for long periods, enabling these tests to be used in the field (Tivey and Butler 1999).

To date, there is scant information in the scientific literature on the use of breath tests on agricultural species of animals. A search of the scientific literature prior to commencement of these studies failed to find any published papers on stable isotope breath testing of birds. One paper (ten Doeschate *et al.* 1995) reported the measurement of $^{14}CO_2$ expired by broiler chickens housed in respiration chambers to study the effect of nutritional status on oxidation of ^{14}C-labelled amino acids injected intraperitoneally or subcutaneously.

In the early stages of the studies reported in this thesis it was anticipated that the most useful breath tests for chickens would be those based on measurement of $^{13}CO_2$ in order to study digestive function of the small intestine in relation to energy metabolism. That is, the initial hypothesis was that variation in energy metabolism in broilers given diets high in soluble NSP with or without glycanase enzymes was attributable mainly to differences in structure and/or function of the upper portion of the small intestine responsible for carbohydrate digestion and absorption. The evidence presented in Chapters 2 and 3 tended to suggest otherwise. Furthermore, during the course of these studies it became increasingly clear from other reports in the scientific literature that gut microflora play a highly significant role in energy metabolism in chickens through effects on gut tissue and rate of passage of digesta (Langhout *et al.* 1999; Bedford, 2000; Bedford and Apajalahti, 2001). Hence it was deemed necessary to put more emphasis on development of hydrogen breath tests for measurement of metabolic activity of gut microflora and transit time of digesta in the latter period of these studies.

This part of the thesis summarises the development of breath tests for chickens for non-invasive measurement of gastro-intestinal function and metabolic activity of gut microflora. The following sections describe the development of equipment and methods for collecting breath samples for $^{13}CO_2$ measurement (section 4.2), measurement of hydrogen and methane in breath samples (section 4.3), and development of breath tests based on hydrogen to measure passage rate of digesta and metabolic activity of gut microflora in chickens (section 4.4).

4.2 Development of equipment and methods for collecting breath samples from chickens

4.2.1 Introduction

Breath testing in adult humans usually involves the collection of samples by asking the patient to blow through a tube into a collection vessel. When it is not possible to get a sample by voluntary means from human or animal subjects, face masks can suffice. An alternative approach is to use a nasal prong to draw breath into a syringe during exhilation.

Initial attempts to construct face masks quickly highlighted some of the practical difficulties associated with this approach to collect breath samples from chickens. It soon became clear that masks needed to fit closely in order to gather a sufficient quantity of breath without contamination from ambient air. Furthermore, the awkward profile of the head and beak of chickens made this difficult to achieve in a mask that could be taken on and off easily without stressing the birds. The alternative of leaving the mask in place for up to three or more hours required either a pressurised air supply or a system of one-way valves to enable the chicken to breath ambient air between the taking of serial breath samples. Other approaches described in the literature such as insertion of a tracheal cannula (Scheid and Piiper 1969) or the gluing of tubes into the nostrils (Itabisashi 1981) were considered extreme from an animal welfare point of view and otherwise impractical or inappropriate for use in this project. The nasal prong approach was also rejected due to the smallness of the nostril openings in chickens.

The idea of using a helmet to collect breath samples came serendipidously when the author and his technician (Mrs Christine Adley) noticed that a cardboard tube from a paper towel

dispenser fitted neatly over the head of a chicken. From there the idea developed into construction of a rigid helmet of the style used by early divers.

4.2.2 Helmets for collecting breath samples from chickens

Plastic helmets were constructed from standard PVC plumbing pipe and caps. A sample of re-breathed air was taken by placing the helmet over the head and neck of the chicken. The helmet was then held firmly against the shoulders and breast of the chicken. After a predetermined period (e.g., 15 seconds) measured by stopwatch, a 10 mL gas sample was drawn through Luer lock fittings into an evacuated tube (Appendix 4).

Helmets of different internal diameter and length were constructed for chickens of different sizes. The choices of helmet size and the period that it was placed over the head of the chicken were based on an estimation of the time taken for re-breathing of air to raise carbon dioxide concentration in the enclosed space to reach at least 2% but not exceed 5% (section 4.2.3). Achievement of these limits was considered important, firstly, to ensure accurate analysis by mass spectroscopy (with CO_2>1%) and, secondly, to avoid disruption to normal respiration by the chicken from excess CO_2 in re-breathed air in the helmet.

4.2.3 Development of methodology for the use of helmets

The period of time that the helmet was placed over the head was determined mainly by the volume of the empty helmet, the space occupied by the head of the chicken, respiratory characteristics of the chicken (Table 8), and the CO_2 content of expired air (Table 9). Preliminary estimates indicated that 30-45 seconds were sufficient. Subsequent trials with $^{13}CO_2$ measurements confirmed these estimates. In contrast, experiments (sections 4.3 and 4.4) involving hydrogen and methane indicated shorter periods (15-30 seconds) of re-breathing were adequate for accurate measurements. The comfort of the chickens was also an important consideration in the decision over re-breathing time. Experience showed that chickens readily tolerated 15 seconds but during longer periods chickens tended to struggle after about 25-30 seconds. This was attributed to an involuntary reflex to raised CO_2 concentration in the headspace. Hence, the shortest possible period was used in experiments.

Table 8. Respiratory characteristics of poultry (Freeman 1984)

	Male	Female
Respiratory frequency (breaths/minute)	12-21	20-37
Tidal volume (mL)	33-46	15-33
Ventilation rate (L/minute)	0.5-0.7	0.5-0.7
Lung capacity (mL)	70	35
Air sacs (mL)	425	260

Table 9. Partial pressure of carbon dioxide in air spaces and blood (McLelland and Molony 1983)

	mm Hg
Arterial blood	29
Venous blood	39
Mean expired air	28
Abdominal air sac	15
Caudal thoracic air sac	24
Cranial thoracic air sac	42
Clavicular air sac	44

4.2.4 Proof of the concept of breath testing chickens

Introduction

Preliminary experiments were conducted to determine whether it was possible to detect $^{13}CO_2$ in the breath of chickens. Two substrates labelled with ^{13}C, a stable isotope of carbon, were chosen for these experiments. The first, ^{13}C-octanoic acid, is given as a liquid meal to measure gastric emptying time for the liquid phase of ingested food in human subjects (Swart and van den Berg 1998). The main reason for choosing ^{13}C-octanoic acid was that it it contains the highest ratio of ^{13}C to ^{12}C of the readily available substrates, and hence, likely to produce a relatively high concentration of $^{13}CO_2$ that could be detected by mass spectroscopy. The second substrate, corn, is naturally enriched in ^{13}C through the metabolic pathway described by Hatch and Slack (1966), and has been used experimentally to examine solid phase gastric emptying and starch hydrolysis in the small

intestine of rats (Symonds *et al.* 1998). This substrate is readily available and cheap compared with labelled probes normally used in clinical practice (Swart and van den Berg 1998).

Materials and methods

Birds, housing and management

Ross broiler chickens were purchased from the Bartter Steggles hatchery, Cavan, SA on Thursday 8 April 1999. These chickens were reared to 15 days of age in separate sex groups in two floor pens in a controlled temperature room. All birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540) during this period.

At 15 days of age, 18 male chickens were transferred in groups of three to metabolism cages in a room maintained at 24-26°C. All birds continued to receive commercial starter crumbles. Birds had free access to feed when not involved in experimental procedures and drinking water was available at all times. Room temperature was adjusted as required to suit the comfort of the chickens.

Experimental procedures

a. $^{13}CO_2$ in expired air following ingestion of ^{13}C-octanoic acid.

At 18 days of age, two chickens in one cage were fasted from 5 pm until 9 am the following day. The third chicken in the cage was transferred to a separate cage in readiness for test procedures involving naturally labelled starch in corn (see section below).

The helmet was constructed from 40 mm PVC tube and a close-fitting cap. A gas sample was taken directly into a 10 mL evacuated tube (Exetainer cat. No. EX10Z10) via a Vacutainer holder fitted with an 18 gauge needle inserted through a luer septum connected by flexible tube to a luer lock fitting attached to the helmet. Each breath sample was taken 45 seconds after a prototype helmet was placed over the head of the chicken and held firmly against the shoulders to minimise loss of expired CO_2. It was assumed that both isotopic forms of CO_2 would diffuse at a similar rate under these circumstances; hence any leakage should not have affected the ratio of $^{13}CO_2$ to $^{12}CO_2$ in the sample.

Following overnight fast, three breath samples taken at 3-minute intervals from the chicken were taken to establish the baseline $^{13}C/^{12}C$ isotope ratio. At 9 am the chicken was

administered a gelatine capsule containing a weighed amount (approximately 95 mg) of vegetable oil containing ^{13}C-octanoic acid (37.8 µg/mg vegetable oil). That is, each chicken received approximately 3.6 mg ^{13}C-octanoic acid. The test chicken was denied feed until completion of breath sampling at 1 pm. The other chicken in the cage was given feed immediately. Breath samples were taken at 10-minute intervals for one hour, followed by samples at 15-minute intervals over the next hour, and then samples at 30-minute intervals for another two hours. After the breath sample was taken the helmet was removed and the chicken was returned to the metabolism cage. Care was taken during handling and breath sampling to minimise any variability associated with stress on the chickens and/or disruption to normal breathing patterns. This set of procedures was repeated on subsequent days using a different chicken each day. Data were obtained from four chickens.

b. $^{13}CO_2$ in expired breath following ingestion of corn.

At 25 days of age, one chicken was fasted from 5 pm to 9 am the following day. Following overnight fast, three breath samples taken at 3-minute intervals from the chicken were taken to establish the baseline $^{13}C/^{12}C$ isotope ratio. At 9 am the chicken was administered with 10 mL of homogenised corn kernel (Edgel brand; high in naturally-occurring ^{13}C labelled starch) via a disposable syringe fitted with a plastic tube that was inserted 4 cm into the oesophagus. Breath samples were taken at 15-minute intervals for two hours followed by samples at 30-minute intervals for another two hours. This set of procedures was repeated on subsequent days using a different chicken each day. Data were obtained from a total of three chickens. It was necessary to resort to use of the feeding tube because chickens refused to eat the corn.

Results and discussion

Enrichment of ^{13}C in breath CO_2 (defined as delta over baseline, the increase in the ratio of ^{13}C to ^{12}C relative to the baseline determined for each chicken) following ingestion of ^{13}C-octanoic acid is shown in Figure 14. The results are consistent with observations in humans and experimental mammals in which ^{13}C-octanoic acid is rapidly absorbed in the intestine, metabolised, then excreted by the lungs (Tivey and Butler 1999). Peak enrichment in chickens between 5 and 30 minutes is comparable with mice (Symonds *et al.* 1998) and considerably less than the 53 minute delay observed in adult humans given a

semi-solid test meal containing sodium [$^{13}C_1$]-acetate to measure emptying of the liquid phase (Braden *et al.* 1995). Presumably, these different intervals of time to reach peak enrichment reflect the relative lengths of the GIT in mice, chickens and adult humans. The flattened, delayed peaks for two chickens (Figure 14) might indicate true variation between birds in terms of gastric emptying time because no problems occurred during administration of the isotope or serial breath sampling.

The enrichment of ^{13}C in breath following ingestion of ^{13}C-starch in corn is shown in Figure 15. Peaks were observed 60-90 minutes post-ingestion. This was much quicker than the mean time of 153 minutes observed in human infants 7-16 months of age with mean weight of 8.6 kg given a test meal made from maize flour (Weaver *et al.* 1995). Hiele *et al.* (1989) reported peaks in $^{13}CO_2$ excretion at 3 and 5 hours in healthy volunteers and patients with pancreatic disease, respectively, following consumption of a test meal made from corn starch suspended in water. As with the results in Figure 14, the main difference between chickens and humans was the shorter time to peak enrichment of ^{13}C in breath CO_2 in chickens.

The degree of enrichment of $^{13}CO_2$ in breath samples from chickens in both experiments was similar to those found with humans and other experimental animals dosed with these labelled substrates. The smooth transitions in Figure 14 and Figure 15 from zero enrichment to peaks and subsequent declines to baseline imply that the sampling procedures produced representative samples of expired breath.

Conclusion

Preliminary experiments using simple PVC helmets to collect breath from individual birds provided proof of the concept of using $^{13}CO_2$ breath tests as non-invasive tools for studying gut physiology in broiler chickens.

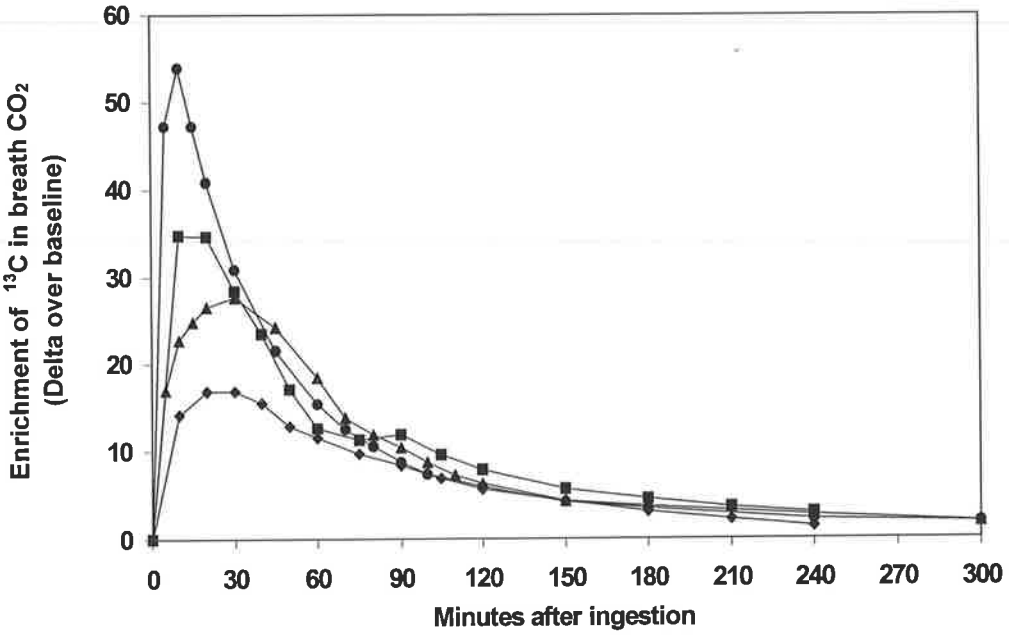


Figure 14. Enrichment of $^{13}CO_2$ in breath following ingestion of a gelatine capsule containing 3.6-3.8 mg ^{13}C-octanoic acid dissolved in vegetable oil. Delta over baseline is the increase in the ratio of ^{13}C to ^{12}C relative to the baseline ratio in each chicken prior to dosing. Each of the four curves represents results from an individual chicken.

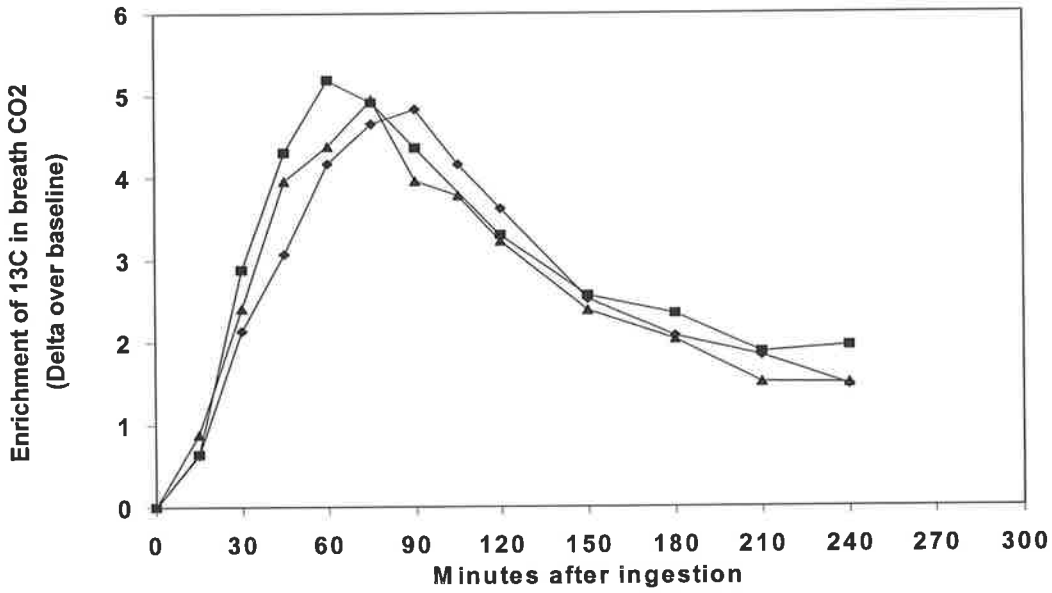


Figure 15. Enrichment of $^{13}CO_2$ in breath following ingestion of cooked corn kernel naturally enriched with ^{13}C-starch. Each of the three curves represents results from an individual chicken.

4.3 Measurement of hydrogen and methane in breath samples from chickens

4.3.1 Introduction

Breath tests based on release of hydrogen and methane are used in medical practice (Robb and Davidson 1987). These tests rely on production of hydrogen by bacterial fermentation of undigested carbohydrate and other nutrients in the large bowel, as hydrogen and methane are not produced by mammalian tissue. A preliminary experiment was conducted with chickens to see whether the commensal gut microflora included bacteria able to synthesise hydrogen and methane to the extent that these gases would emerge in measurable concentrations in breath samples. This experiment tested the hypotheses (a) that hydrogen and/or methane will appear in the breath of chickens given a normal diet, and (b) that an oral dose of lactulose, a synthetic disaccharide used in diagnostic tests in human subjects, would cause an increase in hydrogen concentration in breath of chickens.

4.3.2 Materials and methods

Birds, housing and management

Ross broiler chickens were purchased from the Bartter Steggles hatchery, Cavan, SA on Thursday 15 July 1999. These chickens were reared to 22 days of age in a floor pen in a controlled temperature room. All birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540) to 19 days of age then commercial finisher pellets (Ridley Agriproducts, diet code #504540).

At 22 days of age, 16 chickens (8 male and 8 female) were transferred in pairs to metabolism cages in a room maintained at 24-26°C. All birds continued to receive commercial finisher pellets. Birds had free access to feed when not involved in experimental procedures. Drinking water was available at all times. Excreta trays were cleaned daily. Room temperature was adjusted as required to suit the comfort of the chickens. At 25 days of age, one chicken was removed from each cage and placed alone in another cage, then all chickens were weighed. Chickens continued to have access to feed and water prior to test procedures described in the following sections.

Experimental procedures

Hydrogen and methane in expired air from chickens given a normal diet

Prior to the experiment, Exetainer tubes were opened and flushed with air to remove gaseous hydrocarbons generated in the sterilisation process, then re-evacuated by removal of air via needle and syringe. When chickens were 25 days of age, two 10 mL breath samples were taken from 12 chickens (6 male and 6 female). Chickens were not fasted beforehand. Each breath sample was taken 30 seconds after a helmet (40 mm PVC tube and cap) was placed over the head of the chicken. The second breath sample was taken within 2 - 3 minutes of the first sample, and then the chicken was returned to its metabolism cage for further tests described in the section below. Care was taken during handling and breath sampling to minimise any variability associated with stress on the chickens and/or disruption to normal breathing patterns.

Breath samples were kept cool (0 - 4°C) to minimise gas diffusion during transport and storage prior to analysis. Hydrogen and methane concentrations in breath were measured by gas chromatography (QuinTron Microlyzer).

Hydrogen and methane in expired air following ingestion of lactulose

The chickens were fasted overnight from 5 pm on day 26. Commencing at 8.30 am the following day (27 days of age), each chicken was weighed and breath tested (two 10 mL samples as per the procedures described in the section above) to establish the pre-dosing baseline levels of hydrogen and methane. Each chicken was then administered with 5 mL of diluted lactulose solution via a disposable syringe fitted with a plastic tube that was inserted 4 cm into the oesophagus. A total of 12 chickens (6 male and 6 female) were given approximately 130 mg lactulose in 5 mL water while the other four chickens were given double the dose (260 mg lactulose), or quadruple the dose (520 mg lactulose), in 5 mL water. The lower dose rate (0.1 g carbohydrate per 1 kg body weight) was equivalent to that given to human subjects to assess carbohydrate malabsorption. The higher dose rates (0.2 g/kg and 0.4 g/kg) were used to determine whether carbohydrate loading needed to be higher to achieve measurable levels of hydrogen or methane in expired breath. The lactulose solutions were made from Duphalac syrup (Solvay-Duphar B.V., Holland; 3.34 g lactulose/5 mL) diluted in deionised water.

Each chicken was breath tested (two 10 mL samples) three hours post-dosing with lactulose. The choice of timing was based on a combination of extrapolation from results of a previous experiment with chickens, where the time taken for peak $^{13}CO_2$ output in expired breath was approximately 60 – 90 minutes post-feeding of homogenised corn kernel, and by comparison of results from experiments with rats (Ross Templeman, personal communication).

4.3.3 Results and discussion

There was large between-bird variability in hydrogen (Figure 16) and methane (Figure 17) concentrations (in ppm) in breath samples from non-fasted chickens given a commercial diet, and the same chickens two days later just before and 3 hours after dosing with lactulose. Means, standard deviations (SD), coefficients of variation (CV) and ranges of concentrations of hydrogen and methane in breath samples are summarised in Table 10.

Table 10. Means, standard deviations (SD), coefficients of variation (CV) and ranges of concentrations of hydrogen and methane in breath samples from 12 chickens.

	Mean	SD	CV	Range
		Hydrogen (ppm)		
Fully fed	34.3	37.7	110	7.0 - 115.0
Fasted and before dosing with lactulose	3.6	2.2	61	0.5 - 7.5
Fasted and 3 h after dosing with lactulose	11.3	15.5	136	1.0 - 56.5
		Methane (ppm)		
Fully fed	0.7	0.6	82	0 – 5.5
Fasted and before dosing with lactulose	1.8	1.1	61	0 – 3.0
Fasted and 3 h after dosing with lactulose	1.9	2.3	119	0 – 8.0

In 9 out of 12 chickens studied, there was an increase in hydrogen concentration in the 3-hour period following dosing with lactulose. There was no change in one chicken (number 4 in Figure 16) and the other two chickens (numbers 6 and 7 in Figure 16) showed a decline. Overnight fasting resulted in a reduction in between-bird variability, presumably because of the reduced amount of fermentable material reaching the hindgut.

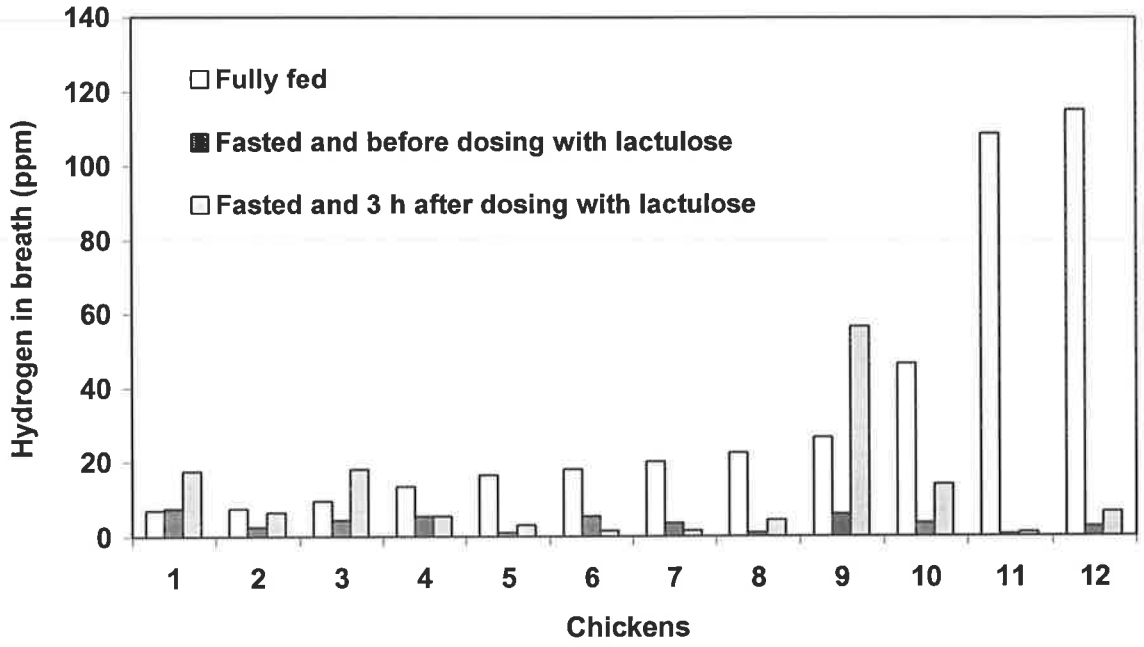


Figure 16. Breath hydrogen concentration (in ppm) in chickens fed *ad libitum* and then two days later from the same chickens fasted overnight immediately before and 3 h after dosing with lactulose (130 mg in 5mL water)

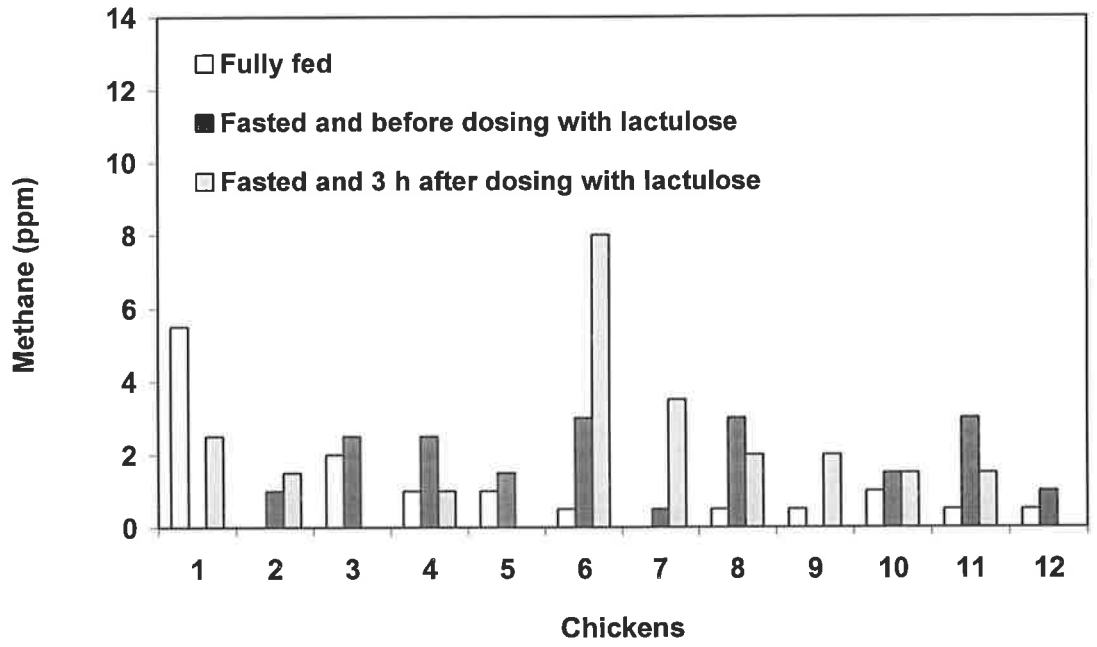


Figure 17. Breath methane concentration (in ppm) in chickens fed *ad libitum* and then two days later from the same chickens fasted overnight immediately before and 3 h after dosing with lactulose (130 mg in 5mL water)

Methane (up to 8 ppm) was detected in the breath of each of the 12 chickens at some stage during this experiment, either before or after dosing with lactulose.

These results imply that additional factors such as the rate of passage of digesta, proliferation of facultative anaerobes (Choct *et al.* 1996*a,b*), and combinations of these may have contributed to the large coefficients of variation in breath hydrogen and methane (Table 10). The hypotheses that digesta transit time and metabolic activity of gut microflora affect energy metabolism are developed in Chapter 5 and Chapter 6, respectively.

4.3.4 Conclusions

All chickens hosted microflora (presumably in the caeca) capable of producing hydrogen and methane from naturally occurring carbohydrate in the diet and a synthetic disaccharide (lactulose) administered orally. Fasting reduced the between-bird variability in breath hydrogen and methane concentrations.

4.4 Development of breath tests based on hydrogen for measuring transit time of digesta and metabolic activity of gut microflora in chickens

4.4.1 Introduction

Breath tests fall into two broad purposes of use; these are (a) the examination of some particular aspect of the metabolic function of host tissue, and (b) the examination of the metabolic activity of the gut microflora. Results of initial experiments (described in sections 4.2 and 4.3) suggest that breath tests involving labelled and non-labelled substrates have potential as analytical tools in broiler nutrition.

Breath hydrogen measurement is used as an indicator of carbohydrate malabsorption in humans and is used for estimating the rate of passage of digesta through the small intestine. These breath tests, used routinely in medical practice, are based on release of hydrogen following microbial fermentation of carbohydrates such as lactulose, which is a synthetic disaccharide not absorbed in the small intestine (Wutzke *et al.* 1997).

Preliminary experiments described in section 4.3 indicated that all of the chickens hosted a microflora able to produce hydrogen and methane from fermentation of undigested carbohydrate but the results were variable. This experiment tested the hypotheses that precision of the hydrogen breath test during serial collections from chickens would be increased by (a) priming the gut microflora with a prior dose of lactulose as a source of fermentable substrate, and (b) fasting the chickens prior to test dosing with lactulose.

4.4.2 Materials and methods

Birds, housing and management

Ross broiler chickens were obtained from the Bartter Steggles hatchery, Cavan, SA on Thursday 8 March 2001. Chickens were raised from hatch in two rearing pens in a controlled temperature room. Male and female chickens were reared separately. All birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540).

At 20 days of age, 24 male chickens were transferred in pairs to 12 metabolism cages located in a controlled-temperature room maintained set at 24-26°C, and given finisher pellets (Ridley Agriproducts, diet code #504540) for the duration of this experiment. Birds had free access to feed and water during the experimental period except for a 3-hour period of fasting immediately before dosing with lactulose. At 22 days of age, one chicken was removed from each cage. Commencing at 25 days of age, four chickens entered the testing cycle that was run over two days. This cycle was repeated twice commencing on subsequent days with a separate set of four chickens entering each 2-day cycle of tests to give a total of 12 chickens in the experiment.

In each 2-day cycle, two fully-fed chickens (primed group) were given a dose of lactulose at 11 am on the day prior to serial breath testing. Each chicken was dosed with approximately 130 mg lactulose in 5 mL of water administered via a disposable syringe fitted with a soft plastic tube that will be inserted 4 cm into the oesophagus. Two other chickens were not dosed at that point. On day 2, two chickens (one prime-dosed and one not dosed) were fasted from 8 am. The other two chickens (also one prime-dosed and one not dosed) had free access to feed. These became the non-fasted group. Commencing at 11 am on day 2, all four chickens were test-dosed with approximately 130 mg lactulose in 5 mL of water.

Sampling of breath

Serial breath testing of each chicken commenced immediately before test dosing at 11 am then at 60, 90, 105, 120, 135, 150, 165, 180, 210, 240 and 300 minutes thereafter. A 50 mL gas sample from the headspace was taken 15 seconds after a helmet was placed over the head of the chicken. Then 10 mL aliquots were transferred to Exetainer tubes for determination of hydrogen and methane concentrations. Breath samples were analysed for hydrogen and methane (QuinTron MicroLyzerl).

Oro-caecal transit time was taken as the time elapsed in minutes from oral dosing until the first of the serial samples showing a consecutive rise in breath hydrogen.

4.4.3 Results and discussion

All chickens showed an increase in breath hydrogen after test dosing with lactulose whether or not they received a priming dose on the previous day (Figure 18 and Figure 19). The size of the standard deviation for treated birds (primed or fasted) expressed as a percentage of the standard deviation for untreated birds (not primed or not fasted) was used to assess whether there was any change in variation. The numbers shown in italics in Figure 18 and Figure 19 represent the relative sizes of the standard deviations at discrete times after test dosing. Fasting prior to test dosing appeared to reduce variation in hydrogen concentration between chickens at each point in time after test dosing. On the other hand, priming appeared to increase variation in the period 90 to 150 minutes, and at 210 minutes after test dosing.

Results of serial breath sampling of fasted chickens are shown in Figure 20. Estimates of oro-caecal transit time for lactulose following fasting ranged from 165 mins for chicken E, 180 mins for chickens B and F, to 210 mins for chickens A, C and D. The mean and standard error for transit time were 193 ± 8 mins. The above estimate is likely to be biased upwards because the interval between serial breath samples was 15 minutes up to 180 minutes post dosing, then 30 minutes thereafter. That is, mean transit time occurred after the period of frequent sampling.

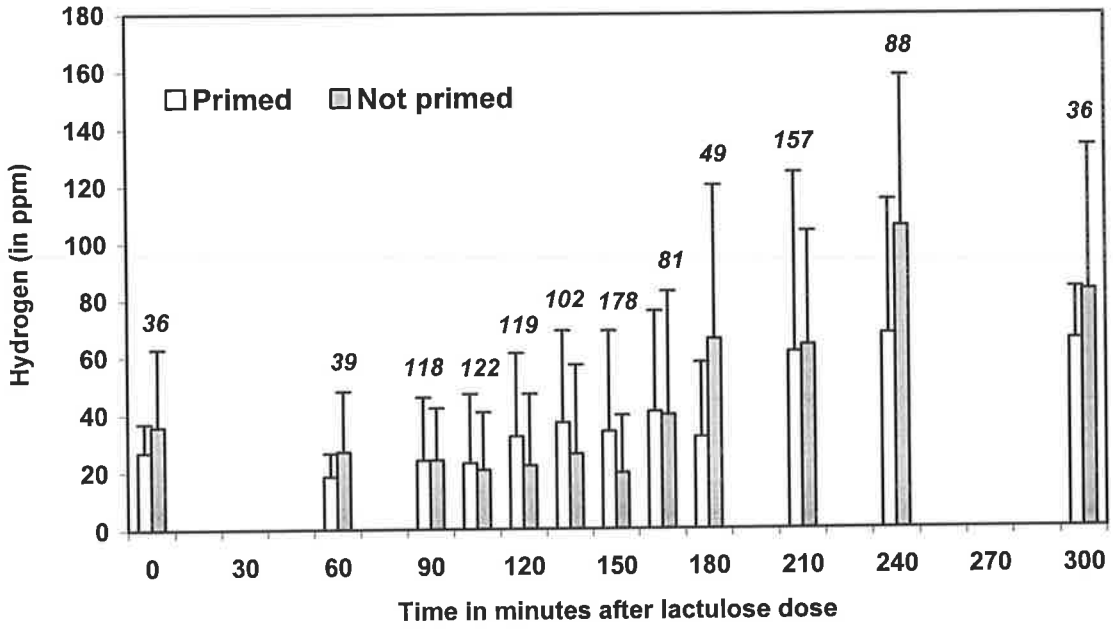


Figure 18. Effects of a priming dose of lactulose (130 mg in 5 mL water) on the previous day on hydrogen concentration in breath after test dosing with lactulose (130 mg in 5 mL water) (means ± SD; n = 6). The numbers in italics show the SD for the primed birds expressed as a percentage of the SD for untreated birds.

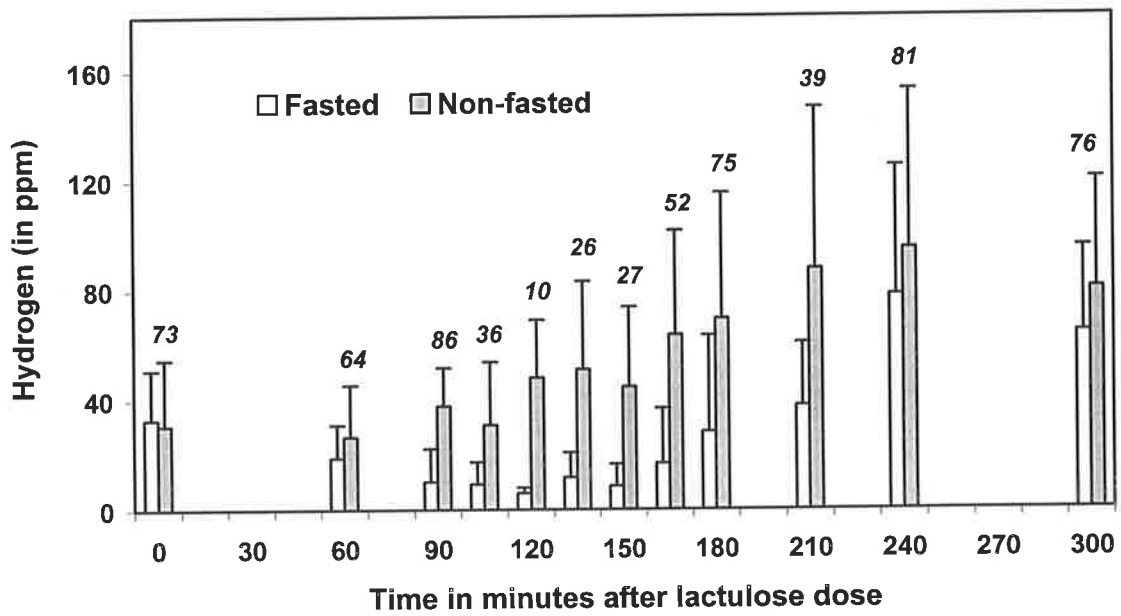


Figure 19. Effects of fasting prior to test dosing with lactulose (130 mg in 5 mL water) on hydrogen concentration in breath (means ± SD; n = 6). The numbers in italics show the SD for the fasted birds expressed as a percentage of the SD for non-fasted birds.

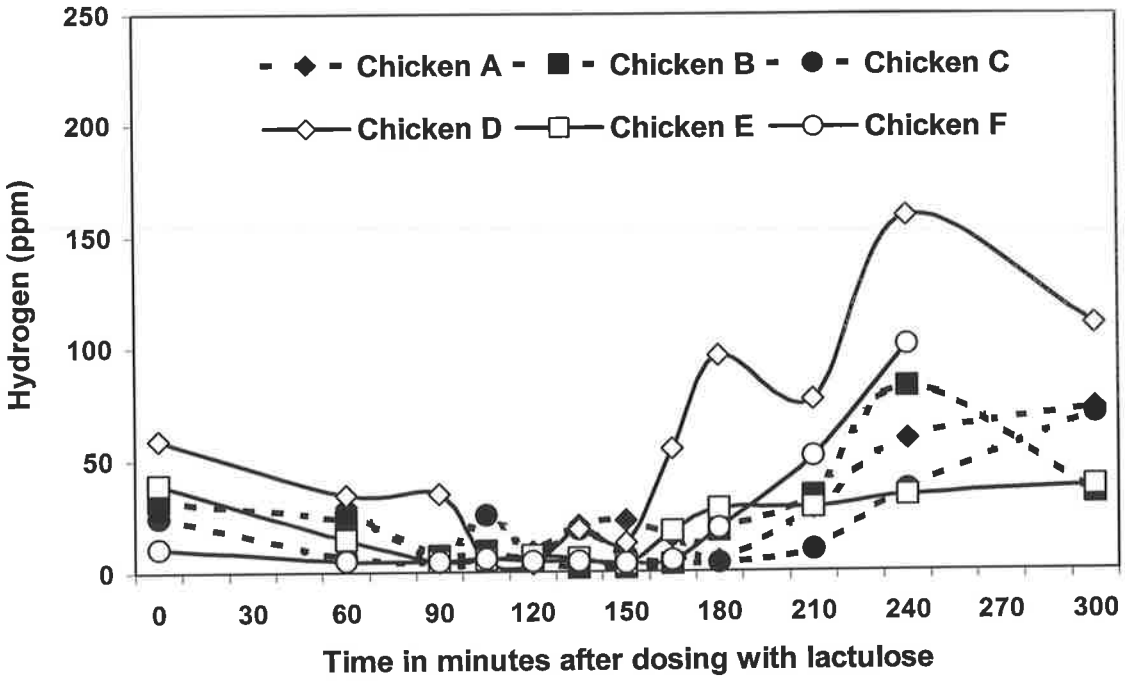


Figure 20. Breath hydrogen concentration (in ppm) in male chickens fasted for 3 h prior to test dosing with lactulose (130 mg in 5mL water).

The hydrogen profile for chicken D with two peaks in hydrogen concentration at 180 and 240 minutes (Figure 20) is very similar in appearance to breath profiles observed in rats and humans with small bowel bacterial overgrowth (proliferation of facultative anaerobes in the small intestine). Choct *et al.* (1996*a*) observed microbial proliferation in the small intestine of chickens associated with an increase in digesta transit time as a result of the gelling properties of soluble non-starch polysaccharides in wheat.

4.4.4 Conclusions

Prior dosing with the synthetic disaccharide lactulose was not necessary, as all chickens appeared to contain hindgut microflora immediately able to utilise lactulose passing out of the small intestine. Fasting for three hours prior to dosing reduced between-bird variability in breath hydrogen concentration. Oro-caecal transit time was 180 minutes (*SE*=8 minutes, *n*=6).

4.5 General discussion and conclusions

Preliminary experiments using simple PVC helmets to collect breath from individual birds provided proof of the concept of using breath tests as non-invasive tools for study of the gastroenterology of broiler chickens.

The patterns of recovery of the stable isotope in the form of $^{13}CO_2$ in breath samples were comparable with those seen in humans, with the exception of the shorter time delay to peak enrichment in breath compared with adult humans. This is not surprising given the shortness of the GIT relative to body weight in chickens compared with mammals (Hill 1971), more rapid transit of digesta in chickens than mammals (Vergara *et al.* 1989), and the higher metabolic rate of chickens compared with adult humans, as indicated by average body temperatures of 42° and 37°C, respectively. The similar recovery patterns suggest that there are no fundamental differences between avian and mammalian species in terms of basic physiological and biochemical processes under-pinning $^{13}CO_2$ breath tests for gastric emptying and pancreatic function. Likewise, the appearance of measurable concentrations of hydrogen in breath collected from chickens, and the comparability of the breath profiles over time with that reported in other animals, indicate the potential for development of breath tests to aid in the diagnosis of maldigestion, malabsorption and dysbacteriosis in commercial flocks.

The hydrogen breath test is an inexpensive, non-invasive way of assessing the overall metabolic activity of the gut microflora without the need to administer additional indigestible carbohydrate to individual chickens. It provides a simple way of tracking changes in metabolic activity of microflora in experimental animals prior to, during and after administration of different dietary treatments and other therapies likely to alter the profile of gut microflora.

Whether or not dosing with lactulose is necessary for accurate and precise measurements of digesta transit time remains to be determined.

Chapter 5. Effects of rate of passage of digesta on the digestion of energy in chickens

5.1 General introduction

Genetic selection for rapid growth and increased feed efficiency over many generations has resulted in the modern broiler chicken having a relatively greater capacity to eat than other breeds of domestic fowl such as egg layer strains (Mahagna and Nir 1996). As the relative differences between broilers and layers in intestinal weight (expressed in g/kg live weight) and length (Mitchell and Smith 1991; Nir *et al.* 1993) are small, it follows that digesta transit time in broilers is shorter than that in layers. Mitchell and Smith (1991) also pointed out that selection for rapid growth rate and increased feed conversion efficiency in domestic fowls was associated with a reduction in the relative mass (in g/kg live weight) of the small intestinal mucosa but, in contrast, absorptive capacity was improved to compensate.

Iskander and Pym (1987) reported that broiler chickens selected for improved feed efficiency had a slower digesta transit time and improved retention of dietary energy and protein compared with broiler chickens selected for increased feed intake. Hence it is possible that the different selection objectives and strategies used over the years to produce the current commercial lines of broiler chickens may well have produced differences between the lines in transit time, and consequently in energy metabolism. There is a lack of published data on the variation that may exist between strains and between individual birds within a strain in the relationship between rate of passage of digesta and digestion of energy. If large differences exist, it would help explain, at least in part, the wide between-bird variability in AME observed by Hughes and Choct (1997). Similarly, there may be differences between males and females in transit time, as well as in gut structure, as was reported in Chapters 2 and 3.

Results of experiments described in Chapter 4 suggest that it is possible to use breath hydrogen to measure the time taken for ingested food to pass from the mouth through the

small intestine into the caeca where undigested carbohydrate is fermented by bacteria to produce hydrogen (oro-caecal transit time).

Two experiments are described in this chapter. The first experiment was designed to validate the use of breath testing as a non-invasive measure of rate of passage of digesta, and the second experiment examined whether rate of passage had any effect on energy metabolism. Both experiments took into account the possibility that transit time differed between the sexes, in an attempt to explain some of the sex-related difference in AME observed in Chapter 3.

5.2 Relationship between oro-caecal transit time and whole tract transit time

5.2.1 Introduction

Whole tract transit time is often measured by serial collection of excreta samples for detection of non-digestible dietary markers that are not normally found in poultry diets in high concentrations. Iskander and Pym (1987) described one such method that involved dosing chickens with ferric oxide (200 mg/kg liveweight) enclosed in a gelatine capsule. They reported mean transit times ranging from 132 to 166 minutes in four selection lines of chickens. This technique has the advantages that a precise starting point in time can be established for individual chickens, it is not necessary to prepare special diets, and does not require serial collection and analysis of excreta to determine the presence or otherwise of non-digestible markers such as chromium oxide (Almirall and Esteve-Garcia 1994), titanium oxide (Hetland and Svihus 2001), or long-chain hydrocarbon (Choct *et al.* 1995). Preliminary trials at PPPI (R.J. Hughes, unpublished data) with ferric oxide indicated that it was always voided with faecal matter and never with material evacuated from the caeca. The easily observed characteristic red appearance usually showed in one or two consecutive faecal dropping only, which suggests that the ferric oxide moved down the tract in a bolus and bypassed the caeca. This is consistent with the report of Vergara *et al.* (1989) that only liquids and soluble compounds enter the caeca in significant amounts. That is, the termination time for passage through the tract could be determined precisely by simply increasing the frequency of observations of faecal droppings after about 2 hours following dosage. Furthermore, the emergence of ferric oxide was assumed to coincide

with the time of arrival of undigested carbohydrate in the caeca of previously fasted animals to replenish the supply of fermentable substrate for the gut microflora.

This experiment tested the hypotheses that (a) oro-caecal transit time (OCTT) determined by rise in breath hydrogen and whole tract transit time (WTTT) determined by appearance of ferric oxide in excreta were similar in duration, and (b) estimates of transit time measured by either method differed between males and females.

5.2.2 Materials and methods

Birds, housing and management

Ross broiler chickens obtained from the Bartter Steggles hatchery, Cavan, SA on Friday 11 May 2001 were raised from hatch in two rearing pens in a controlled temperature room. Male and female chickens were reared separately. All birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540).

At 14 days of age, a total of 32 chickens were transferred in single-sex pairs to 16 metabolism cages (with males and females in alternate cages) located in a controlled-temperature room kept at 25-27°C initially, and given starter crumbles for the duration of this experiment. The temperature setting in the room was reduced daily until it was 22°C at the end of the experiment.

At 17 days of age, one chicken was removed from each cage, leaving a total of 16 chickens for this experiment. Birds had free access to feed and water during the experimental period except for a 3-hour period of fasting immediately before dosing with lactulose. The results presented in Chapter 4, which suggested that it may not be necessary to administer additional non-digestible carbohydrate, were not available when the current experiment commenced, hence it was thought that lactulose was needed to produce sufficient hydrogen for measurement in breath.

Commencing at 18 days of age, eight chickens of the 16 chickens were fasted for three hours from 8 am. At 11 am, all chickens were administered with a gelatine capsule containing ferric oxide (Fe_2O_3, 200 mg/kg liveweight) as described by Iskander and Pym (1987). Six of the eight chickens were dosed also with approximately 130 mg lactulose in 5 mL of water administered via a disposable syringe fitted with a plastic tube that was

inserted 4 cm into the oesophagus. The two chickens not dosed with lactulose (controls) provided a measure of base-line variation in hydrogen production from undigested carbohydrate by gut microflora. These procedures were repeated on day 19 with the remaining seven chickens (one female chicken died prior to testing). No chicken was fasted or dosed with lactulose and ferric oxide more than once.

Estimation of whole-tract transit time and oro-caecal transit time

Excreta trays were examined at 5-minute intervals or more frequently for signs of ferric oxide in voided droppings. Whole-tract transit time for each chicken was taken as the time elapsed (in minutes) from time of administration of ferric oxide in a gelatine capsule to time of first observation of the distinctive red colouration in droppings. Oro-caecal transit time was taken as the time elapsed in minutes from oral dosing until the first of two or more consecutive samples showing a numerical rise in breath hydrogen.

Sampling of breath

Serial breath testing of each chicken commenced immediately before test-dosing at 11 am then at 120, 150, 165, 180, 195, 210 and 240 minutes thereafter. A 50 mL gas sample from the headspace was taken 15 seconds after a helmet (50 mm diameter) was placed over the head of the chicken. The gas sample was drawn into a pre-evacuated 60 mL syringe attached to the helmet. Then 10 mL aliquots were transferred to two Exetainer tubes. Care was taken during handling and breath sampling to minimise any variability associated with stress on the chickens and/or disruption to normal breathing patterns. Breath samples were analysed for hydrogen and methane (QuinTron MicroLyzer).

Statistics

Wilcoxon's test was used to determine whether the data were normally distributed. The difference (TTDIFF, in minutes) between WTTT and OCTT was calculated for each chicken. WTTT and TTDIFF data required log $_e$ transformation to normalise the distribution prior to further analysis. OCTT data were also log tranformed for comparative purposes. Analysis of variance (by GLM procedure) was used to examine the effects of sex, dosing with the lactulose, and the sex by dosing interaction on transit time. The combined effects of sex and dosing on transit times were examined by the LSMEANS

procedure. The relationship between log transformed WTTT and OCTT was examined by regression analysis.

5.2.3 Results

Sex of the chicken and dosing with lactulose had no effect ($P>0.05$) on WTTT, OCTT or TTDIFF, nor was the sex by dosing interaction significant ($P>0.05$). These results are summarised in Table 11. Breath hydrogen profiles for individual male and female chickens dosed with lactulose are shown in Figure 21 and Figure 22, respectively. Breath hydrogen concentrations at various times after dosing with lactulose are shown separately for male and female chickens in Figure 23.

Methane was not detected in the breath from any chicken at any stage, which is in contrast with results presented in section 4.2.2, and in other experiments described later in this thesis.

Table 11. Oro-caecal transit time (OCTT in minutes), whole tract transit time (WTTT in minutes), and the difference (TTDIFF in minutes) between WTTT and OCTT in male and female chickens dosed with lactulose (130 mg in 5 mL water) or not dosed after a 3 hour fast.

			OCTT		WTTT		TTDIFF	
Lactulose	Sex	n	Mean	SE	Mean	SE	Mean	SE
Dosed	Female	5	166	10.6	184	19.1	18	17.2
Dosed	Male	6	165	9.7	164	17.4	-1	15.7
Control	Female	2	150	16.8	177	30.2	27	27.2
Control	Male	2	163	16.8	168	30.2	5	27.2
	Overall	15	163	5.6	173	10.0	10	9.2

Results in Table 11 are expressed as untransformed data for simplicity. Statistical analyses were done on log transformed data. The mean difference of 10 minutes between OCTT and WTTT was not significant ($P>0.05$).

One female chicken (shown as J in Figure 22) had a long WTTT (285 minutes) in comparison with other chickens. When the data for chicken J were omitted, WTTT was 158 ± 13.4 minutes and TTDIFF was -8 ± 9.0 minutes for the remaining four female

chickens dosed with lactulose. OCTT was unchanged (166 minutes). The overall mean values for WTTT and TTDIFF then became 165 ± 6.5 minutes, and 2 ± 5.2 minutes, respectively, and the effects of sex and dosing remained non-significant (P>0.05). That is, the removal or inclusion of data for chicken J did not affect the outcome of statistical analysis.

The linear relationship between log transformed WTTT and OCTT was not significant (P=0.06, R^2=0.25). On removal of data for chicken J as mentioned above, the relationship became statistically significant (P<0.01), but remained weak (R^2=0.45).

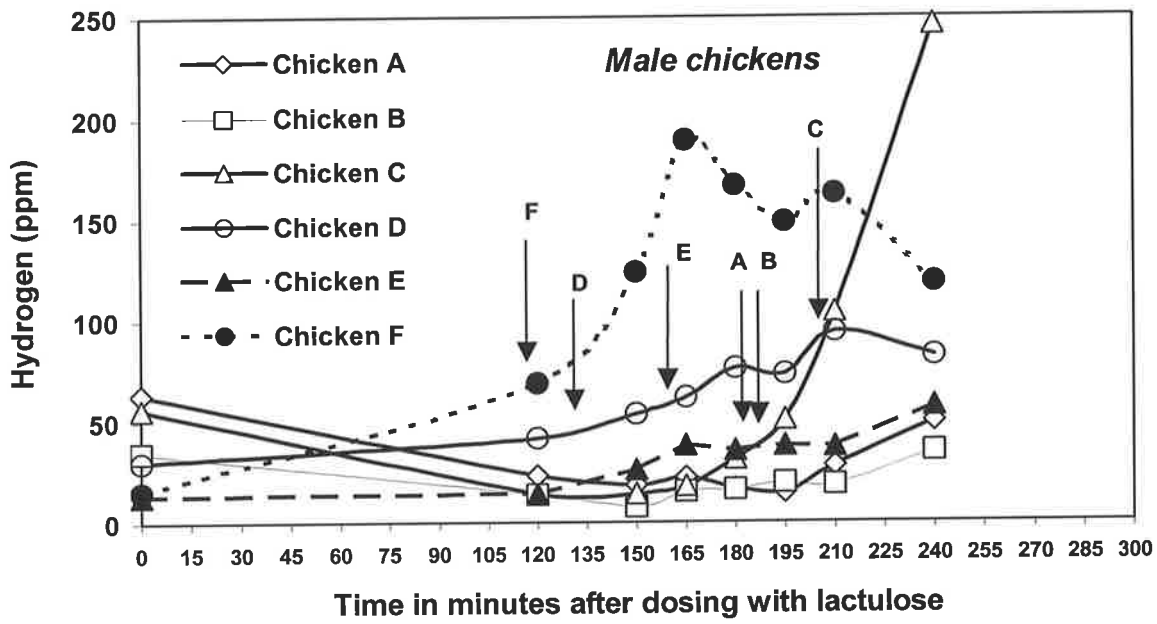


Figure 21. Breath hydrogen concentration (in ppm) in male chickens (n = 6) fasted for 3 h prior to test dosing with lactulose (130 mg in 5mL water). The vertical arrows indicate whole tract transit time for ferric oxide marker.

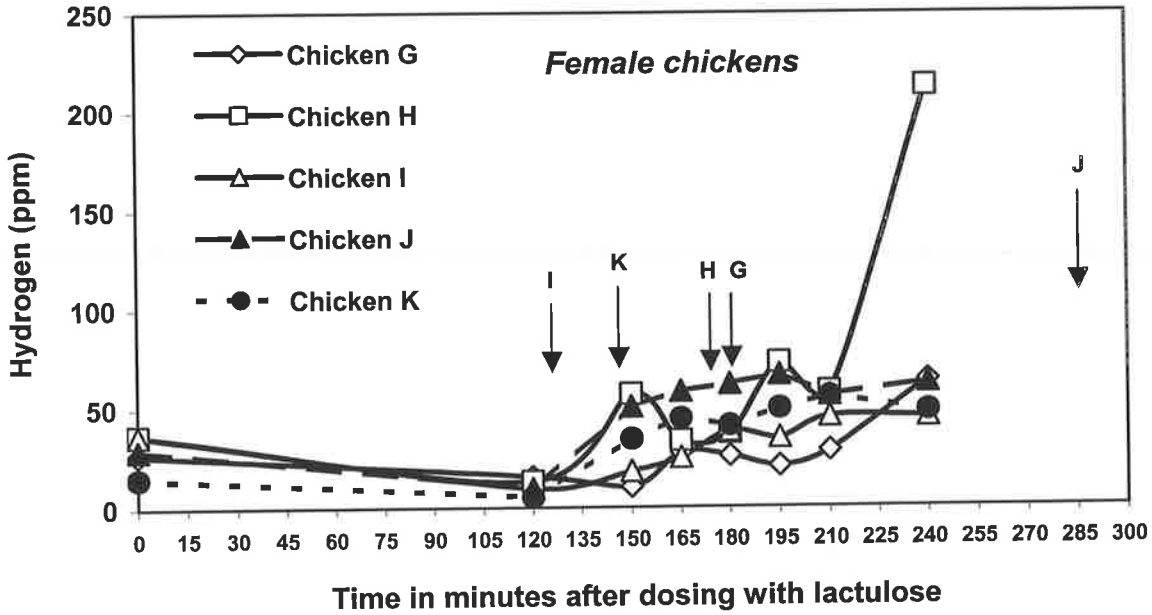


Figure 22. Breath hydrogen concentration (in ppm) in female chickens (n = 5) fasted for 3 h prior to test dosing with lactulose (130 mg in 5mL water). The vertical arrows indicate whole tract transit time for ferric oxide marker.

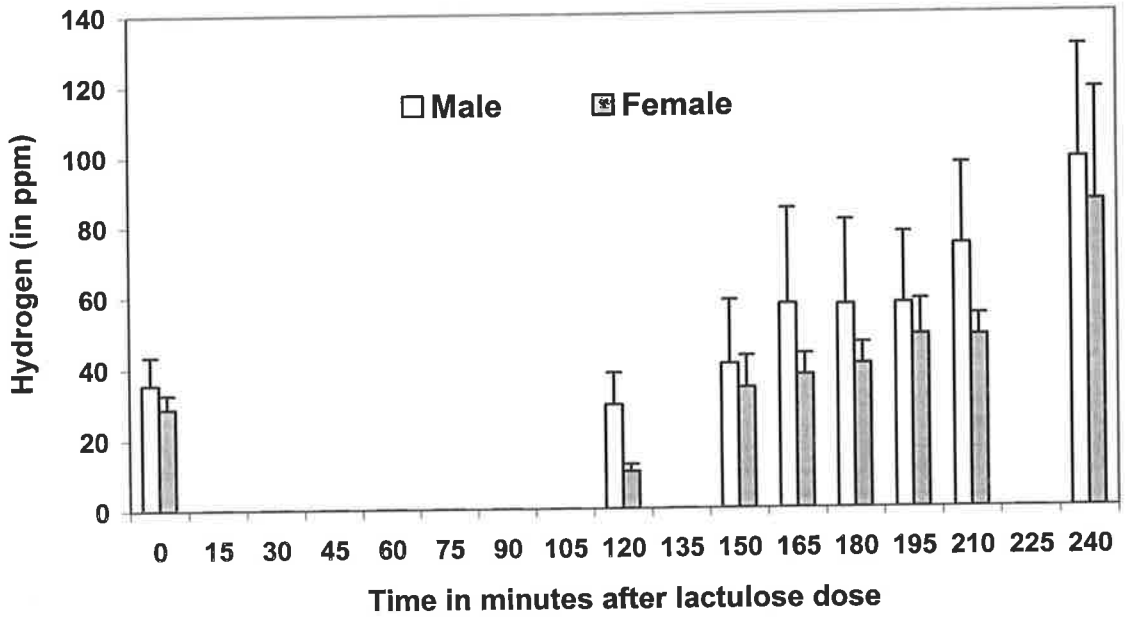


Figure 23. Concentrations of hydrogen in breath of male (n=6) and female chickens (n=5) at various times after dosing with lactulose (means ± SE).

5.2.4 Discussion

Oro-caecal transit time (mean ± SE) was 163 ± 5.6 minutes and whole tract transit time was 173 ± 10.4 minutes. Neither measurement was affected by the sex of the chicken or by dosing with lactulose. The lack of effect from dosing indicates that it is possible to measure OCTT by breath analysis without the need to add indigestible carbohydrate to the diet, which supports the conclusion reached in Chapter 4.

WTTT appeared to be approximately 10 minutes longer than OCTT (Table 11), but that mean difference was not significant and became negligible when the results for chicken J (Figure 22) were omitted. In fact, for 6 out of 15 individual chickens, whole tract transit time was 4 to 28 minutes shorter than oro-caecal transit time, whereas it might be expected that whole tract transit time would be slightly longer, assuming that the passage of ferric oxide was not delayed by entering the caeca. A possible explanation for this anomalous observation is that there was a lag phase between commencement of bacterial fermentation of substrate on arrival of digesta in the caeca and emergence of hydrogen in breath.

Hence, on the basis of these results, the estimates of OCTT given by rise in breath hydrogen and WTTT by excretion of ferric oxide can be regarded as comparable. However, neither estimate of transit time differed between male and female chickens.

There was a significant but weak positive correlation between oro-caecal transit time determined by rise in breath hydrogen and whole tract transit time measured by appearance of ferric oxide marker in excreta. The possibility that ferric oxide and lactulose moved through the tract in a manner atypical of normal chyme cannot be dismissed. In humans, lactulose acts as laxative but this was not indicated in this experiment as chickens produced droppings with normal appearance. Presumably then, transit time was not shortened by any laxative properties of lactulose. However, ferric oxide may have irritated the gut, thereby resulting in a shortened period of residence. In contrast, the delay in excretion of ferric oxide by one female chicken in particular may have arisen from the reason noted by Tuckey *et al.* (1958) that the rate of passage of ferric oxide can be retarded if birds become agitated during the experiment. This seems unlikely here as none of the birds seemed agitated during the breath collection period. However, the mean value for all other birds (165 minutes) was at the high end of the range (138 to 162 minutes) reported by Tuckey *et al.* (1958) and the range (132 to 166 minutes) reported by Iskander and Pym (1987), but

within the range (111 to 190 minutes) reported by Golian and Polin (1984) for 3-week old chickens fed diets without added fat. If there was a delay in WTTT due to agitation or stress in the present study, then it was reflected in OCTT also. The implication is that the total times for passage of ferric oxide and lactulose were similarly affected by peristaltic movements in the gut.

The hydrogen profile for one female chicken (represented as H in Figure 22) showed two peaks in hydrogen concentration (at 150 and 195 minutes), a pattern which is similar to that seen in humans with small bowel bacterial overgrowth (R.N. Butler, personal communication). It is also consistent with proliferation of facultative anaerobes in the small intestine as noted by Choct *et al.* (1996*a*) in chickens fed a diet with high soluble NSP content. The first peak occurred well before appearance of ferric oxide in excreta.

The results shown in Figure 21 to Figure 23 suggest that the output of hydrogen from males exceeded that of females. It may mean that the overall metabolic activities of gut microflora differed between males and females, and also between individual chickens irrespective of gender. This could help explain why no chicken in this study had a measurable concentration of methane at any stage, and also why breath hydrogen concentrations tended to be higher, in contrast to the results presented in section 4.3. The relative importance of gut microflora activity is discussed in the next chapter.

5.2.5 Conclusions

The rate of passage of digesta appears not to be the reason for differences between males and females in the digestion of energy. However, differences between males and females in breath hydrogen concentrations point to sex-dependent differences in fermentation patterns in the hindgut.

A non-invasive breath test for estimation of oro-caecal transit time can be devised without the need to dose chickens with lactulose. That is, caecal microflora can produce measurable concentrations of hydrogen in breath by fermentation of existing levels of complex carbohydrates in typical broiler feed.

5.3 Relationship between whole tract transit time and AME value of wheat

5.3.1 Introduction

As pointed out in section 5.1, modern broilers have a prodigous capacity to eat (Mahagna and Nir 1996), a relatively short retention time for ingested food, and greater absorptive capacity (Mitchell and Smith 1991) compared with layers. Among broilers, a selection line with slower transit time retained more energy and protein than another line with faster transit time (Iskander and Pym 1987). Therefore, it can be hypothesised that slower transit times are associated with higher AME values in individual broiler chickens of the same strain given the same feed.

A relatively slow transit time, however, is not always associated with comparatively faster growth and more efficient feed conversion. For example, barley β-glucan slowed the rate of passage of digesta in chickens and depressed AME, whereas dietary addition of β-glucanase ameliorated the problem (Salih *et al.* 1991; Almirall and Esteve-Garcia 1994; Attia *et al.* 1996). The anti-nutritive effects of soluble NSP such as arabinoxylans in wheat and β-glucan in barley are associated with the formation of viscous gels in the lumen of the gastrointestinal tract which impedes the diffusion of ingested food particles, digestive enzymes and bile salts, and hinders the interaction between digested particles and absorption sites on the intestinal mucosa (Annison 1993*a*; Bedford and Morgan 1996; Smits *et al.* 1997). Hence, it is possible that soluble NSP in concentrations commonly found in broiler diets can slow the passage of digesta and reduce AME, thereby interfering with the relationship between transit time and energy retention.

A wheat-based diet with and without feed enzymes was chosen for this experiment in the expectation that individual chickens would respond in a variable manner to the gelling properties of soluble NSP and that enzymes would ameliorate any negative effects of the NSP. The wheat was grown at Narrabri under relatively warm, dry conditions, which were linked previously with occurrence of low AME wheats as a result of the raised concentration of soluble NSP and associated increase in digesta viscosity (Hughes and Choct 1999).

This experiment tested the hypotheses that (a) AME values and WTTT were related, and (b) the relationship between AME and WTTT differed between males and females.

The hypothesis in respect to gender differences was retained for this study because, despite the lack of evidence in section 5.2 to support this, it remained possible that a sex-related difference in transit time could help explain a difference between males and females in AME as was observed in Chapter 3.

5.3.2 Materials and methods

Birds, housing and management

Ross broiler chickens obtained from the Bartter Steggles hatchery, Cavan on Friday 11 May 2001 were raised from hatch in two rearing pens in a controlled temperature room. Male and female chickens were reared separately, and maintained on commercial starter crumbles (Ridley Agriproducts, diet code #503540).

At 17 days of age, a total of 32 chickens were transferred in single-sex pairs to 16 metabolism cages located in a controlled-temperature room set at 25-27°C initially, and given starter crumbles. The temperature setting in the room was reduced daily until it was 22°C at the end of the experiment. Birds had free access to feed and water prior to and during the experimental period. Chickens were placed one per cage in 24 cages for the experiment when they were 19 days old, and continued to receive the starter crumbles.

Experimental diets and AME procedures

The basal diet comprised (in g/kg), 800 wheat (variety Oxley grown at Narrabri, NSW in 2000), 155 casein, 20 dicalcium phosphate, 11 limestone, 7 DL-methionine, 2 vitamin and mineral premix, 3 salt, and 2 choline chloride (60%). Enzyme products with xylanase activity were added to the basal diet to provide four dietary treatments comprising control (no enzyme), Avizyme 1300 (1kg/tonne), Kemzyme W1 (1kg/tonne), and Bio-Feed Wheat CT (200g/tonne). In addition to xylanase activity, Avizyme 1300 had protease activity and Kemzyme W1 had β-glucanase, protease, amylase, cellulase and lipase activities. The four experimental dietary treatments were replicated six times (three female and three male chickens) in a randomised complete block layout. Experimental diets were pelleted (4 mm diameter and 6 mm length) in a cold-press to avoid selective feeding.

The 7-day metabolism experiment commenced when the chickens were 21 days of age. AME values for diets were determined in a classical AME study involving measurements of total feed intake and total excreta output and subsequent measurement of gross energy values of feed and excreta by isoperibol bomb calorimetry. The values for AME of wheat were calculated by subtraction of energy contributed by casein (assumed to be 20.1 MJ/kg dry matter) from the overall energy content of the experimental diet which was measured directly from total energy intake in the feed and total energy output in excreta (see Appendix 3).

Estimation of whole-tract transit time

Results from the previous experiment (section 5.2) indicated the potential of breath testing as a non-invasive measure of rate of passage of digesta. However, because the estimates of whole tract transit time and oro-caecal transit time were not different, it was decided that the ferric oxide method would be used in this experiment to examine the effect of rate of passage of digesta on energy metabolism. The rationale for this choice at that time was that ferric oxide was simpler to apply and considerably quicker and cheaper than the breath hydrogen technique when time and costs of analysis were taken into account.

On day 6 of the experiment commencing at 8.15 am, chickens (26-days of age) were administered with a gelatine capsule containing ferric oxide (Fe_2O_3 200 mg/kg live-weight). Chickens were given 2-3 mL of water via a disposable syringe fitted with a plastic tube inserted 4 cm into the oesophagus to ensure that the capsule was not regurgitated. Excreta trays were examined frequently for signs of red colouration from ferric oxide in voided droppings. Whole-tract transit time for each chicken was taken as the time elapsed (in minutes) from time of administration of ferric oxide in a gelatine capsule to time of first observation of red colouration in droppings.

Statistics

Wilcoxon's test was used to determine whether the data were normally distributed. Whole tract transit time data required $\log_e$ transformation to normalise the distribution prior to further analysis. Analysis of variance (by GLM procedure) was used to examine the effects of diet and sex, and the diet by sex interaction. Analysis of covariance (by GLM

procedure) was used to determine whether the linear coefficients of regression for energy excreted versus gross energy intake between the diets and between males and females.

5.3.3 Results

Effects of diet, and the interaction between diet and sex, were not significant ($P>0.05$) for any measurement (Table 12). Males were significantly greater in live weight (in g/bird) than females at the start (948 vs 831), and at end of the experiment (1425 vs 1217), gained more weight (476 vs 386) and ate more feed (99.1 vs 84.6 g/bird/day), respectively. The mean AME value for the wheat was extremely high (15.56 MJ/kg dry matter) and ranged from 14.4 to 16.3 MJ/kg DM for individual birds.

Table 12. Summary of the effects of diet and sex on the performance, AME, dry matter digestibility and digesta transit time.

Variable	Mean	Diet (D)	Sex (S)	DxS
Live weight at start (g/bird)	890	NS	***	NS
Live weight at end (g/bird)	1321	NS	***	NS
Growth (g/bird)	431	NS	***	NS
Feed intake (g/bird)	91.8	NS	***	NS
Feed conversion (g feed: g gain)	1.506	NS	NS	NS
AME of wheat (MJ/kg DM)	15.56	NS	NS	NS
Dry matter digestibility (g/g)	0.751	NS	NS	NS
Whole tract transit time (WTTT, minutes)	206	-	-	-
Log transformed WTTT	5.27	NS	NS	NS

*** $P<0.0.001$; NS not significant ($P>0.05$)

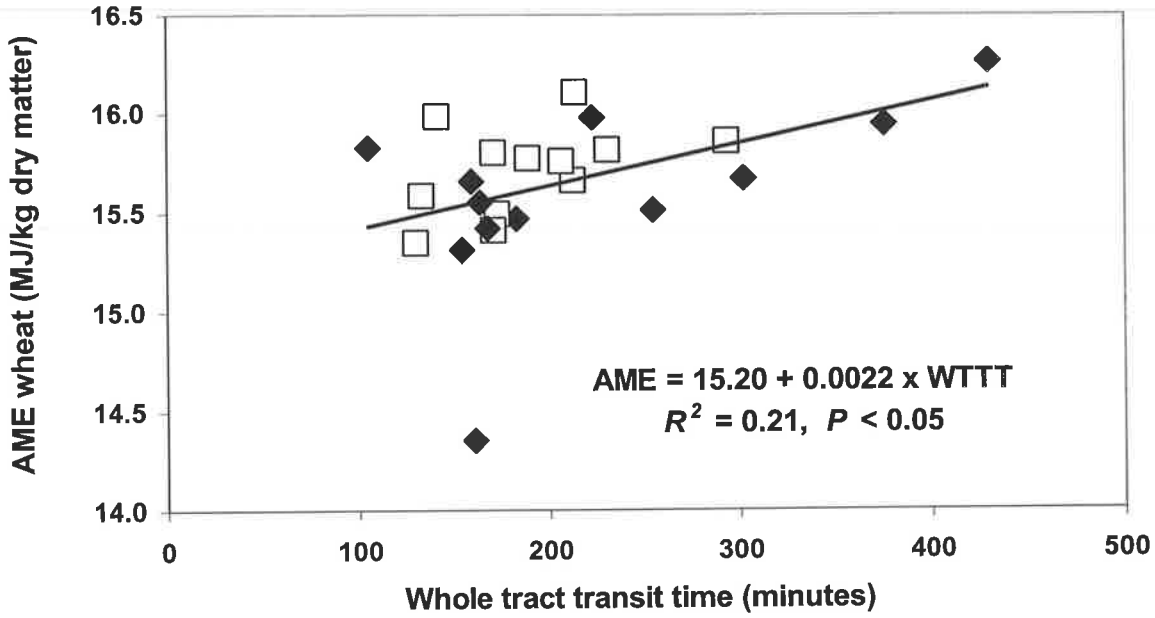


Figure 24. Plots of values for AME of wheat and whole tract transit time (WTTT) for individual chickens (n = 24). Males are represented by □ and females by ◆. Data shown here are untransformed values for WTTT. Similar statistical results were obtained for $\log_e$ transformed WTTT.

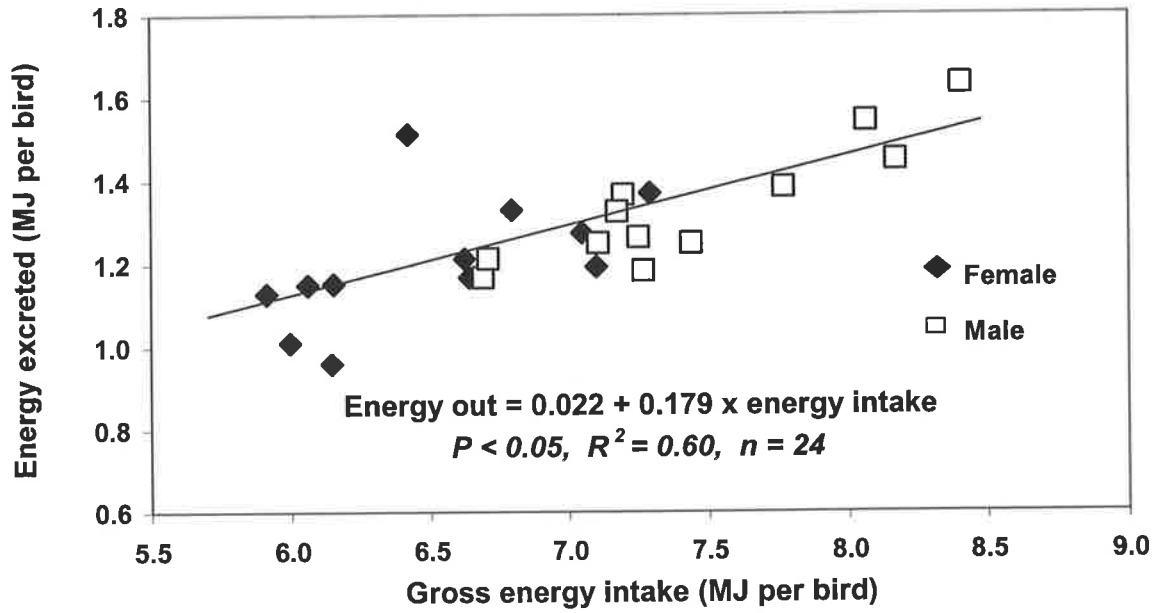


Figure 25. Relationships between energy excreted and gross energy intake for male (□) and female (◆) chickens.

The relationship between AME of the wheat and whole tract transit time is shown in Figure 24. Analysis of covariance indicated that the linear relationship between AME and WTTT shown in Figure 24 was the same for males and females. The omission of data from one female bird with the lowest AME value (14.4 MJ/kg DM) shown in Figure 24 did not alter the overall result. The two chickens with longest transit time were females and are shown in Figure 25 as the points with lowest energy excretion values. The chicken with the lowest AME value in Figure 24 was also a female and had a higher energy excretion value than any other female (Figure 25).

Analysis of covariance indicated that the linear relationship between energy intake and energy excretion shown in Figure 25 was the same for males and females. Endogenous energy loss estimated by extrapolation to zero energy intake was 22 kJ/bird/day, which is considerably less than 46 and 101 kJ/bird/day estimated for females and males, respectively, in section 3.4, for chickens given a diet based on low AME wheat.

5.3.4 Discussion

AME increased linearly with whole tract transit time. This relationship was unaffected by the sex of the chicken.

The lack of response to enzymes by chickens given the wheat-based diet was probably due to the unusually high AME value of the wheat. An AME value of 15.6 MJ/kg DM is at the high end of the ranges for Australian wheats reported by Mollah *et al.* (1983) and Rogel *et al.* (1987). Repeated measurements of AME values for this wheat in subsequent experiments (data not shown) confirmed this high value.

In this experiment, chicken were not fasted prior to dosing but continued to eat cold-pressed pellets containing 800 g/kg wheat which was not milled prior to pelleting. The resulting pellets were easily broken during feeding activity by the chickens. Svihus and Hetland (2001) pointed out that ileal starch digestibility was increased when cold-pressed pellets were crushed to mash prior to feeding, and when some of the wheat component of the diet was fed as whole grain. Hence, it is possible that the physical form of the feed used in the present experiment contributed to the high AME value.

Although not measured, it is highly likely that this particular wheat had a relatively low concentration of soluble NSP given its very high AME value. This could explain why energy excretion by both males and females in this experiment increased linearly with increasing energy intake, and why it was comparatively lower than that for most of the chickens fed a diet based on low AME wheat as shown in Figure 13 (section 3.3). This adds strength to the conclusion reached in section 3.4 that some key findings reported in recent publications ought to be verified with chickens of both sexes in order to maximise the value of this information for application in commercial industry practice. The example given was the work of Svihus and Hetland (2001) who showed that overloading the digestive tract of male chickens with starch can result in incomplete digestion of starch, and impaired feed conversion, with the possibility that microbial fermentation in the hindgut could result in large losses of energy. There was no indication in the present study that a high starch intake overloaded the digestive tract of either sex in contrast to the observations made on male chickens by Svihus and Hetland (2001). This tends to suggest that it is the soluble NSP component of a high grain intake rather than the starch content *per se* that causes the impairment in starch digestion and leaves the chickens vulnerable to microbial overgrowth of the small intestine as noted by Choct *et al.* (1996*a*).

The relatively long WTTT of 206 minutes observed here with a wheat diet compared with WTTT of 165 minutes for a commercial broiler feed reported in section 5.2.3 is difficult to explain on the basis of what is known about the effects of soluble NSP and addition of enzyme to the diet on transit time (Salih *et al.* 1991; Almirall and Esteve-Garcia 1994; Attia *et al.* 1996; Bedford and Morgan 1996; Smits *et al.* 1997). The commercial broiler diet used previously and the experimental wheat diets contained recommended dosages of enzymes so it seems unlikely that digesta viscosity would contribute to a difference in transit time. Similarly, it seems unlikely that soluble NSP was the cause because this would require the wheat diet to contain a higher concentration of soluble NSP than the commercial diet which is not consistent with the very high AME value of the wheat. Perhaps the difference in transit time was associated with fasting prior to dosing with ferric oxide in the previous experiment (section 5.2) but not in the present experiment, or the physical form of the feed offered (relatively hard, steam-treated starter crumbles compared with soft cold-pressed pellets used in the present experiment), or the physical and chemical attributes of the different diets. A further possibility is the insoluble NSP content of the

respective diets. Hetland and Svihus (2001) recently pointed out that inclusion of oat hulls in the diet shortened digesta transit time but did not affect nutrient digestibility. Insoluble NSP in flour mill offal added to the commercial broiler diet may have reduced the transit time.

5.3.5 Conclusions

Because the AME value of wheat used in this experiment was high, the work ought to be repeated with wheat of lower energy value (e.g., in the range 13 to 14 MJ/kg DM) in order to determine the relative importance of rate of passage of digesta on degree of completeness of digestion and absorption of nutrients in individual chickens of both sexes.

5.4 General discussion and conclusions

Whole tract transit time measured by first appearance of marker in faecal droppings and oro-caecal transit time by first rise in breath hydrogen provide a useful basis for studying the comparative effects of treatments such as dietary concentrations of NSP (Almirall and Esteve-Garcia 1994) and fat (Mateos *et al.* 1982) which can influence the rate of passage of digesta and, hence digestibilities of nutrients. However, some workers have preferred to use serial collection of faeces to derive cumulative excretion curves to estimate T_{50} (time taken for 50% of excretion of marker) and the mean retention time (Almirall and Garcia 1994; Danicke *et al.* 1999). A more sophisticated approach may also be required with hydrogen breath testing. This would serve the dual purpose of measuring mean retention time of digesta, and indicate whether there were any signs of microbial overgrowth of the small intestine.

A major disadvantage of any method involving a indigestible marker is that it provides an estimate of whole tract transit time, including an unknown period in the hindgut, which would affect the extent of bacterial fermentation of undigested carbohydrate, fat and other nutrients. Danicke *et al.* (1999) observed a decrease in mean retention time due to xylanase supplementation in different segments of the digestive tract. The most pronounced effects of the enzyme were in the jejunum and ileum where starch digestion and absorption take place. A non-invasive estimate of time of residence in the small intestine would be more advantageous as this is where most of the energy and other nutrients are digested and absorbed (Levin 1984) and because it does not require serial

killing of animals to determine residence time in any given segment of the tract. Hence, it is desirable to develop a hydrogen breath test based on serial collection of samples to derive an accurate estimate of oro-caecal transit time (i.e., digesta residence time in the upper part of the gastrointestinal) to avoid any confounding effects on retention of nutrients associated with proliferation of microflora in the hindgut. On the other hand, a case can be made for a hydrogen breath test based on a single sample to assess overall metabolic activity of gut microflora.

In conclusion, the rate of passage of digesta is an important determinant of energy metabolism in chickens, but it does not explain why females have any advantage over males as observed in Chapter 3. Also, it is clear that sex-dependent differences in gut microflora profiles warrant further study

Chapter 6. Influence of gut microflora on digestive function of broiler chickens

6.1 General introduction

Results presented in previous chapters indicate the likelihood that the gut microflora profile is a key factor in variation in energy digestion by chickens. An exponential increase in energy excretion with increasing energy intake by males, but not by females, was observed in Chapter 3. This is consistent with the observations of Svihus and Hetland (2001) that excessive feed intake can overload the digestive tract with starch, which can result in large losses of energy due to microbial fermentation, as described by Choct *et al.* (1996*a*). Large differences between individual chickens in concentrations of hydrogen in exhaled breath were observed in sections 4.3 and 5.2. Prior to breath testing, both flocks of chickens were reared in a similar manner in the same room, but at different times, and were fed commercial broiler feed. As explained in Chapter 4, hydrogen in the breath is produced by microbial fermentation of undigested feed reaching the hindgut. It follows that variation in hydrogen concentration in the breath is indicative of individual chickens hosting commensal microflora differing widely in fermentation patterns. In addition, variable concentrations of methane were detected in the breath of chickens in one experiment (section 4.3), but methane was not detected in the breath of chickens in a later experiment (section 5.2). These results point to the existence of differences in the range of species and/or numbers of organisms within each species, and hence differences in the overall metabolic activity of the gut microflora, as a result of previous rearing and feeding conditions. Furthermore, differences between males and females in breath hydrogen concentrations indicate sex-dependent differences in gut microflora profiles in addition to wide between-bird variation within each sex.

The gut of the newly hatched broiler chicken is free of organisms (Yamauchi *et al.*, 1990) and relatively immature in terms of absorptive capacity and immunocompetence compared with older animals (Jin *et al.*, 1998; Uni *et al.*, 1999). Shortly after hatching, chickens are exposed to ubiquitous micro-organisms in the hatchery and on arrival on the farm. These

organisms proliferate as they compete for various niches in the micro-environment of the gut (Ewing and Cole 1994). In addition to maturation of intestinal function and the immune system in the first two weeks of life (Vieira and Moran 1999), the profile of the gut microflora is also likely to change with the age of the chicken in response to ingestion of various types and amounts of substrates such as carbohydrate in the diet. Also, as chickens age they will continue to ingest a range of organisms, some of which could be pathogenic (Ewing and Cole 1994), from a rapidly changing environment as viable organisms are shed by other birds and possibly introduced to the farm from neighbouring properties. McBurney *et al.* (2003) reported that the profile of organisms in the small intestine stabilised at about three weeks of age, with lactobacilli predominating in healthy chickens.

The opportunity for proliferation of microflora in the small intestine increases as the viscosity of digesta increases. Glycanase enzyme products that depolymerise NSP such as arabinoxylan in wheat and β-glucan in barley can reduce the viscosity of digesta and thus control microbial overgrowth of the small intestinal contents by facultative anaerobes (Choct *et al.* 1996*a,b*). Feed enzymes have proven to be a very effective tool for increasing the digestible energy value of grains for poultry and for improving the uniformity of growth and feed efficiency of broiler flocks.

This chapter describes the effects of the strain, sex and age of the chicken, type of grain used in the diet, and dietary addition of NSP-degrading enzymes and antibiotics, on microbial fermentation patterns in the gut, and whether the overall metabolic activity of the gut microflora influenced the digestion of energy. Hydrogen and methane breath testing (as described in Chapter 4 and Chapter 5) was used as a non-invasive way of gauging changes in the overall or net metabolic activity of the gut microflora.

6.2 Metabolic activity of gut microflora in male and female chickens of two different strains given a diet based on low AME wheat

6.2.1 Introduction

AME values were on average 4% higher in females compared with males when given a diet based on low AME wheat, but there was no difference observed between two

commercial strains of chickens (Chapter 3). The possibility that the overall metabolic activity of the gut microflora influenced these results was examined by analysis of breath samples taken during the course of the experiment described in Chapter 3. This analysis tested the hypotheses that (a) a decrease in AME value was associated with an increase in breath hydrogen from microbial fermentation in the intestinal tract, and (b) metabolic activity of the gut microflora, as indicated by increased expiration of hydrogen in the breath, differed according the sex and strain of chickens.

6.2.2 Materials and methods

A total of 24 Cobb and 24 Ross chickens (22 or 23 days of age) were used in this 7-day metabolism study. Other details, including the composition of the wheat-based diet (Table 6), are described in section 3.2.

Each chicken was breath tested on days 0 and 6 of the 7-day study period as per the procedures described in Chapter 4, in order to quantify initial differences between chickens due to prior rearing conditions, as well as any effects induced by the wheat-based diet during the course of the experiment. Hydrogen and methane concentrations in breath were measured by gas chromatography (QuinTron Microlyzer).

6.2.3 Statistics

Wilcoxon's test was used to determine whether the data were normally distributed. Hydrogen and methane concentrations in breath at the start and end of the experiment, and the change in concentrations during the experiment required $\log_e$ transformation to normalise the distribution prior to further analysis. Analysis of variance (by GLM procedure) was used to examine the effects of sex, strain, and the sex by strain interaction. The relationships between $\log_e$ transformed hydrogen concentration on day 6 and AME, feed intake, growth and feed conversion were examined by regression analysis. Analysis of covariance (by GLM procedure) was used to determine whether the linear coefficients of regression differed according to sex and strain.

6.2.4 Results

Hydrogen and methane concentrations in breath were highly variable between birds within each strain by sex combination (Table 13, Figure 26 and Figure 27). Breath hydrogen and methane concentrations at the start and end of the experiment, and changes during the experiment, were not affected ($P>0.05$) by sex or strain of the chicken, or the sex by strain interaction.

Table 13. Means, standard deviations (SD), coefficients of variation (CV) and ranges of concentrations of hydrogen in breath samples taken from male and female chickens of two commercial strains on days 0 and 6 of the 7-day experiment.

Strain	Sex	n	Mean	SD	CV	Range
			Hydrogen (ppm) on day 0			
1	Female	12	29	20.8	72	7-72
	Male	12	36	32.2	89	7-125
2	Female	12	31	19.4	63	5-76
	Male	12	32	14.3	45	16-63
			Hydrogen (ppm) on day 6			
1	Female	12	24	18.8	79	2-69
	Male	12	45	62.8	138	5-234
2	Female	12	23	15.2	67	9-59
	Male	12	12	9.7	79	1-31

There were no significant relationships ($P>0.05$) between breath hydrogen concentration on day 6 of the 7-day feeding period and AME, but the relationship between feed conversion ratio and breath hydrogen was highly significant (Figure 28). Growth rate and breath hydrogen were negatively correlated ($P<0.01$) but the relationship was not as strong ($R^2=0.15$) as that for feed conversion ($R^2=0.44$; Figure 28). There was no relationship ($P>0.05$) between feed intake and hydrogen concentration, nor were there any differences in the relationships due to sex or strain of chickens.The parabolic relationship between feed conversion and breath hydrogen remained significant ($P<0.01$) but was weaker ($R^2=0.11$) when the result for one chicken with the highest breath hydrogen concentration (234 ppm) was omitted from the analysis.

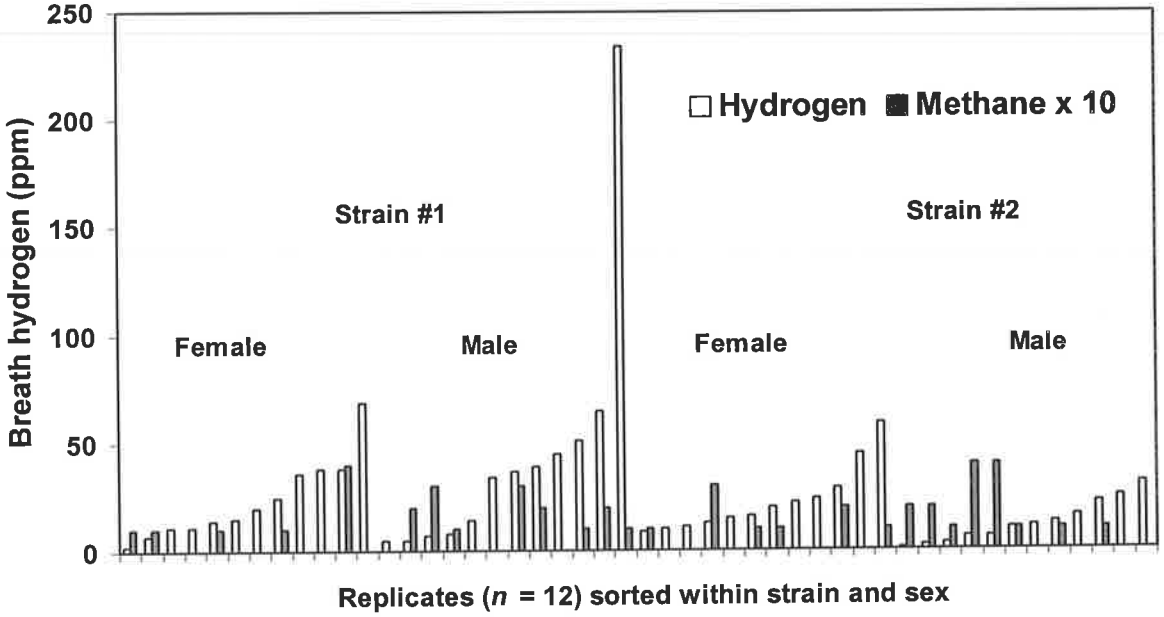


Figure 26. Hydrogen and methane (x10) concentrations in breath samples taken on day 6 from chickens given a low AME wheat diet for seven days. Each bar in the figure is the result for one chicken.

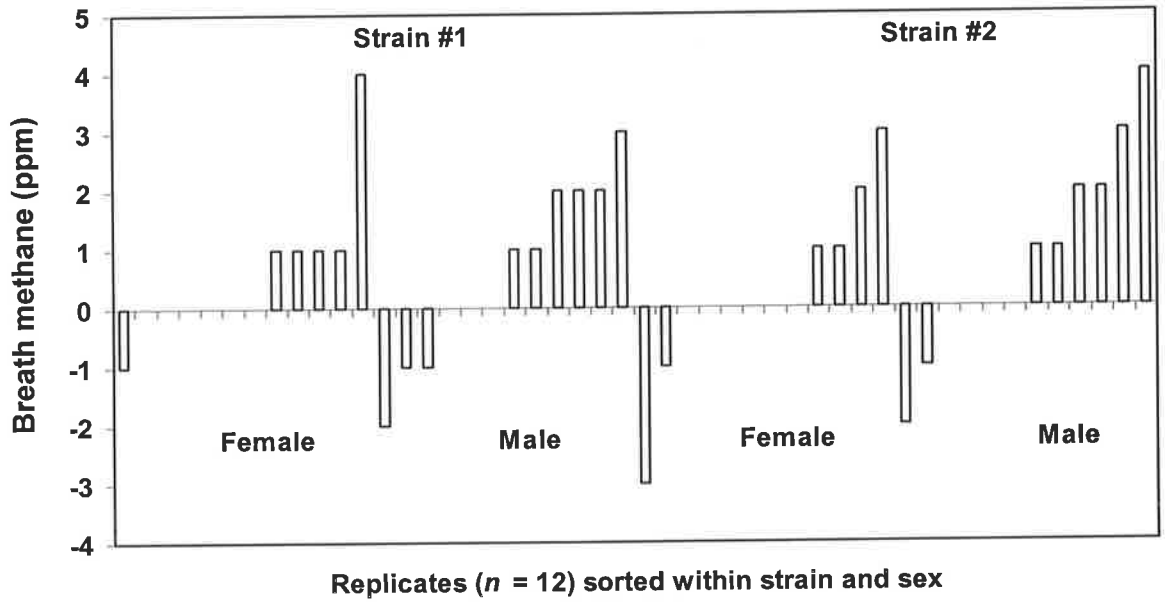


Figure 27. Change in methane concentration in breath from day 0 to day 6 in chickens given a low AME wheat diet for seven days. Each bar in the figure is the result for one chicken. Zero values indicate no change in breath methane between days 0 and 6 (n=4 chickens) or no methane in breath on days 0 and 6 (n=15).

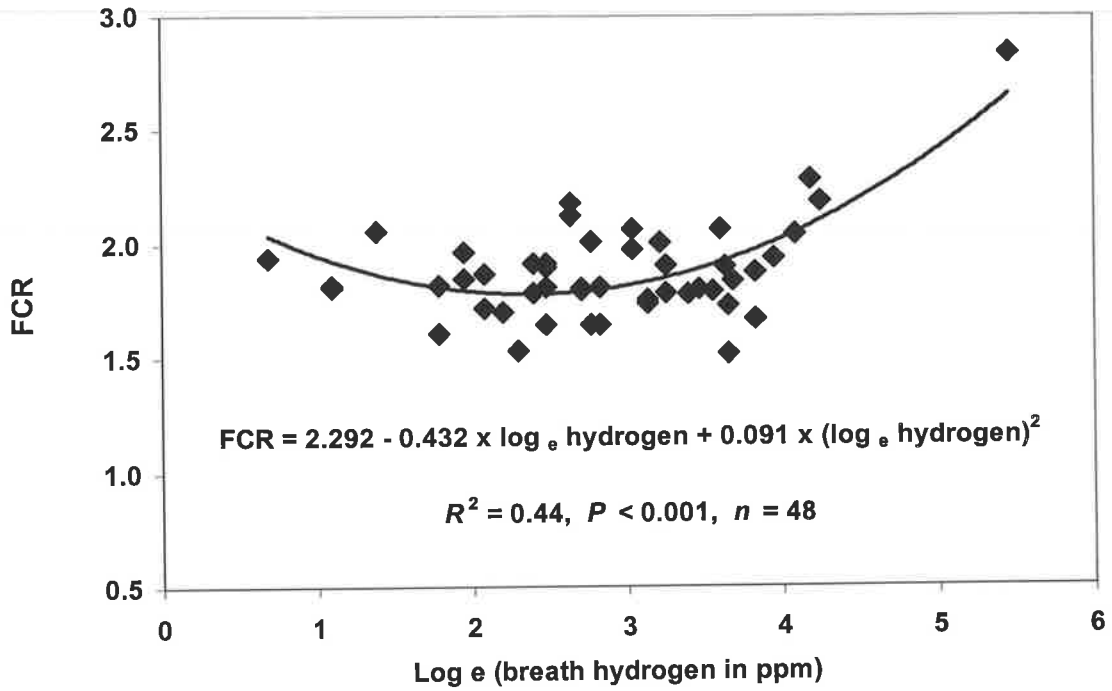


Figure 28. Relationship between feed conversion ratio (FCR) and hydrogen concentration in samples taken on day 6 from chickens given a low AME wheat diet for a 7-day feeding period. Each point in the figure is the result for one chicken.

6.2.5 Discussion

There was no evidence to support the first hypothesis that AME value of the wheat-based diet and concentration of hydrogen in the breath were inversely correlated. However, it was apparent that when breath hydrogen concentration exceeded about 10 ppm ($\log_e$=2.3 in Figure 28), feed conversion rate increased (i.e., feed efficiency was reduced), as growth was reduced but feed intake was unchanged. These observations are consistent with the chicken and its gut microflora competing for energy and possibly other nutrients thus slowing the rate of growth and reducing feed efficiency in the chicken. Likewise, there was no evidence to support the second hypothesis that the relationships between breath hydrogen and growth and feed efficiency were influenced by the sex or strain of the chickens.

The results are in general agreement with the findings of Bird *et al.* (2002) who questioned whether gut bacteria, beneficial or otherwise, stimulated responses by the host animal that increased its energetic needs through immune reactions and repair of gut damage. Their

conclusion was based on studies with mice that indicated that the gut microflora did not alter whole-animal energy balance. That appears to be the case in the current experiment as there was no detectable increase in feed intake with increasing expiration of hydrogen, nor was AME affected. An alternate possibility is that the microflora influenced the partitioning of energy in the host animal. Klasing (1996) explained how a homeorhetic response to pathogenic bacteria involved the partitioning of dietary nutrients to support the immune system at the expense of growth, skeletal muscle accretion and reproduction. He also pointed out that there was a significant cost in terms of energy and certain nutrients to repair tissue damage and to support compensatory growth post-challenge. Whether repartitioning of energy could occur in response to sub-acute inflammation of mucosal tissue as a result of proliferation of non-pathogenic species in healthy chickens remains to be seen. Taylor (2003) reported that an abrupt change in the type of grain used in the diet of laying hens at peak production led to a decrease in luminal pH and a colitis-like condition accompanied by the presence of orange-coloured mucus, blood and particles of undigested grain in the droppings. Feed intake and egg production were unaffected by the change in the diet.

Kelly and King (2001) noted that in pigs up to 6% of net energy in the diet could be lost due to microbial fermentation in the stomach and small intestine. The possibility that losses of short chain fatty acids from fermentation of undigested carbohydrate leaving the small intestine were as variable as the production of hydrogen and methane production needs further study in chickens. Although uptake of energy through absorption of short chain fatty acids in the hindgut of chickens is not as effective as that in pigs and rats (Jorgensen *et al.* 1996), nevertheless, it could amount to a small but relevant source of variation in AME. In addition, losses of volatile products could reduce the gross energy content of excreta and hence result in an overestimation of the AME value. Furthermore, variation in these volatile losses could contribute to variation in AME values obtained with individual chickens.

As hydrogen is a precursor for methane formation by certain species of gut bacteria, then it could be assumed that increased methane concentration would be associated with reduced hydrogen concentration in breath. The low concentrations of methane relative to hydrogen in individual chickens shown in Figure 26 suggests that this was not the case in the current

study. A decline in production of hydrogen independently of methane production could indicate that numbers of and/or activities of hydrogen producers declined, or that hydrogen usage by other species increased.

Large changes in methane production were observed in some chickens (Figure 27), which may indicate a shift in fermentation patterns in the hindgut, such as the formation of acetate and other short chain volatile fatty acids, and in the metabolic activities of bacteria which utilise sulphate. Changes such as these could alter pH of the luminal environment, which in turn could favour some species of bacteria over others. Furthermore, variation in the subsequent losses of volatile fermentation products from voided excreta prior to collection in energy balance experiments could lead to variation in the errors associated with measurement of gross energy, and hence, variation in AME values obtained with different chickens. This possibility is explored further in section 6.6.

6.2.6 Conclusions

The observations in regard to expiration of hydrogen and methane point to remarkably different metabolic activities of gut microflora in birds reared on commercial diets with the same nutrient specifications (but not necessarily the same ingredient composition), and to wide variation between chickens in the metabolic activity of the gut microflora following a change in the composition of the diet.

6.3 Effects of NSP-degrading enzymes on AME of wheat and metabolic activity of gut microflora

6.3.1 Introduction

Despite the huge success of feed enzyme technology, questions remain about the specific modes of actions of enzymes (Smits and Annison 1996; Williams 1997), and why enzymes can reduce but do not eliminate variation in energy values for grains (Bedford 1996; Kocher *et al.* 1997). It is plausible that some of the variation in AME remaining after treatment by exogenous enzymes is due to indigestible fragments from partial depolymerisation of NSP providing a food source for commensal gut microflora.

The lack of an association between AME value of the diet and expiration of hydrogen in the breath (reported in section 6.2) may have arisen because the gut microflora had already reached a stable equilibrium as the chickens were four weeks of age at the time of breath testing (McBurney *et al.* 2003). Hence, it was considered worthwhile to repeat aspects of the previous study, this time with chickens 15 days of age at the start of the experiment, and to use a feed enzyme treatment in an attempt to alter the amounts of fermentable substrate reaching the distal part of the intestine.

This experiment was a further test of the hypotheses examined in section 6.2 that (a) decrease in AME value is associated with an increase in breath hydrogen from microbial fermentation in the intestinal tract, and (b) the inverse relationship between AME and breath hydrogen differs between male and female chickens.

6.3.2 Material and methods

A total of 24 Ross chickens were obtained from the Bartter Steggles hatchery, Cavan, SA on Thursday 6 July 2000 and raised from hatch in two rearing pens in a controlled temperature room. Male and female chickens were reared separately. All birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540). At 12 days of age, chickens were transferred in pairs to 24 metabolism cages located in a controlled-temperature room set at 25-27°C initially, and given starter crumbles (Ridley Agriproducts, diet code #503540) for 3 days while they adapted to the cages. At 14 days of age, one bird was removed from each of the 24 cages. The chickens were 15 days of age at the start of the 7-day metabolism study. The temperature setting in the room was reduced daily until it was 22°C at the end of the experiment.

The composition of the basal diet is shown in Table 6. The two experimental diets were unsupplemented basal diet as the control and the basal diet supplemented with a feed enzyme product (Avizyme 1302 added at 500 g/tonne) as the treatment. This product had xylanase and protease activities according to the manufacturer's product safety sheet.

The AME value of the wheat-based experimental diet was determined in a conventional energy balance study involving measurements of total feed intake and total excreta output,

and subsequent measurement of gross energy (GE) values of feed and excreta, as per the procedures described in section 2.2.2.

Each chicken was breath tested on days 0 and 6 of the 7-day study period as per the procedures described in Chapter 4 in order to quantify any initial differences between chickens due to prior rearing conditions, as well as any effects of dietary treatments during the experiment. Hydrogen and methane concentrations in breath were measured by gas chromatography (QuinTron Microlyzer).

6.3.3 Statistics

Distribution of data, transformation if required and subsequent analyses of variance and covariance were conducted as described in section 6.2.3.

6.3.4 Results

Results of statistical analysis are summarised in Table 14. Responses to enzyme in growth approached significance (P=0.09). The sex by diet interaction was significant (P<0.05) for AME of the diet and dry matter digestibility. Enzyme inclusion significantly increased AME for males (15.0 vs 15.7 MJ/kg DM; P<0.05) but there was no response in females (15.3 vs 15.4 MJ/kg DM; P>0.05). Similarly, enzyme inclusion significantly increased dry matter digestibility for males (0.77 vs 0.74; P<0.05) but there was no response in females (0.76 vs 0.75; P>0.05). The sex by diet interaction approached significance (P=0.06) for feed conversion ratio. Enzyme inclusion improved feed conversion in males (1.53 vs 1.64) but there was no response in females (1.65 vs 1.65). The change in breath hydrogen from day 0 to day 6 tended (P=0.10) to be greater in females than males but in opposite directions, i.e., breath hydrogen increased in males, but decreased in females, during the 6-day period between tests.

Eight chickens (six female and 2 male) of the 24 in the study had no detectable methane in breath at the commencement of the study (day 0). However, all 24 chickens produced methane on day 6, which points to a shift in the metabolic activity of the gut microflora as discussed in section 6.2.5.

There were no significant relationships ($P>0.05$) between breath hydrogen and AME, feed intake or growth. This is in contrast to results described in section 6.2, where there was an indication that microflora competed for energy. Despite improvements in AME, growth, and feed conversion due to addition of enzyme to the diet, there were no effects of enzyme on hydrogen or methane concentrations in breath on day 6 of the 7-day experiment.

Table 14. Summary of main effects in analysis of variance.

Variable	Diet [3]		Sex [3]		
	Control	Enzyme	Female	Male	SEM
Live weight at 15 days of age (g)	525 a	524 a	497 b	551 a	8.3
Growth (g/bird)	321 a	340 a	298 b	363 a	7.3
Rate of gain (g/kg) [1]	611 a	650 a	599 b	662 a	16.6
Feed intake (g/bird/day)	75.4 b	76.7 a	70.1 b	82.0 a	1.46
Feed conversion (g feed: g gain)	1.646 a	1.589 b	1.650 a	1.585b	0.0210
AME of diet DM (MJ/kg)	15.19 b	15.53 a	15.38 a	15.34 a	0.086
Dry matter digestibility (g/g)	0.747 b	0.765 a	0.755 a	0.757 a	0.0046
Breath hydrogen on day 0 (ppm) [2]	59 a	81 a	91 a	49 b	12.4
Breath hydrogen on day 6 (ppm) [2]	52 a	70 a	55 a	66 a	13.9
Change in breath hydrogen (ppm)	-7 a	-11 a	-35 a	17 a	21.5

[1] Rate of gain = Growth × 1000 ÷ Live weight at start of experiment

[2] Data were $\log_e$ transformed to normalise the distribution prior to analysis

[3] Means with the same letter within a main effect are not significantly different ($P>0.05$)

6.3.5 Discussion

There was no evidence to support the hypothesis that AME value of the wheat-based diet and concentration of hydrogen in the breath were inversely correlated, or that the relationship differed in male and female chickens. In contrast to the results reported in section 6.2, there was no indication in the current experiment that the gut microflora competed with the chicken for dietary energy.

The difference between females and males in breath hydrogen at the start and end of the experiment support earlier conclusions that metabolic activity of gut microflora is sex-dependent. Metabolic activities of microflora, as indicated by breath hydrogen, appeared to be independent of enzyme addition to the feed. The enzyme product used in this experiment is understood to work primarily by cleaving the xylose backbone of arabinoxylan, the main soluble NSP in wheat (Bedford 1995). It is possible that a substantial reduction in viscosity occurred as a result of only a limited number of cleavages of the xylose backbone, but that this reduction in molecular weight was sufficient to reduce the anti-nutritive effects of NSP (as discussed in section 1.3.2), thus enabling an increase in AME in this experiment. Nevertheless, under these circumstances in both control and treated chickens, it is likely that similar amounts of undigested fragments of arabinoxylan reached the hindgut to be fermented by gut microflora, hence the lack of a difference on hydrogen concentration in the breath from chickens fed the control diet compared with those given the diet supplemented with the enzyme product.

6.3.6 Conclusions

The feeding of a commercial starter diet led to the development of different metabolic activities of gut microflora in 15-day old male and female chickens, with female chickens exhaling almost twice the concentration of hydrogen compared with males. These patterns changed when chickens were given a diet containing a high level (700g/kg) of wheat. Presumably, the viscosity reducing action of exogenous enzymes contributed to enhanced starch digestion in the small intestine, but did not alter the amounts of complex carbohydrate reaching the hindgut.

6.4 Effects of age of chicken and exogenous enzymes on AME and metabolic activity of gut microflora of chickens given wheat and barley diets

6.4.1 Introduction

The results in sections 6.2 and 6.3 showed that chickens reared in a single group on litter and fed a commercial broiler feed hosted gut microflora with different fermentation patterns as indicated by large between-chicken differences in exhalation of hydrogen and methane. Breath hydrogen from chickens at 15 days of age was 70 ppm and fell to 61 ppm after consuming an experimental wheat diet for six days (section 6.3). In older chickens (22 days of age), breath hydrogen was 32 ppm initially then fell to 26 ppm after six days on an experimental wheat diet (section 6.2). Also, breath hydrogen concentrations differed between male and female chickens 15-days of age but not in chickens 22-days of age. These observations are consistent with the findings of McBurney *et al.* (2003) that the bacterial profiles in healthy chickens stabilise with age, if not in overall metabolic activity, as noted previously.

The beneficial effects of feed enzymes are thought to be partially dependent on the age of the chickens, with the best responses often being seen in older chickens (Bedford and Morgan 1996). AME value of a wheat-based diet was improved by the use of a viscosity-reducing enzyme product in the previous experiment (section 6.3), but there was no apparent effect on the amount of undigested carbohydrate reaching the hindgut. It was considered worthwhile to repeat some aspects of the previous experiment, this time with an enzyme product with a wider range of activities in an attempt to release other NSP from the cell wall structure of the grain in the diet, in addition to viscosity reduction. Wheat and barley were chosen as these contain two different types of soluble NSP, mainly arabinoxylan and β-glucan, respectively, in an attempt to alter the flow of undigested carbohydrate into the hindgut to promote different patterns of microbial fermentation.

This experiment tested the hypotheses that (a) AME values increased with age of the chicken, (b) hydrogen concentration in breath decreased with age of the chicken.

6.4.2 Materials and methods

Birds, housing and management

Ross broiler chickens were obtained from the Bartter Steggles hatchery, Cavan, SA on Thursday 11 January 2001. Chickens were raised from hatch in two rearing pens in a controlled temperature room. Male and female chickens were reared separately. All birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540).

At 15 days of age, chickens were transferred in groups of five to 24 metabolism cages located in a controlled-temperature room set at 25-27°C initially, and given experimental diets. One chicken in each pen was selected at random, weighed, and identified by a leg-band for subsequent breath testing on days 0 and 6 of the 7-day experimental period. The temperature setting in the room was reduced daily until it was 22°C at the end of the experiment.

The above procedures were repeated commencing at 22 and 29 days of age with new cohorts of chickens taken from the original rearing groups housed in floor pens.

Experimental procedures

The basal diets comprised (in g/kg) 800 new season wheat or barley (Janz or Chebec cultivars, respectively, grown on the Roseworthy Campus), 152 casein, 20 dicalcium phosphate, 11 limestone, 7 DL-methionine, 5 vitamin and mineral premix, 3 salt, and 2 choline chloride (60%). Kemzyme W1 provided by Kemin Australia Pty Ltd was added to each basal diet at the rate of 1000 g/tonne to provide a total of four dietary treatments. The enzyme product had β-glucanase, protease, amylase, cellulase and lipase activities according to the manufacturer. Each dietary treatment was allocated to three cages of male chickens and three cages of female chickens in a randomised complete block design.

Weighed experimental diet was given on day 0 after breath sampling was completed. The first three days enabled the chickens to adapt to the feed. Feed intake was measured during this period. During the following four days feed intake was measured and all excreta collected and dried daily. Moisture content of excreta collected on day 5 of the metabolism study was measured. Birds were weighed at the start and end of the 7-day period. The

respective values for AME of wheat and barley in the diets were calculated by subtraction of energy contributed by casein (assumed to be 20.1 MJ/kg dry matter) from the overall energy content of the experimental diet which was measured directly from total energy intake in the feed and total energy output in excreta.

Breath samples were collected from the selected chickens commencing at 8.30 am on days 0 and 6 of each of the 7-day metabolism studies. A breath sample was taken 15 seconds after a prototype helmet (50 mm diameter) was placed over the head of the chicken as per the procedures described previously.

Statistics

Distribution of data, transformation if required and subsequent analyses of variance and covariance were conducted as described in section 6.2.3.

6.4.3 Results

The only significant ($P<0.05$) interaction involving sex was with age on live weight at the start of each 7-day experiment, feed intake, and feed conversion. These interactive effects were significant because the respective differences between males and females differed with age (Table 15). Males were significantly ($P<0.05$) heavier and ate more feed than females in each 7-day experimental period, but there were no significant ($P>0.05$) differences between the sexes in feed conversion. For simplicity, all data were re-analysed with a reduced linear model without interactions involving sex. The results of this analysis of variance are summarised in Table 16.

Female chickens had significantly higher ($P<0.001$) values for AME of the diet (14.44 vs 14.21 MJ/kg DM; $SE=0.03$) and dry matter digestibility (0.718 vs 0.709; $SE=0.002$) compared with males when data were pooled across age of chicken, grain type and enzyme addition.

The combined effects of age of chicken, grain and enzyme on AME value of the grain, feed intake, and growth are summarised in Figure 30, Figure 29 and Figure 31, respectively. Dry matter digestibility showed a similar pattern of results as AME of grain (results not presented). The AME value of the wheat diet was greater than barley for each age of

chicken, and the response to enzymes increased as chickens aged. However, the AME values for barley and wheat not supplemented with feed enzymes were significantly (P<0.05) lower for chickens four weeks of age compared with those two weeks of age (Figure 29). Addition of enzyme to the barley diet significantly (P<0.05) increased feed intake of chickens 2- and 3-weeks of age, but not in chickens 4-weeks of age (Figure 30). In contrast, addition of enzyme to the wheat diet significantly (P<0.05) increased feed intake in 4-week old chickens, but not in younger chickens (Figure 30). Significant (P<0.05) improvement in growth was observed when chickens were given the barley diet from 2- and 3-weeks of age (Figure 31). Growth was significantly (P<0.05) reduced by enzyme addition to the wheat-based diet given to 3-week old chickens (Figure 31).

Feed conversion was significantly (P<0.05) affected by the interaction between grain type and enzyme addition (Figure 32). Enzymes significantly (P<0.05) improved feed conversion in chickens given the barley diet, whereas feed conversion in chickens given wheat was not affected (P>0.05) by enzyme addition.

Hydrogen (114 ppm; SE=11) and methane (32 ppm; SE=2.4) concentrations in breath at the start of each 7-day metabolism experiment did not differ with age of the chickens (Table 16). Hydrogen concentration in breath at the end of each 7-day experiment was significantly affected by the interaction between age and enzymes, and there was a trend (P=0.11) towards an effect from the age by enzyme interaction. The combined effects of age of chicken, grain and enzyme on breath hydrogen concentration on day 6 are shown in Figure 33. Addition of enzyme to the barley diet resulted in significant (P<0.05) reduction in breath hydrogen, but there was no effect (P>0.05) in chickens fed the wheat diet. For both the wheat and barley diets not supplemented with enzymes, breath hydrogen concentrations increased significantly (P<0.05) from week 2 to week 4.

Methane concentration in breath on day 6 of each 7-day experimental period was not affected (P>0.05) by sex of the chicken, grain type or enzyme addition. However, there was a highly significant effect of age of the chicken (Table 16). Chickens 4 weeks of age at the start of the experimental period had a significantly (P<0.001) higher methane concentration on day 6 (57 ppm; SE=3) than chickens 2- and 3-weeks of age (20 and 13 ppm, respectively; SE=3).

Table 15. Effects of age and sex of chicken on initial live weight, and feed intake and feed conversion ratio during the 7-day experimental period

Age (in days)	Sex	Live weight (g/bird)	Feed intake (g/bird)	Feed conversion (g feed: g gain)
15-22	Female	506	67.3	1.678
	Male	530	74.9	1.639
22-29	Female	918	83.4	1.855
	Male	1074	100.2	1.923
29-36	Female	1452	103.5	2.315
	Male	1534	114.8	2.208
	Pooled SEM	5	1.4	0.051

Table 16. Reduced model analysis of variance of data showing significance of main effects of sex (S) and age (A) of chicken, type of grain in the diet (G) and dietary addition of enzyme (E), and interactions between age, grain and enzyme.

Variable	S	A	G	E	AxG	AxE	GxE	AxGxE
Live weight (g/bird)	***	***						
Weight gain (g/bird)	***	***	***	*			**	*
Feed intake (g/bird/day)	***	***	*	**			*	*
Feed conversion ratio		***	***	*			*	
AME diet (MJ/kg DM)	***	***	***	***	***	**		
Dry matter digestibility	***	***	***	***	***	***		
Breath H_2 day 0 (ppm) †								
Breath H_2 day 6 (ppm) †		***				**		
Breath CH_4 day 0 (ppm) †								
Breath CH_4 day 6 (ppm) †		***						

† All statistical analyses were done on $\log_e$ transformed data

* $P<0.05$, ** $P<0.01$, *** $P<0.001$

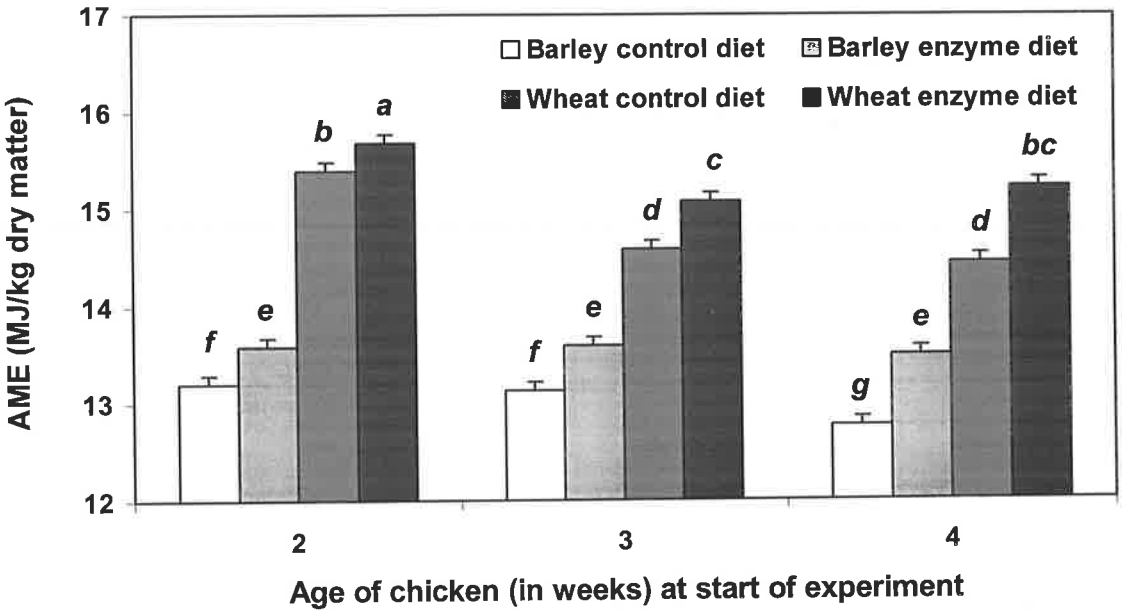


Figure 29. Effects of age of chicken, grain type and addition of enzyme to the diet on AME of the grain (means ± SE). Means with the same letter are not significantly different (P>0.05).

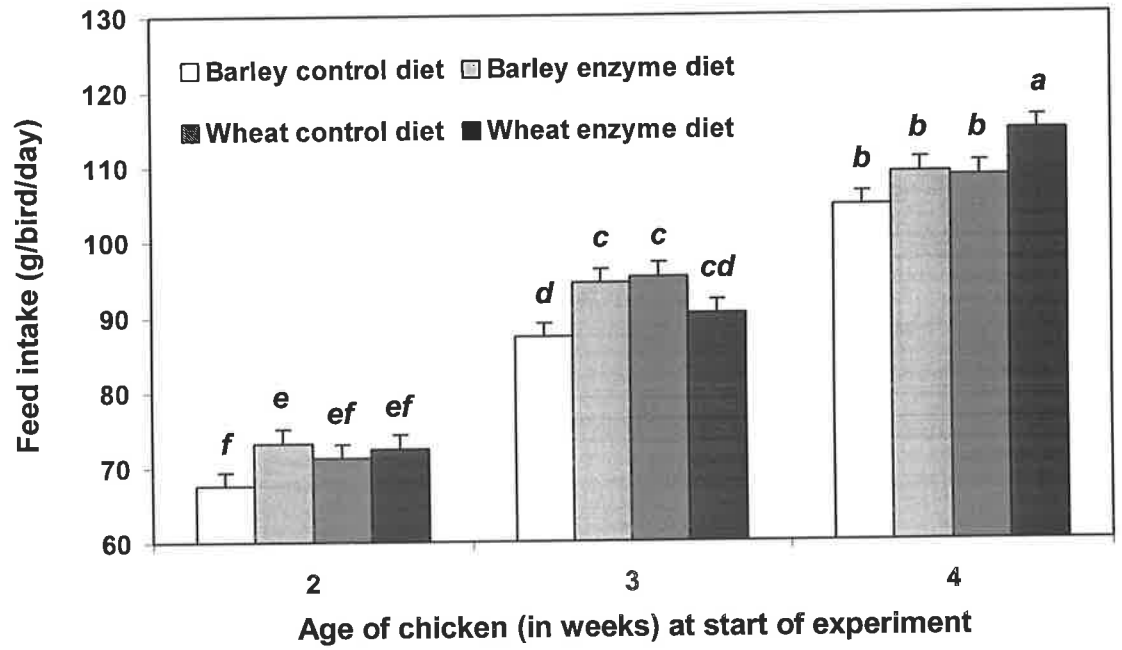


Figure 30. Effects of age of chicken, grain type and dietary enzyme on feed intake (means ± SE). Means with the same letter are not significantly different (P>0.05).

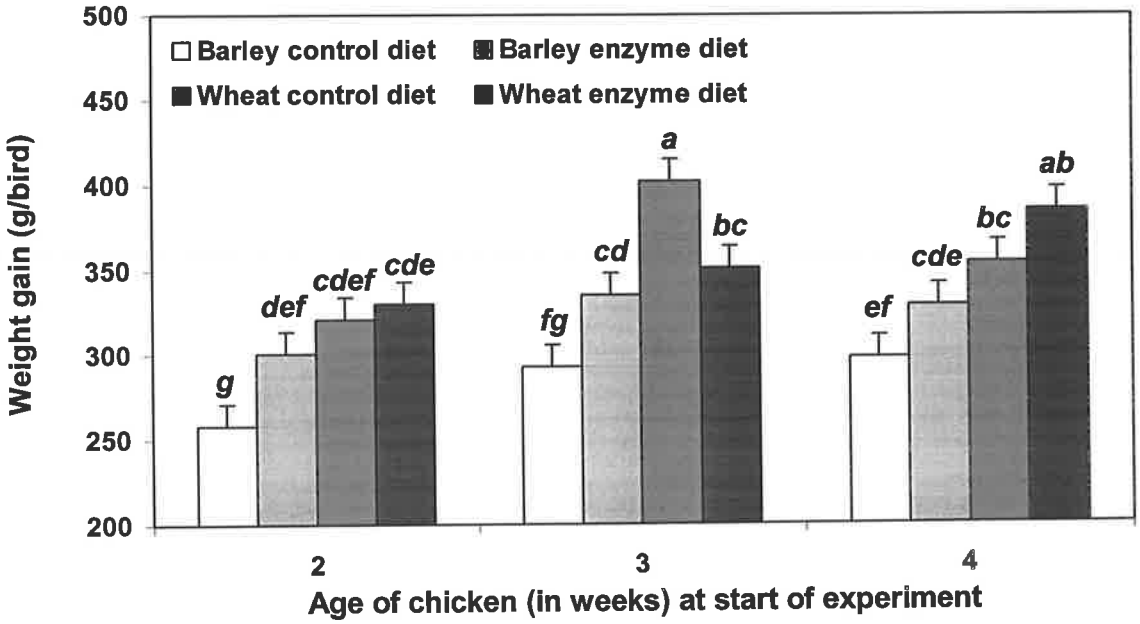


Figure 31. Effects of age of chicken, grain type and dietary enzyme on weight gain (means ± SE). Means with a common letter are not significantly different (P>0.05).

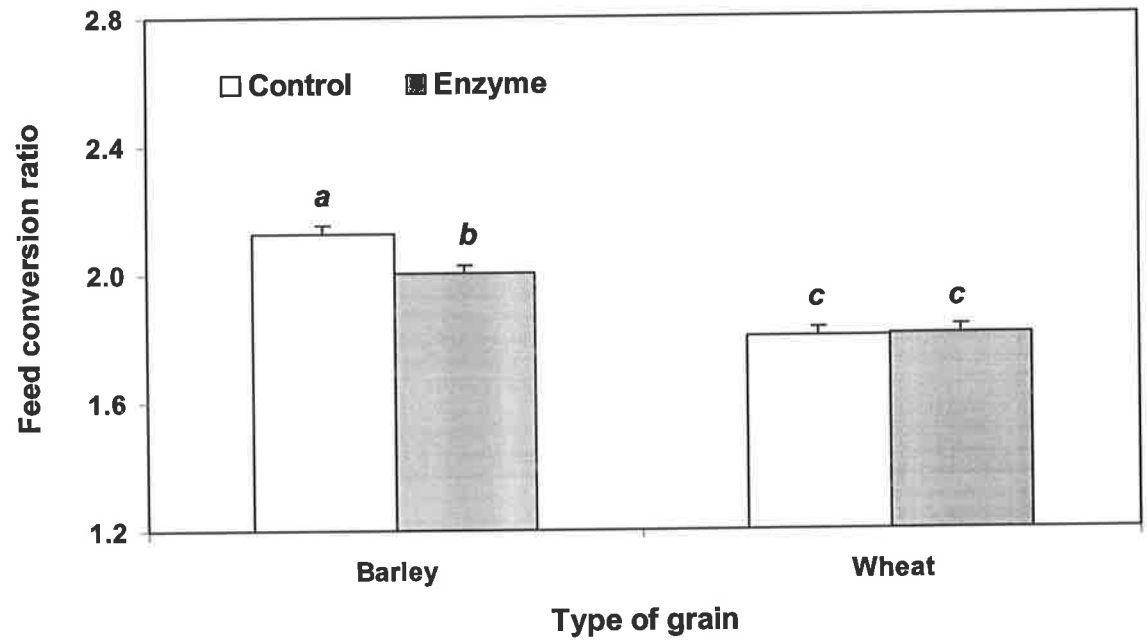


Figure 32. Effects of grain type and addition of enzyme to the diet on feed conversion (means ± SE). Means with a common letter are not significantly different (P>0.05).

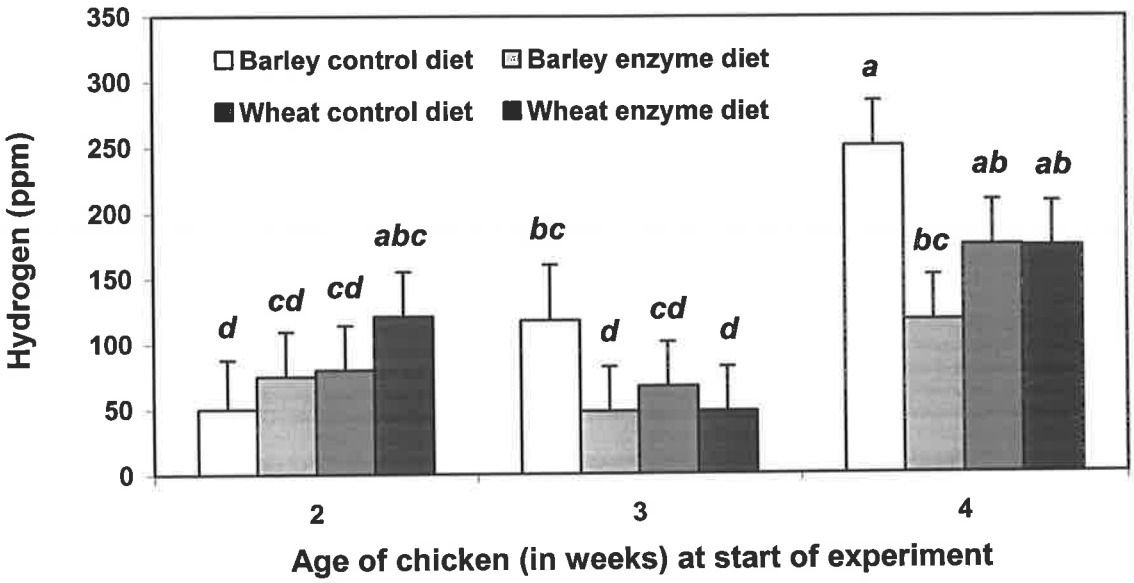


Figure 33. Effects of age of chicken, grain type and addition of enzyme to the diet on hydrogen concentration in breath on day 6 (means ± SE). Means with a common letter are not significantly different (P>0.05). All statistical analysis was done on $\log_e$ transformed data.

6.4.4 Discussion

The hypotheses that AME values increase with age of the chicken, and that the flow of undigested carbohydrate into the hindgut decreases with age, are not supported by the results of this study. In fact, the direct opposites occurred. The significant reductions with age of chicken on AME value of the diet, and increases in hydrogen and methane concentrations, imply a greater uptake of energy by the small intestine and a reduction in the amount of undigested substrate for fermentation by microflora in the hindgut.

The large increases in hydrogen and methane in breath from older chickens after consuming experimental diets for one week (from 29-36 days of age) are not consistent with the findings of McBurney *et al.* (2003) that gut microflora profiles stabilise by about four weeks of age.

The beneficial effects of feed enzymes in improving the AME values of barley and wheat (Bedford and Morgan 1996) were observed in this study also, but the relative improvement seemed to arise from enzymes limiting the reduction in AME with age observed in the

unsupplemented control group. The sparing effect of enzymes on AME with age appears to be related to significant reduction in hydrogen concentration in breath from chickens fed the barley diet, whereas no changes at any stage were evident in chickens fed wheat.

It is possible that the β-glucanase activity of the enzyme product used in this study resulted in release of absorbable sugars from the β-glucan in the barley diet (Bedford 1995). This would allow both an increase in energy uptake by the chicken, and a reduction in the substrate available for bacterial fermentation in the hindgut, thus lowering breath hydrogen concentration. In the case of wheat-fed chickens, the beneficial effects of enzymes on AME are likely to have arisen through a different mode of action as the branched structure of arabinoxylan is more difficult to degrade than the simpler structure of β-glucan Bedford 1995). In this case, the amount of complex carbohydrate reaching the hindgut would be unaffected by enzyme treatment and, hence, breath hydrogen would be similar in both control and treated groups, as was observed in section 6.3.

Hydrogen in breath prior to feeding the experimental diets (day 0) was higher in older chickens, but methane content on day 0 was not affected by age of the chicken. On the other hand, breath hydrogen and methane concentrations did not differ between males and females given commercial broiler feed which is in contrast to results reported in sections 6.2 and 6.3. Nevertheless, the observations in this experiment support the general conclusion from the two experiments described in sections 6.2 and 6.3 that remarkably different metabolic activities of gut microflora can occur in birds reared on starter diets with the same nutrient specifications (but not necessarily the same ingredient composition).

6.4.5 Conclusions

Different types of NSP in grains fed to chickens will influence the metabolic activity of the gut microflora. The specific activities of exogenous enzymes used to improve digestibility of energy will also have a bearing on the fermentation profiles of gut microflora.

6.5 Effects of antibiotics in the feed on the site of digestion of carbohydrate from different types of grain

6.5.1 Introduction

Different types of NSP in grains fed to chickens can influence the fermentation profiles the gut microflora, and exogenous enzymes used to improve digestibility of energy can also have a bearing on the metabolic activity of gut microflora, depending on the type of grain (section 6.4).

Hughes *et al.* (2001) measured AME and ileal DE values for a selection of samples of Australian barley, oats, sorghum, triticale and wheat. They noticed that for each sample of barley and oats AME exceeded ileal DE by about 0.4 MJ/kg, whereas for sorghum samples, ileal DE was approximately 0.3 MJ/kg higher than AME. They concluded that for barley and oats, microbial proliferation in the hindgut utilised energy in the form of otherwise non-digestible carbohydrates. This was thought to lower the gross energy content of the excreta through losses of volatile fermentation products, as discussed by Choct (1999). In the case of sorghum, there was little loss of energy through microbial proliferation in the hindgut, and the difference between DE and AME was assumed to represent endogenous energy losses.

It follows that if microbial overgrowth of viscous digesta in the small intestine can be avoided by use of feed enzymes in order to reduce variation in energy metabolism (Kocher *et al.* 1997), then therapeutic use of antibiotics in the feed ought to reduce variation in energy metabolism by retardation of growth of bacteria, particularly facultative anaerobes (Choct *et al.* 1996*a*), in the distal region of the small intestine. This experiment tested the hypothesis that dietary addition of antibiotics reduced the difference between measured values for ileal DE and AME.

6.5.2 Materials and methods

Ross broiler chickens were obtained from the Bartter Steggles hatchery, Cavan, SA on Thursday 9 March 2000. Chickens were raised from hatch in two floor pens in a controlled

temperature room. Male and female chickens were reared separately. All birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540).

At 22 days of age, chickens were transferred in weighed, single-sex groups of six to 48 metabolism cages located in a controlled-temperature room set at 25-27°C initially. The temperature setting in the room was reduced daily until it was 22°C at the end of the experiment. Chickens had free access to the experimental diets (Table 17) and water.

Table 17. Composition of the experimental basal diets

Ingredient	g/kg
Cereal grain (sorghum, barley, triticale or wheat)	773
Casein	152
Dicalcium phosphate	20
Limestone	11
Sodium chloride	3
DL-methionine	7
Vitamin and mineral premix	2
Choline chloride (60%)	2
Digestibility marker (Celite)	30

The grains were barley (control FG96B.3815), sorghum (FG99S.7832), triticale (frosted FG98T.6805) and wheat (low AME, LS97W.1906) from the GRDC Premium Grains for Livestock Program.

Emtryl (500 ppm) and Lincospectin (44 ppm) were included in the premix of ingredients for the antibiotic treated diets. These antibiotics were selected for their broad spectrum activity against a wide range of gut bacterial species. The intention was to reduce the bacterial population as much as possible by using readily available antibiotic products in a prescribed manner. Each of the eight experimental diets was replicated six times (three groups of female and three groups of male chickens).

The AME values of the experimental diets were determined in a conventional energy balance study involving measurements of total feed intake and total excreta output and subsequent measurement of gross energy values of feed and excreta as per the procedures described in previous section .

One chicken in each cage was selected at random and tagged for repeated breath testing. Hydrogen content of breath was measured on days 0 and 6 of the 7-day experimental period to gauge the changes in the metabolic activity of the gut microflora in response to antibiotics and type of grain in the diet. A breath sample was taken 30 seconds after a helmet was placed over the head of the chicken and held firmly against the shoulders as per the procedures described in previous sections.

At the end of the 7-day metabolism study, three chickens from each cage were weighed then killed by intravenous injection of pentobarbitone. The ileum (from 1 cm below Meckel's diverticulum to 4 cm above the ileo-caecal junction) was dissected from each bird. Ileal digesta were pooled and stored on ice. Ileal digesta was centrifuged at 12,000 g for 12 minutes at 4°C for viscosity measurement of fresh supernatant using a Brooffield DVIII model viscometer with a CP40 cone and shear rate of 5-500 s^{-1}. Then samples were re-constituted, frozen and stored at -20°C pending analyses to determine GE and acid-insoluble ash (digestibility marker) for calculation of ileal DE (Appendix 3).

Statistics

Distribution of data, transformation if required and subsequent analyses of variance and covariance were conducted as described in section 6.2.3.

6.5.3 Results

The inclusion of antibiotics in the feed did not significantly affect AME values for the diet or the cereal grain, ileal DE, or AME and DE as fractions of gross energy (GE) of the diets (Table 18 and Figure 34).

Table 18. Reduced model analysis of variance of data showing significance of main effects of type of grain in the diet (G), dietary addition of antibiotics (A), sex of the chicken (S), and interactions between grain, antibiotics and sex.

Variable	G	A	S	GxA	GxS
Feed intake (g/bird/day)	***		***	0.10	
Weight gain (g/bird)	***	***	**	0.09	*
Rate of gain	***	***	**		
Feed conversion ratio	***	***	***	***	***
Excreta moisture content (%)	***		*		
AME diet (MJ/kg DM)	***		***		***
AME grain (MJ/kg DM)	***		***		***
Dry matter digestibility	***		***		***
Ileal digestible energy (DE, MJ/kg DM)	***				
Ratio of AME to gross energy	***		***		***
Ratio of DE to GE	***				
Ratio of DE to AME	*		0.10		
Log_e transformed ileal viscosity (cP) †	***				
Log_e transformed breath hydrogen day 6 (ppm) †	***			**	
Log_e transformed breath hydrogen change (ppm) †	**			*	

† All statistical analyses were done on log_e transformed data

$P<0.05$, ** $P<0.01$, *** $P<0.001$ -- numerical values are shown where $0.10>P>0.05$

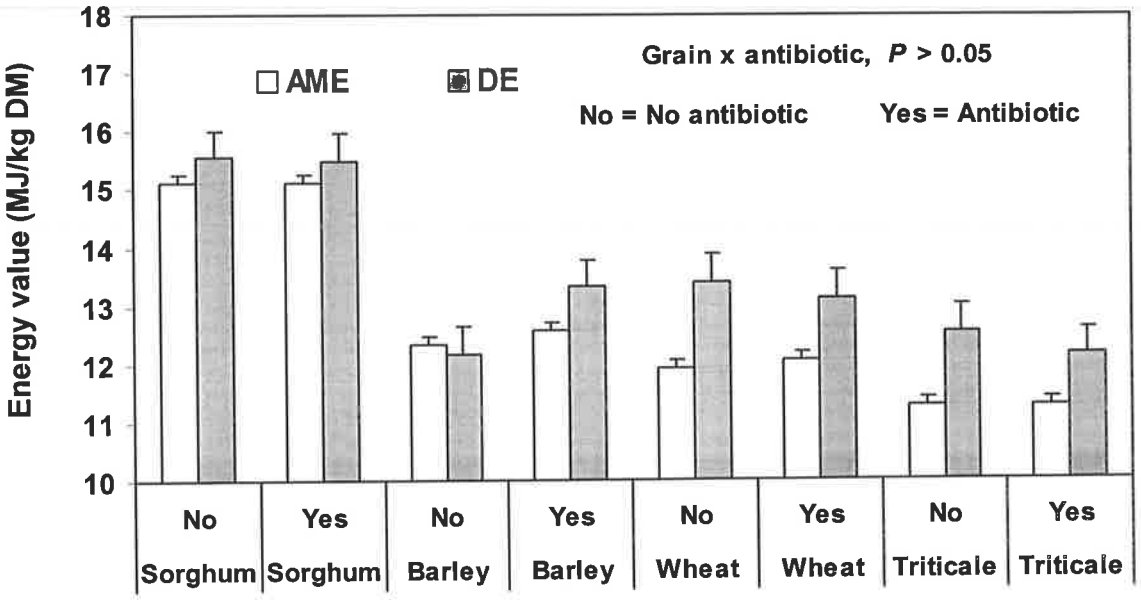


Figure 34. Effects of grain and antibiotic on AME and ileal DE of the diets (means ± SE).

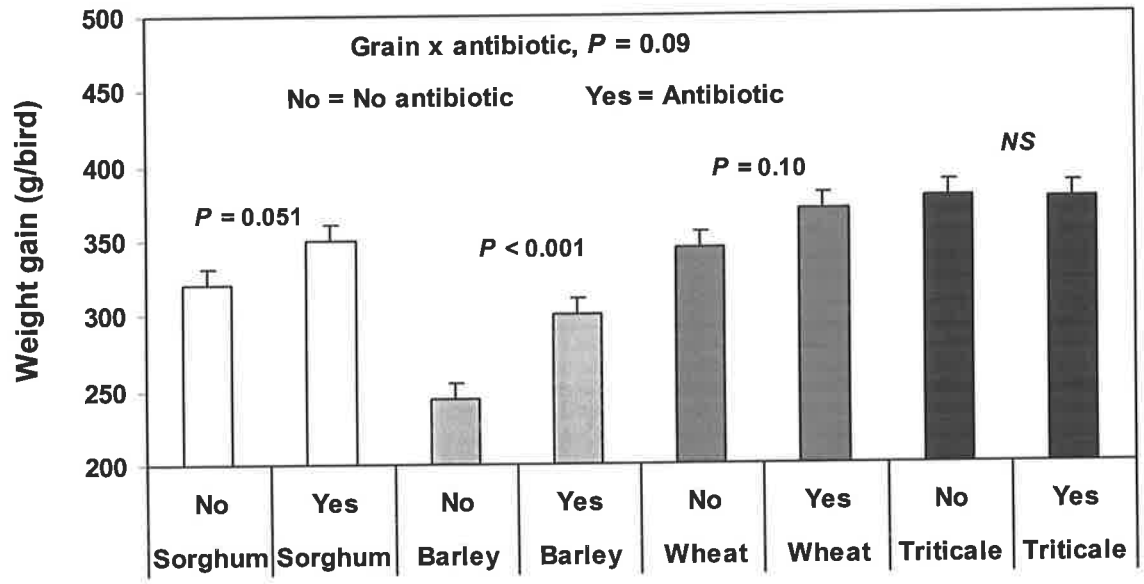


Figure 35. Effects of grain and antibiotic on live weight gain (means ± SE).

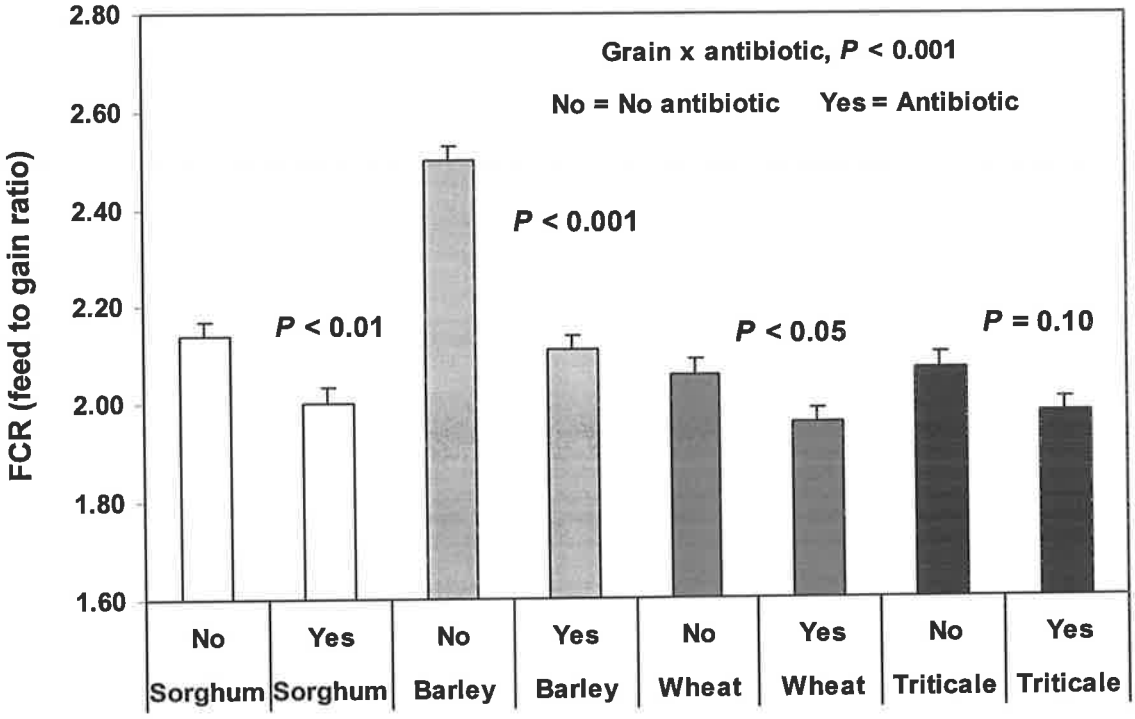


Figure 36. Effects of grain and antibiotic on feed conversion ratio (means ± SE).

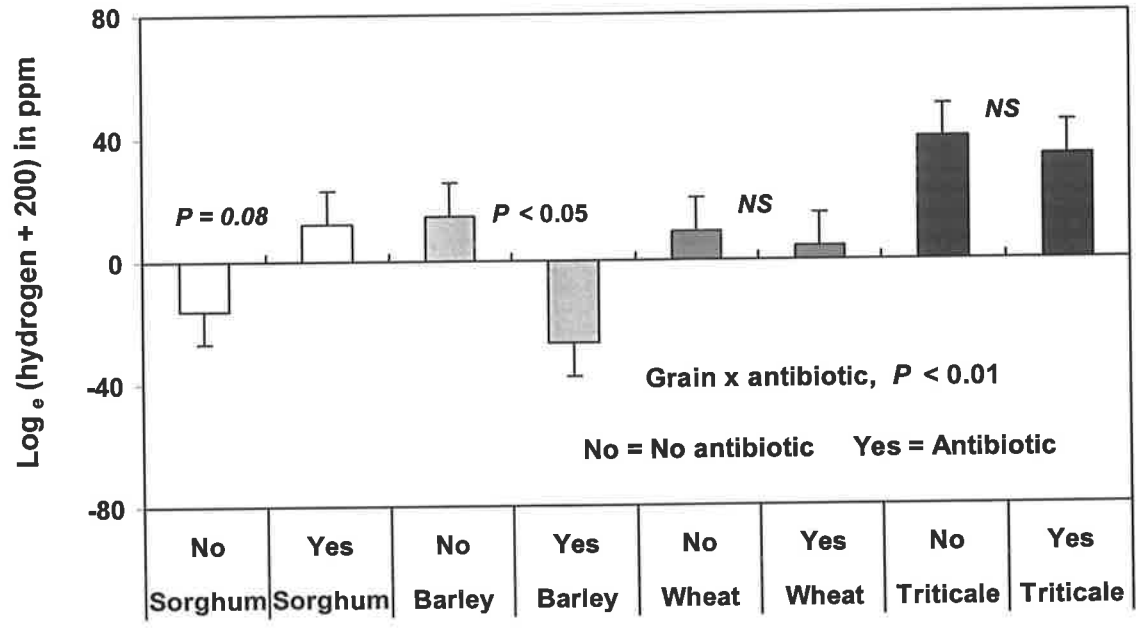


Figure 37. Effects of grain and antibiotic on change in hydrogen concentration in breath from day 0 to day 6 of the 7-day metabolism study (means ± SE).

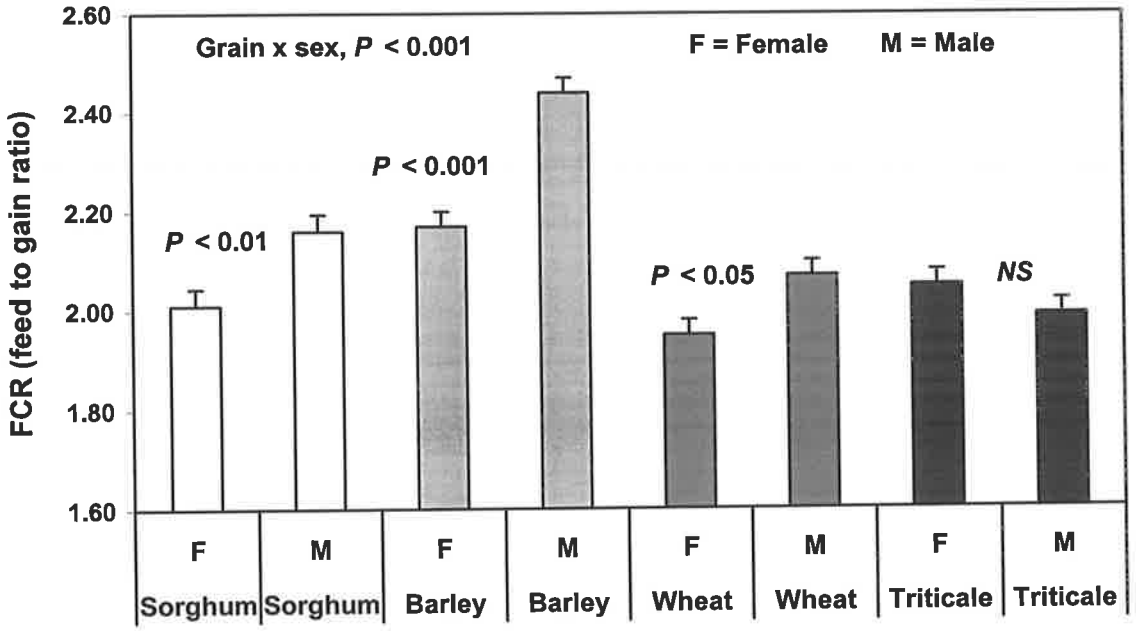


Figure 38. Effects of grain and sex of chicken feed conversion ratio (means ± SE).

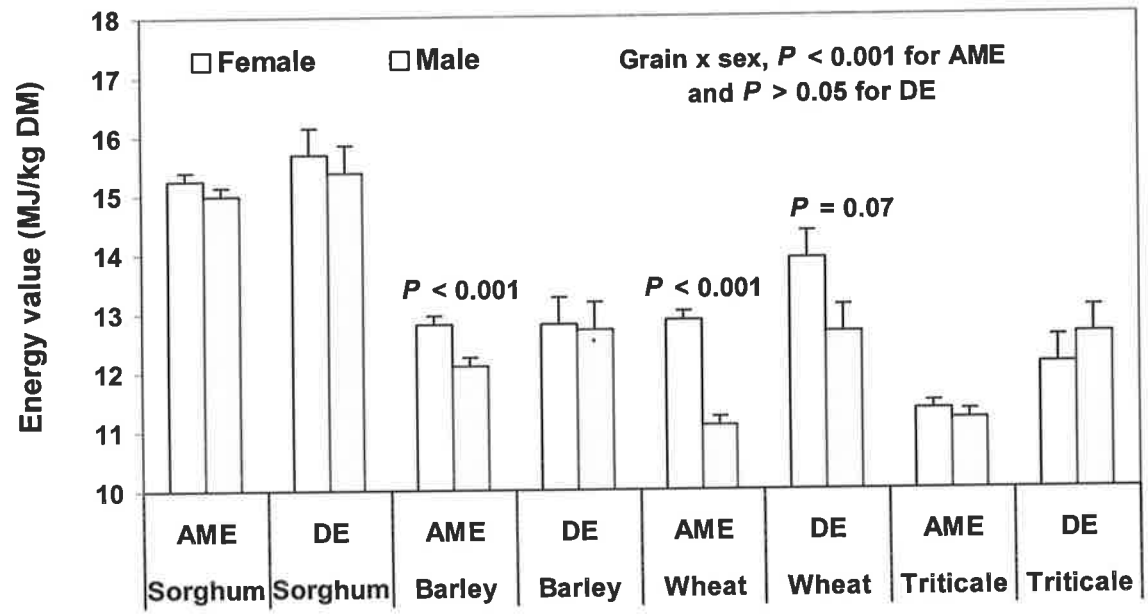


Figure 39. Effects of grain and sex of chicken on AME and ileal DE of the diets (means ± SE).

However, antibiotic treatment improved, or tended to improve, weight gain (Figure 35) and feed conversion (Figure 36) for each cereal grain except triticale, but had no effect on feed intake. The effects of antibiotic treatment on metabolic activity of the microflora as indicated by breath hydrogen measurements differed across grain types (Table 18). Antibiotic treatment resulted in a significant ($P<0.05$) decrease in breath hydrogen from chickens given barley compared with the respective control diet, but there was no significant ($P>0.05$) response to antibiotics observed in chickens fed triticale or wheat (Figure 37). There was a tendency ($P=0.08$) for an increase in breath hydrogen due to antibiotic supplementation of the sorghum diet (Figure 37).

Feed conversion, live weight gain and energy uptake on different cereal grains were significantly ($P<0.05$) influenced by the sex of the chicken (Table 18). Males ate more feed and grew faster than females on the same grain type, but converted less efficiently than females when given sorghum, barley or wheat diets (Figure 38). Male chickens had significantly ($P<0.05$) lower AME values than females when given barley and wheat diets (Figure 39). Ileal DE values for wheat tended ($P=0.07$) to be lower for males than females (Figure 39).

6.5.4 Discussion

It was evident that antibiotics did not reduce the differences between AME and DE values for any of the grains, hence the hypothesis was not supported. The lack of an effect of antibiotics on excreta moisture content suggests that administration of therapeutic levels of antibiotics did not induce scouring, which may otherwise have influenced energy metabolism.

Antibiotic treatment resulted in a numerical increase in hydrogen concentration in breath of chickens given sorghum but a decrease in chickens given barley (Figure 37) compared with the respective control diets. Any increase in breath hydrogen in chickens given a sorghum diet supplemented with antibiotics would tend to suggest that there was an increase in fermentable substrate reaching the distal part of the intestinal tract. This is difficult to explain on the basis of an increased amount of carbohydrate or NSP reaching the hindgut because AME and DE were unaffected by dietary addition of antibiotics. The decrease in breath hydrogen in chickens fed the barley diet supplemented with antibiotics looks similar

to the response to enzyme addition to a barley diet reported in section 6.4. In that case, it was suggested that the reduction in breath hydrogen was related to the reduction in the amount of β-glucan reaching the hindgut and an associated increase in AME. This is not a plausible explanation for the present response to antibiotics unless one of the end products of bacterial fermentation included an enzyme able to degrade β-glucan (Choct and Kocher (2000). The possibility that gut microflora can produce NSP-degrading enzymes is explored in section 6.6.

Differences in the amounts of hydrogen produced are indicative of changes in the numbers and/or species of bacteria in the gut population, and/or changes in the metabolic activities of those bacteria. Furthermore, these changes in hydrogen concentration in response to antibiotics were also dependent on the type of grain used in the diet. Presumably, the types and amounts of undigested nutrients flowing into the hindgut provided different substrates for growth of bacterial species surviving antibiotic treatment. As pointed out in section 6.2.5, shifts in the production of volatile fatty acids and sulphates could affect the pH of the luminal environment.

The lack of a significant difference ($P>0.05$) in the DE:GE ratio between males and females on sorghum, barley, wheat and triticale diets (Figure 39) implies that digestive and absorptive processes in the small intestine were unaffected by the sex of the chicken. On the other hand, male chickens had significantly lower AME values than females when given barley and wheat diets (Figure 39). Therefore, the differing effects of sex on DE and AME values for these grains suggest that post-intestinal processes and events associated with gut microflora were influenced by the sex of the chicken.

6.5.5 Conclusions

The influence of gut microflora on energy metabolism differed according to the type of grain, and is at least partially dependent on the sex of the chicken. This implies "communication" between the host and gut microflora that affects the metabolic activity of the bacteria, and possibly the function of the host mucosal tissue. Alteration of the balance between the host and its commensal microflora by feeding different grains or antibiotics is likely to result in patterns of growth and nutrient utilisation that are difficult to predict.

6.6 Synthesis of NSP-degrading enzymes by gut microflora in chickens given wheat and barley diets

6.6.1 Introduction

Choct and Kocher (2000) reported that the ability of gut microflora to produce enzymes able to degrade NSP influenced between-bird variation in AME. They found that xylanase activity produced by microflora degraded NSP in the caeca and lowered the viscosity of excreta below that of ileal digesta collected from the same bird. However, they did not observe an inverse relationship between AME and viscosity of ileal digesta. Kocher and Choct (2000) concluded that excreta viscosity was a non-invasive indicator of microbial enzyme activity in the hindgut. It seems reasonable to believe that some of the between-bird variation in AME reported in Chapter 3, and an increase in breath hydrogen observed in chickens given a sorghum diet supplemented with antibiotics (section 6.5), could have arisen from microbial production of NSP-degrading enzymes, and hence worthy of further investigation.

The possibility was raised in section 6.2.5 that losses of volatile products could reduce the gross energy content of excreta and hence result in an overestimation of the AME value. Also, variation in any volatile losses could contribute to variation in measured values for AME depending on the metabolic activity of the microflora in different chickens.

The experiment tested the hypotheses that (a) AME was inversely correlated with viscosity of ileal digesta, and with viscosity of freshly-voided excreta, and (b) that losses of volatile fatty acids from excreta were variable.

6.6.2 Materials and methods

Birds, housing and management

Ross chickens were obtained from the Bartter Steggles hatchery, Cavan, SA on Thursday 18 November 1999 and raised from hatch in floor pens in a controlled temperature room. Male and female chickens were reared separately. All birds were given commercial starter crumbles (Ridley Agriproducts, diet code #503540).

At 18 days of age, chickens were transferred in pairs to 96 metabolism cages located in a controlled-temperature room set at 25-27°C initially, and given commercial starter crumbles (Ridley Agriproducts, diet code #503540) for 2-3 days while they adapted to the cages. The temperature setting in the room was reduced daily until it was 22°C at the end of the experiment.

At 21 days of age, one bird was removed from each of the first 48 cages. The 48 chickens remaining were weighed, and then returned to the cage. Chickens had free access to the experimental diet (Table 19) and water throughout the study. These chickens were killed on day 7 (at 28 days of age) for digesta and tissue samples. Chickens in the other 48 cages were treated in the same manner in the period 22 to 29 days of age.

Experimental diets and AME measurement

Table 19. Composition of the basal diet

Ingredient	g/kg
Grain (wheat or barley)	700
Meat and bone meal	76
Soybean meal	170
Vegetable oil	40
Sodium chloride	2.5
L-lysine HCl	2.5
DL-methionine	3
Vitamin and mineral premix with millrun diluent	5
Choline chloride (60%)	0.8

Dr Mingan Choct (UNE) supplied the wheat soon after harvest in the 1996/97 growing season. A subsequent experiment in August 1997 involving 32 individually housed chickens at PPPI verified that it had a low-AME value (13.1 MJ/kg DM). The wheat was kept in a cool room (4°C) between AME testing and its subsequent use in this experiment to avoid any post-harvest change in AME (Choct and Hughes 1997). Similarly, a sample of barley (FG96B.3815) with AME (12.9 MJ/kg DM) was chosen from the collection of grains used in the GRDC Premium Grains for Livestock Program. The AME value of barley was measured in an experiment conducted at PPPI in October 1999 (Hughes *et al.*

2001). The diets were cold pressed into pellets approximately 6 mm in length and 4mm in diameter.

The AME values of the experimental diets were determined in a conventional energy balance study involving measurements of total feed intake and total excreta output and subsequent measurement of gross energy (GE) values of feed and excreta by isoperibol bomb calorimetry (Parr Instrument Company).

Collection and measurement of digesta and excreta

On day 5 of the metabolism study, fresh excreta (12 g) samples were collected on plastic sheets placed in the excreta collection trays for 1-2 hours. Approximately 10 g samples were weighed in tared plastic screw-cap jars, treated with 0.5 M sulphuric acid (0.5 mL per g excreta), then securely sealed in a plastic jar to minimise the loss of volatile components, and kept on ice until placed in a freezer at -20°C. Approximately 2 g fresh excreta samples was placed in untared tubes and centrifuged at 12,000 g for 12 minutes at 4°C. Viscosity of supernatant was determined on 0.5 mL of supernatant using a Brookfield DVIII model viscometer at 25°C with a CP40 cone and shear rate of 5-500 s^{-1}.

At the end of the 7-day metabolism study, each chicken was weighed then killed by intravenous injection of pentobarbitone. The ileum, from 1cm below Meckel's diverticulum to 4cm above the ileo-caecal junction, and the caeca was dissected. Approximately 3 g ileal digesta was weighed in a tared centrifuge tube, and stored on ice pending viscosity measurement of fresh supernatant.

Excreta samples were sent to the University of New England for measurement of volatile fatty acids by gas liquid chromotography as described by Kocher (2000).

Statistics

Distribution of data, transformation if required and subsequent analyses of variance and covariance were conducted as described in section 6.2.3.

6.6.3 Results

The results of analysis of variance are summarised in Table 20.

Table 20. Analysis of variance of data showing significance of main effects of sex of the chicken (S), and of type of grain in the diet (G), and the interaction between sex and grain.

Variable	S	G	SxG
Live weight at start (g/bird)	***		
Feed intake (g/bird/day)	***	***	
Weight gain (g/bird)	**	***	*
Feed conversion ratio	*	***	**
Excreta moisture content (%)			
AME diet (MJ/kg DM)	*	***	*
Dry matter digestibility		***	*
Log $_e$ transformed ileal viscosity (cP) †		***	
Log $_e$ transformed excreta viscosity (cP) †			
Difference between excreta viscosity and ileal viscosity (cP)		***	

† All statistical analyses were done on log $_e$ transformed data

$P<0.05$, ** $P<0.01$, *** $P<0.001$

Male chickens were significantly heavier than females at the start of the experiment (938 vs 827 g/bird; $P<0.001$), and ate more feed (108 vs 95 g/bird; $P<0.001$). There were no differences ($P>0.05$) due to sex in excreta moisture (74%), viscosity of ileal digesta (24.8 cP) viscosity of fresh excreta (7.8 cP), or change in viscosity 17cP). Chickens given the barley diet ate less feed (94 vs 109 g/bird; $P<0.001$), gained less weight (291 vs 432 g/bird; $P<0.001$), had higher excreta moisture (74.6 vs 73.1%; $P<0.001$), and more viscous ileal digesta (30.1 vs 19.3 cP; $P<0.001$) compared with chickens fed wheat.

The grain by sex interaction was significant for live weight gain, feed conversion ratio, and dietary AME. For weight gain there was no difference ($P>0.05$) due to sex for chickens given the barley diet (291 g/bird) whereas males given the wheat diet were significantly heavier than females (466 vs 397 g/bird; $P<0.001$). In contrast, feed conversion (1.78 g feed: g gain) and AME (14.8 MJ/kg DM) were unaffected ($P>0.05$) by sex of chicken given wheat, whereas there were large differences in favour of females given barley for

feed conversion (2.16 vs 2.48 g feed: g gain; $P<0.001$) and AME (12.8 vs 12.0 MJ/kg DM; $P<0.01$).

There was no significant (P>0.05) statistical relationship between AME and excreta viscosity in contrast to the earlier work of Choct and Kocher (2000). However, there were statistically significant ($P<0.01$) but weak inverse relationships between AME and viscosity of ileal digesta (AME = 16.0 – 0.79 x $\log_e$ transformed ileal viscosity; $R^2=0.10$) and between AME and the difference between viscosities of ileal digesta and fresh excreta (AME = 14.1 – 0.028 x change in viscosity; $R^2=0.09$, Figure 40).

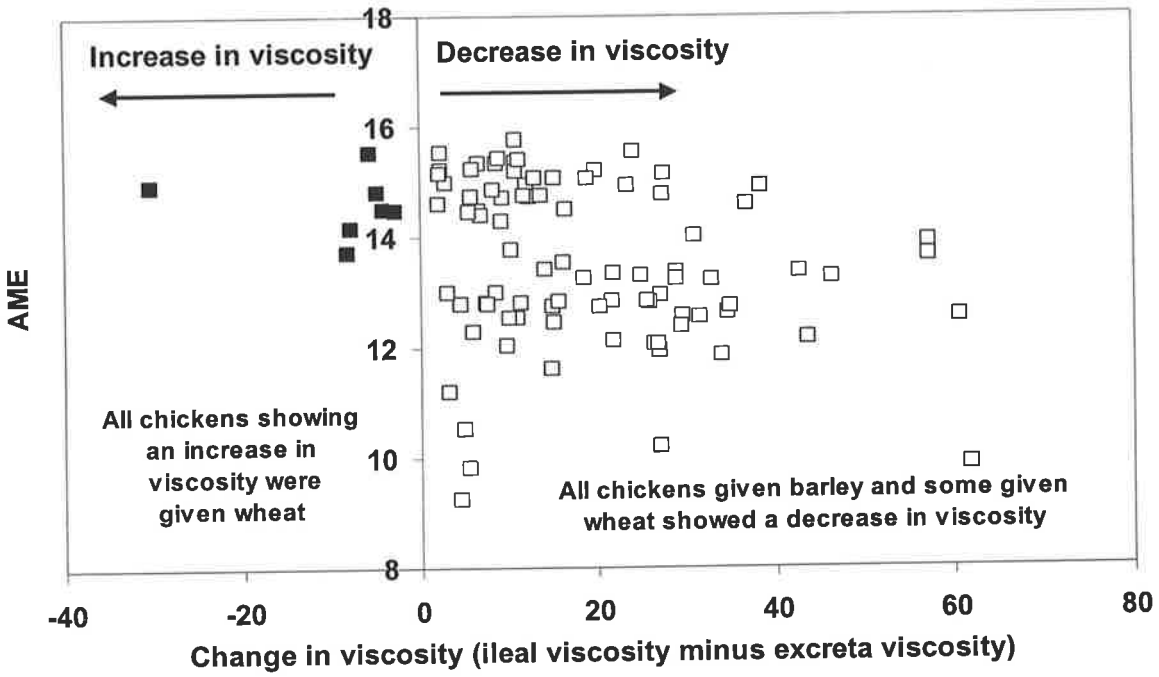


Figure 40. Association between AME (MJ/kg DM) of diet and change in viscosity as digesta passed through the hindgut.

The data for acetic acid and butyric acid concentrations in excreta were so highly skewed that it was not possible to transform these sufficiently to conduct analysis of variance. The raw data are plotted in Figure 41. The two male chickens with the highest concentrations of acetic acid in Figure 41 each had an AME value of 12.8 MJ/kg DM for the barley diet compared with a mean value of 12.0 MJ/kg DM for male chickens.

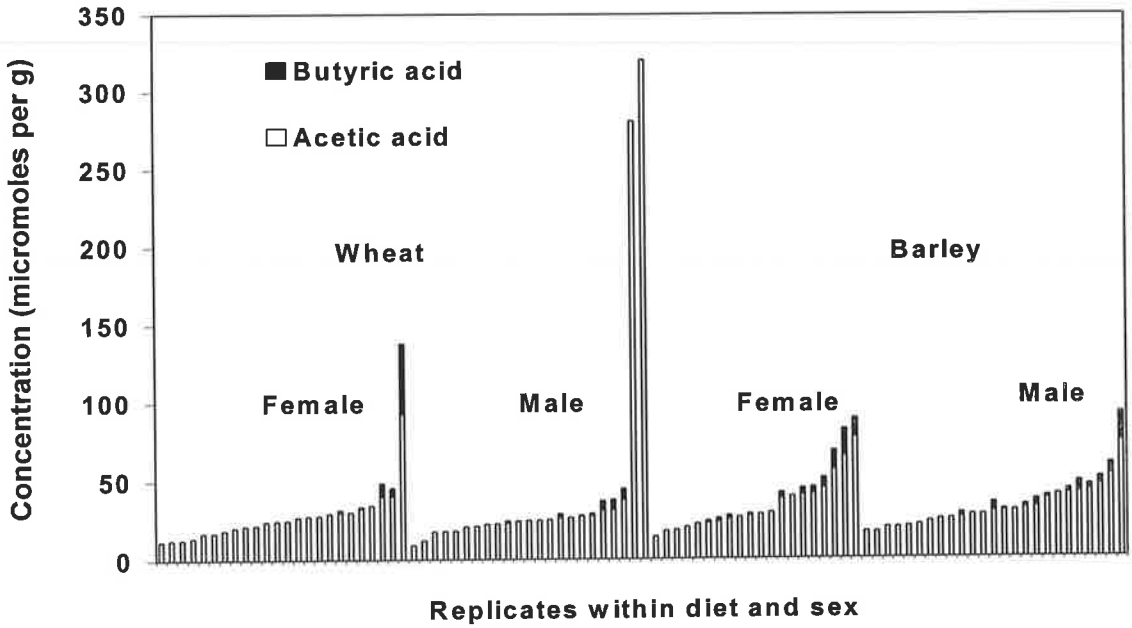


Figure 41. Concentrations of acetic acid and butyric acid in fresh excreta. Data are sorted by acetic acid concentration within each combination of grain type and sex of chicken. Each bar represents results from one chicken.

6.6.4 Discussion

The hypothesis that AME is inversely related to ileal viscosity is supported, whereas the hypothesis concerning the relationship between AME and excreta viscosity is not supported. An increase in viscosity of excreta relative to viscosity of ileal digesta (Figure 40) could be explained by the release of viscosity-inducing NSP from cell walls from wheat, but not from barley, through the action of microbial enzymes on undigested remnants leaving the small intestine. Clearly, this response was not evident in all chickens given the wheat diet. Also, production of acetic and butyric acids in fresh excreta differed widely between individual male and female chickens and between chickens fed the wheat and barley diets (Figure 41). If these observations are indicative of changes in the profiles of bacterial populations in these chickens then it follows that variation in production of microbial enzymes could also contribute to the variability in measurements of AME.

6.6.5 Conclusions

Viscosity of excreta was a poor predictor of the AME value of the diet. An increase in viscosity of excreta relative to viscosity of ileal digesta could be explained by microbial

production of enzymes capable of cleaving NSP from cell walls in wheat but not in barley. Hence, the *in situ* microbial synthesis of NSP-degrading enzymes could add to the variability in measurements of AME.

6.7 General discussion and conclusions

Large differences in concentrations of hydrogen and methane occurred between individual chickens which had been hatched and reared under the same conditions. This was noted in several different experiments reported in this thesis. Between-chicken variation in composition of the breath points to the possibility of other differences in the fermentation patterns in the hindgut, such as the formation of acetate and other short chain volatile fatty acids, lactic acid, and in the metabolic activities of bacteria which utilise sulphate. Changes such as these could alter pH of the luminal environment, which in turn could favour some species of bacteria over others. When chickens were subjected to abrupt dietary changes, large changes in breath composition were observed which indicates a further shift in the metabolic activities of the microflora. Between-chicken variation in concentrations of acetate and butyrate in freshly-voided excreta was a further indication of variable shifts in the metabolic activities of the microflora and/or changes in the bacterial population.

The influence of gut microflora on the nutritive value of different cereal grains is at least partially dependent on the sex of the chicken. This implies "communication" between the host and gut microflora that affects the metabolic activity of the bacteria, and possibly the host tissue. The site of receival of chemical messages broadcast by microflora could be distally remote from the site of transmission. Conversely, in the case of proliferation of microflora in the small intestine (Choct *et al.* 1996*a*), it is feasible that the influence is felt in upper regions of the tract, which could be detrimental to the uptake of energy and other nutrients, thus affecting growth and feed efficiency. Alteration of the balance between the host and its commensal microflora by feeding different grains or antibiotics is likely to result in patterns of growth and nutrient utilisation that are difficult to predict.

Bacterial overgrowth of the gut is likely to have detrimental effects partly due to significant losses of nutrients. In addition, increased concentrations of toxic waste products and cell

wall antigens from proliferating commensal organisms could ultimately lead to health problems through general inflammation of the gut, thus paving the way for invasion of tissue by other organisms pathogenic to the bird.

During the course of these studies, the overall or net metabolic activity of the gut microflora was assessed indirectly through non-invasive methods such as measurement of breath hydrogen and methane, and volatile acids in voided excreta. The non-invasive approach could be taken further through *in vitro* studies involving metabolic inhibitors of bacteria taken from excreta to elucidate the effects of different dietary components on fermentation patterns of the commensal bacteria in the hindgut. If faeces and caecal contents were separated then it may be possible to distinguish between the activities of microflora from the lower small intestine and those from the caeca. These non-invasive methods need to be validated against direct measurements of various fermentation products in different regions of the intestinal tract.

In summary, a fuller understanding of the role of the gut microflora on energy metabolism of the chicken is required. This will be an essential step towards knowing how to control the colonisation of the gut in newly hatched chicks and to how to maintain an ideal microflora for the life of the bird, which would be beneficial for the health, welfare and performance of commercial flocks.

Chapter 7. General discussion and summary

7.1 Introduction

A diet for broiler chickens that provides all of the nutrients essential for maintenance and growth of the flock as a whole may also have other chemical and physical properties that are detrimental to the processes of ingestion, digestion, absorption, transport and utilisation of nutrients (Hughes and Choct 1999). The Australian chicken meat industry is highly dependent on supply of energy from cereals such as wheat and barley that are known to vary widely in apparent metabolisable energy (Choct 1999). In contrast, sorghum is generally assumed to be a relatively consistent source of energy. To date, triticale has proven to be a relatively reliable source of energy not subject to the low AME phenomenon observed in some samples of wheat (Hughes and Cooper, 2002).

Cereal grains are a major source of anti-nutritive components that are likely to have significant bearing on how effectively all dietary components are utilised by poultry. Of the known causes of variation in energy value of grains, soluble NSP stand out as a major determinant of the availability of energy and other nutrients (Hughes and Choct 1999).

One of the modes of action of soluble NSP is to form a viscous gel in the gut, which in turn affects the rates of digestion and absorption of nutrients. Also, rates of gastric emptying and transit time of digesta are thought to be influenced by increased digesta viscosity, thereby providing hind-gut microflora with an opportunity to colonise the small intestine to the detriment of the host in terms of diminished use of nutrients and reduced ability to ward off ingested pathogens (Choct 1999). Exogenous glycanases are able to depolymerise NSP thereby reducing the deleterious effects resulting from increased viscosity of digesta (Choct *et al.* 1996a). Feed enzymes have proven to be a very effective tool for not only increasing the energy values of grains for poultry but also for improving the uniformity of growth and feed efficiency of broiler flocks. Despite the global success of feed enzyme technology, questions remain about the specific modes of actions of enzymes (Smits and Annison 1996; Williams 1997), and why enzymes can reduce but do not eliminate variation in energy values for grains (Bedford 1996; Kocher *et al.* 1997).

The broad objective of this project was to develop a clearer understanding of the physiological limitations to digestive function of broiler chickens particularly in relation to energy metabolism. The general hypothesis examined in this project was that feed-related factors such as NSP induce variable responses among individual chickens within the flock in the usage of carbohydrate from cereal grains as a source of energy. The work carried out during the course of these studies is described in five chapters, and is summarised in the following sections.

The most important findings in these studies were that the sex of the chicken and the overall or net metabolic activity of the gut microflora were major contributors to variation in energy digestion. Furthermore, it became apparent from the results of several different experiments described in this thesis that gut microflora profiles differed between male and female chickens.

These and other findings concerning the effects of interactions between bird- and feed-related factors on energy metabolism are summarised in the following sections.

7.2 Influence of gut structure on digestion of energy by broiler chickens

The relative importance of the structure of the small intestines of chickens as a potential source of variation in energy metabolism was examined in Chapter 2. Gross morphology of the small intestine and the finer structure of the intestinal mucosa differed between male and female chickens. Females appear to have more potential ability than males for digestion and absorption of carbohydrate and fat as a result of them having a relatively larger pancreas and jejunum than males. However, between-bird variation in AME could not be fully explained by differences in the weights of duodenum, jejunum and ileum relative to metabolic body weight, or by changes in villus height or crypt depth in these sections of the small intestine. It was concluded that factors other than gut morphology were responsible for up to 67% of the variation observed in AME.

7.3 Variable responses in digestion of energy from a wheat-based diet are associated with sex and strain of chicken and morphology of the small intestinal mucosa

AME values for females were 4% higher than for males, with such a difference being highly significant in commercial terms. The patterns of AME values obtained with individual chickens and the corresponding coefficients of variation for the strain by sex combinations, indicated that females are more uniform than males. Some individual chickens, particularly males, responded poorly to the wheat diet compared with others. On the other hand, some males responded just as well as the best performing females. This is despite chickens being reared in the same room from hatch, and receiving the same rearing diet prior to the experiment. It seems that female chickens were better adapted than males to handle a poor quality diet, but there was no difference between males and females when a high quality feed was given. These results point to the existence of a fundamental difference between males and females in overall energy metabolism, irrespective of the strain, possibly associated with differences in energy costs to repair and maintain the gut, endogenous losses, or responses to inflammatory effects of microflora.

7.4 Breath tests as non-invasive indicators of digestive function of broiler chickens and metabolic activity of gut microflora

A specific objective in this project was the adaptation of breath tests used for clinical diagnosis of intestinal disorders in humans for use as non-invasive methods for assessing digestive functions in chickens. It was expected that breath tests would provide more insight than the usual snapshot view obtained in conventional nutrient balance studies, and that these non-invasive tools would allow the study of the dynamic interplay between various factors affecting energy uptake in broilers. It was shown in Chapter 4 that with breath tests, it was possible to identify certain characteristics of individual chickens prior to experimentation with soluble NSP and endogenous enzymes in order to follow the resulting changes in gastro-intestinal functions such as digesta transit time, and microbial proliferation in the small intestine.

7.5 Effects of rate of passage of digesta on the digestion of energy in chickens

There was good agreement between oro-caecal transit time determined by rise in breath hydrogen and whole tract transit time measured by appearance of ferric oxide marker in excreta, although whole tract transit times were generally shorter than oro-caecal transit times. Other results obtained in the work described in this chapter were inconclusive probably because the concentration of NSP in the wheat used in this experiment was too low to produce a viscous digesta. Despite the wheat having a very high AME value overall, some chickens with longer transit times seemed able to extract more energy from the diet than those with shorter transit times.

The rate of passage of digesta appears not to be the reason for differences between males and females in the digestion of energy. However, differences between males and females in breath hydrogen concentrations pointed to sex-dependent differences in fermentation patterns in the hindgut.

A non-invasive breath test for estimation of oro-caecal transit time can be devised without the need to dose chickens with lactulose. That is, caecal microflora can produce measurable concentrations of hydrogen in breath by fermentation of existing levels of complex carbohydrates in typical broiler feed.

7.6 Influence of gut microflora on digestive function of broiler chickens

In Chapter 6, hydrogen and methane breath tests were used as indicators of the metabolic activity of the gut microflora in a series of five experiments. Responses to low AME wheat by individual chickens were highly variable in terms of acetic acid and butyric acid concentrations in freshly voided excreta in one experiment, and in hydrogen and methane concentrations in breath in two subsequent experiments with different batches of chickens.

Observations in regard to expiration of hydrogen and methane pointed to remarkably different metabolic activities of gut microflora in birds reared on commercial diets with the same nutrient specifications (but not necessarily the same ingredient composition), and to

wide variation between chickens in the metabolic activity of the gut microflora following a change in the composition of the diet.

The feeding of a commercial starter diet led to the development of substantially different gut microflora profiles in 15-day old male and female chickens, with female chickens exhaling almost twice the concentration of hydrogen compared with males. These patterns changed when chickens were given a diet containing a high level (700g/kg) of wheat. Presumably, the viscosity reducing action of exogenous enzymes contributed to enhanced starch digestion in the small intestine, but did not alter the amounts of complex carbohydrate reaching the hindgut. That is, feed enzymes did not appear to influence the fermentation patterns of the gut microflora in this experiment.

The effect of age of chickens on responses to enzyme addition to wheat and barley diets were examined in the fourth experiment described in Chapter 6. Enzymes improved growth, feed conversion and AME for each age of chickens. Hydrogen and methane in breath prior to the feeding of experimental diets did not change with the age of chicken. However, large increases in hydrogen and methane in breath from older chickens during the 7-day experiment point to an increase in carbohydrate escaping digestion in the small intestine leading to increased metabolic activity of the gut microflora. Different types of NSP in grains fed to chickens influenced the metabolic activity of the gut microflora. The specific activities of exogenous enzymes used to improve digestibility of energy also had a bearing on the fermentation profiles of gut microflora, in contrast to the result described in the paragraph above.

The influence of gut microflora on the nutritive value of different types of cereal grains was studied in the fifth experiment described in Chapter 6. Antibiotics were added to the feed in an attempt to reduce the population of bacteria in the hindgut and thereby promote more uniform usage of carbohydrate from the grains. The effects of antibiotic treatment on metabolic activity of the microflora was indicated by breath hydrogen measurements differing across grain types. Antibiotic treatment resulted in an increase in hydrogen production in chickens given sorghum but a decrease in chickens given barley. On the other hand, antibiotic treatment failed to affect either ileal DE or AME.

The influence of gut microflora on the site of digestion of carbohydrate differed between male and female chickens, and according to type of grain used in the diet. Ileal DE values for wheat and barley were unaffected by sex, whereas AME values were lower in male chickens compared with females. These contrasting effects strongly imply that post-intestinal processes and events associated with gut microflora were critically influenced by the sex of the host chicken.

This poses questions as to what occurs at the organ or cellular level that enables the gut microflora to change so radically in terms of numbers, species or activities according to the nature of the feed consumed and the sex of the host animal. Kelly and King (2001) remarked that the molecular basis for how the gut distinguishes between commensal and pathogenic bacteria was poorly understood but that there was "bi-directional communication" between epithelial cells, cells in the immune system, and gut bacteria. Likewise, Bedford and Apajalahti (2001) referred to a "two-way negotiated process" between host tissue and microflora resident in the small intestine. The likely signalling compounds involved in cross-talk, and deserving of further study, are cytokines and polyamines (Kelly and King 2001).

Differences in the amounts of hydrogen produced are indicative of changes in the numbers and/or species of bacteria in the gut population, and/or changes in the metabolic activities of those bacteria. Furthermore, these changes in hydrogen concentration in response to antibiotics were also dependent on the type of grain used in the diet. It is also evident from these results that antibiotics did not bridge the gap between AME and DE values. Presumably, the differential flow of undigested nutrients into the hindgut created different growth media for those hydrogen-producing species of bacteria surviving antibiotic treatment.

The influence of gut microflora on the digestive function of the chicken was partially dependent on the composition of the diet. Inherent characteristics of grains induced different responses in energy metabolism in male and female chickens. The likelihood that requirements for nutrients other than energy are also sex-dependent warrants investigation.

It seems reasonable to suggest that some of the between-bird variation in AME observed in this and other studies could have arisen from microbial production of NSP-degrading

enzymes, and that high concentrations of arabinoxylans and ß-glucans in wheat and barley can induce gene expression for xylanase and ß-glucanase activities in gut bacteria.

Hydrogen and methane concentrations in the breath of chickens were highly variable within and between experiments described in this thesis. It is possible that some of this variation can be attributed to the movement of digesta in the entire gastrointestinal tract, and how faecal matter from the intestine and caecal matter are voided.

Sklan *et al.* (1978) observed a shuttling of digesta between the gizzard and duodenum in 4- and 7-week old male chickens. Godwin and Russell (1997) demonstrated a reverse flow of caecal material to the crop in 6-week old chickens fasted for as little as four hours, but not in fully-fed birds. The observations of Godwin and Russell (1979) are at odds with those of Akester *et al.* (1967) who claimed that retrograde movement of material from the cloacal region was a mechanism for caecal filling but that no material passed into the ileum. Clarke (1979) noted that caecal evacuation could cause fluctuations in the gut environment by altering factors such as oxygen tension and concentration of fermentative substrates. He observed that in healthy birds given a 12-h light-dark cycle there was a periodicity in caecal emptying with caecal faeces passed frequently at certain times during the light period and never passed at night. Perhaps variation in breath hydrogen, methane and other fermentation products can be attributed to a reduction in fermentation immediately following caecal evacuation and a subsequent increase in metabolic activity following the filling of the caeca with a re-newed supply of fermentable substrate.

7.7 Key determinants of variation in the digestion of energy by broiler chickens

The sex of the chicken and the overall or net metabolic activity of the gut microflora were the major contributors to variation in energy digestion. Furthermore, gut microflora profiles differed between male and female chickens.

Two broad conjectures can be developed in relation to these findings to indicate directions for future research. These are termed the "nutrient flow conjecture" and the "cross-talk conjecture".

The "nutrient flow conjecture" is based on observations that male and female chickens from feather-sexable crosses have different rates of growth of feathers *in ovo* which indicates that the sexes utilise differently the same nutrient stores in eggs during incubation. After hatch, they continue to have different rates of feather growth and, hence, different utilisation of nutrients during the first 2-3 weeks post-hatch at least. The conjecture is that this results in sex-related differences in the composition of undigested material reaching the hindgut which in turn results in differences in the population profiles of gut bacteria.

The "cross-talk conjecture" is based on the assumption that the nature of chemical signalling between host mucosal tissue and gut microflora differs between males and females, particularly during the two week post-hatch period when the digestive and immune systems are maturing, and the gut microflora populations are reaching a stable equilibria.

If the principle of Occam's razor is taken into account then the nutrient flow conjecture would seem to be the simpler way of explaining sex-dependent differences in gut microflora profiles. Nevertheless, recent advances in our understanding of the role of cytokines in mucosal immunity in chickens (Lowenthal *et al.* 2000; Muir *et al.* 2000; Bean *et al.* 2003) increases the appeal of the cross-talk conjecture as an avenue of research into the effects of gut microflora on energy metabolism in chickens.

It seems reasonable to propose that if evidence can be found in chickens to support either or both of these conjectures, then it is likely that sex-dependent differences such as these will be present also in other animal species, including humans.

7.8 Summary and Conclusions

It is apparent that gender can influence the digestive capacity of chickens through endogenous energy losses, gut structure and function, and metabolic activity of gut microflora. This raises the question "Is there sexual dimorphism in other physiological and biochemical systems also?" There are important scientific and commercial implications should such differences exist. Firstly, future research should include an examination of any gender-related influences. Secondly, the commercial implications are that males and

females may have different nutrient requirements, and may respond differently to feed additives such as prebiotics, probiotics and feed enzymes.

The effect of the gender of the individual chicken on its functional capacity to digest and absorb nutrients has received little attention by researchers until recently. Hughes (2001) noted that much of our current knowledge of nutrient utilisation and nutrient requirements of broiler chickens was gained by study of males only. Experiments designed around chickens of the same sex may have some advantages, however, it is possible that only one half of the true story will be revealed, or less, if underlying interactions involving sex go undetected.

Bacterial overgrowth of the gut is likely to have detrimental effects partly due to significant losses of nutrients. In addition, microbial proliferation could ultimately lead to health problems through general inflammation of the gut, thus paving the way for invasion of intestinal tissue by organisms pathogenic to the bird, such as *Eimeria* spp and *Clostridium perfringens*.

The influence of gut microflora on the nutritive value of different cereal grains is at least partially dependent on the sex of the chicken. This implies a form of "communication" between the host and its bacterial population which influences the metabolic activities of both the bacteria and host tissue. Alteration of the balance between the host and its commensal microflora by feeding different grains, and various dietary additives such as antimicrobials, enzymes, betaine, acidifiers, prebiotics and probiotics, is likely to result in patterns of growth and nutrient utilisation that are difficult to predict.

A fuller understanding of the role of the gut microflora is required, particularly in regard to what induces shifts in fermentation patterns, and the consequences on the integrity of the mucosal barrier. Further work is needed to determine the fundamental reasons why bacterial colonisation of the gut is variable and why it can differ substantially between male and female chickens. This is a necessary step towards being able to control the colonisation of the gut in newly hatched chicks and to maintain an ideal microflora for the life of the bird, which will be beneficial for the health, welfare and performance of commercial flocks, and for enhancement of product uniformity, quality and safety.

7.9 Recommendations

A key conclusion reached in this investigation was that the sex of the chicken strongly influenced digestive physiology and energy metabolism. Several practical recommendations can be made as a result:-

For all intents and purposes, male and female chickens should be regarded as if they are entirely different "strains" and hence should be fed and managed differently, as is the usual practice for current commercial strains such as Cobb and Ross chickens, which are known to differ substantially in growth rate and carcass yield.

Placement of males and females in separate sheds in order that sex-specific feeding and management programs can be applied, in order to optimise growth, carcass yield, and composition within each sex.

Undertake segregated rearing on a trial basis on commercial farms to validate the experimental findings observed in this study.

During large-scale commercial trials, it is recommended that records be kept on any sex-dependent differences in environmental control settings needed for flock comfort, susceptibility to disease, behavioural changes, water consumption, litter condition, hock burn and breast blisters, uniformity of live weight with age, uniformity of carcass yield, and incidence of condemnations.

The importance of sex on gut structure and function and bacterial colonisation of the gut in otherwise healthy, high performing chickens observed in this study leads to the following recommendation for further research:-

The relative importance of gut epithelial function needs to be established more clearly in experimental work in which other key determinants of digestive capacity such as digesta transit time and gut microflora size and activity are measured simultaneously.

During this study, all experimental work was done with feather-sexable strains of chickens. Clearly, a differential rate of feather growth in ovo implies that male and female embryos utilise the same supply of nutrients in eggs differently. Furthermore, differential feather growth persists long after hatch. It follows that males and females utilise feed differently, and that the flow of specific undigested nutrients into the hindgut will differ between males and females. This may be a feasible explanation for why males and females hosted gut microflora with the widely differing metabolic activities observed in this study. Similar work to that described in this report should be undertaken to:-

Determine whether sex-dependent differentiation occurs in the gut microflora in non-feather sexable crosses of broiler chickens in order to better understand the relative importance of the gut microflora in the productivity and health of chickens. Such a study should compare a feather sexable cross with the non-feather sexable reciprocal cross, and should include simultaneous measurements of other key determinants of the digestive capacity of chickens.

Preliminary experiments using simple PVC helmets to collect breath from individual birds provided proof of the concept of using breath tests as non-invasive tools for study of the gastroenterology of broiler chickens. Breath testing of chickens prior to use in experiments highlighted the fact that colonisation of the gut with commensal bacteria differed substantially from batch to batch, and that the gut microflora had a big impact on subsequent productivity of chickens in experimental flocks. These observations lead to two avenues for further research. The first involves:-

Characterisation of the gut microflora of chickens and its changes in relation to feed composition and gut health, and study of the nature of interactions between microbes and the mucosal immune system. Further work in this area should take into account recent advances made in our understanding of cytokines and it will require development of DNA-based tools for identification and quantification of anaerobic organisms that are difficult to enumerate using conventional microbiological techniques.

Investigation of the factors that influence how the gut of newly hatched chickens is colonised initially and how early nutrition influences this process.

The other avenue of approach involves exploitation of breath testing techniques for experimental and commercial use. However, it is likely that breath testing will have most use as a research tool initially. Further research is needed to:-

Develop breath tests for detection of specific gastrointestinal disorders such as pancreatic insufficiency, maldigestion, and malabsorption, and for semi-quantification of gastrointestinal functions such as rate of passage of digest, and bacterial proliferation in the small intestine. Breath tests need to be validated against conventional techniques involving slaughter of animals for collection of tissue and digesta samples.

Breath analysis has potential for commercial applications also:-

Examine the feasibility of monitoring productivity and health of entire flocks by analysis of air exhausted from the shed. Initially, samples of air can be collected for analysis with specialised laboratory instruments housed offsite. For example, samples could be analysed for hydrogen and methane to indicate gut microbial activity, for stable isotopes to gauge catabolic reactions, or for short chain hydrocarbons to monitor oxidative stress in chickens. In the long term, it is envisaged that in situ probes will be installed in computer hardware for controlling the shed environment

Finally, chickens were raised in clean and dirty environments in this study in an attempt to magnify differences within and between sexes. The chickens appeared to be unaffected by immuno-suppressive viruses that cause Marek's disease, infectious bursal disease and chick anaemia, or by bacterial infections. There was, however, clear evidence that chickens reared in a dirty environment grew slower and were less uniform in weight than chickens reared in a clean environment. Furthermore, male chickens fared far worse than females in terms of uniformity. These observations indicated that there were significant levels of potentially harmful agents in the environment, such as feather scurf and dander,

dust from feed, and non-pathogenic microorganisms with antigenic or inflammatory properties. It is recommended that applied research be undertaken to:-

Compare used versus fresh litter from both an economical and a biological point of view. These investigations should measure effects on the health and welfare of animals and humans who work in the sheds and near surrounds.

Develop recycling processes that reduce or eliminate potentially harmful properties of re-used litter. These investigations should identify the source and nature of hazardous components of litter materials, and should determine the number of cycles litter can go through before disposal is necessary, and how disposal should be carried out.

Appendices

Appendix 1. Descriptions of rearing pens

The modular pen system consists of three floor modules each 1800 x 1200 mm, with 600 mm high mesh sides and 300 mm floor height. The system is easy to assemble, clean and operate with self-locking height adjustment mechanisms for the feeders and drinkers. The main floor section comprises three sub-sections each 1800 x 1200 mm with two legs 300 mm high and covered with 25 x 25 mm plastic floor mesh. Sawdust and wood shavings can be spread over stiff brown paper to provide bedding material. End wall panels are 1800 mm long x 600 mm high with the bottom 150 mm made from solid steel sheet and the remainder made from 25 x 25 mm steel mesh. Side panels are 1200 mm long x 600 mm with the bottom 150 mm made from solid steel sheet and the remainder made from 25 x 25 mm steel mesh. All steel components were hot dipped galvanised after welding

Drinker units are 1000 mm long with seven nipple drinkers and splash trays complete with pressure regulators, end sight tube, hose and adjustable rail support. Hopper feeders are plastic and supported from a steel cross rail that fits onto the top of side panels. The heater assembly comprises three 240V AC heater elements with reflectors and power cord, and is mounted on a steel cross rail that fits onto the top of side panels.

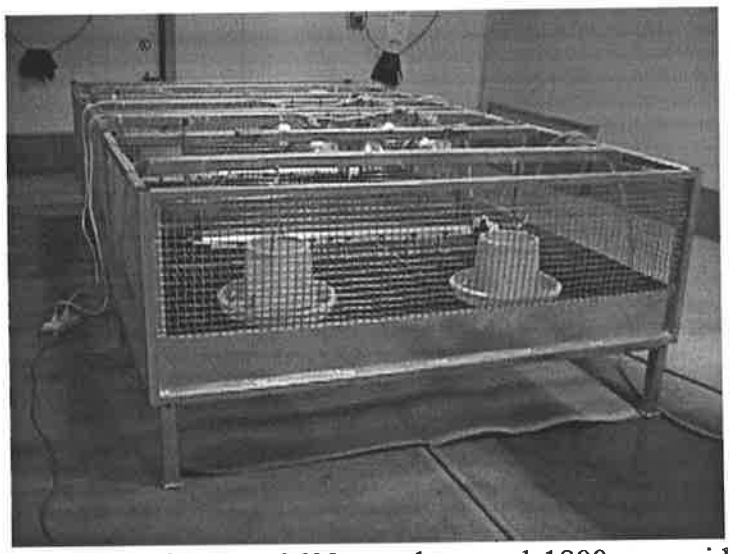


Figure 42. Experimental rearing pen 3600 mm long and 1800 mm wide split cross-wise into three sub-sections

Appendix 2. Description of metabolism cages

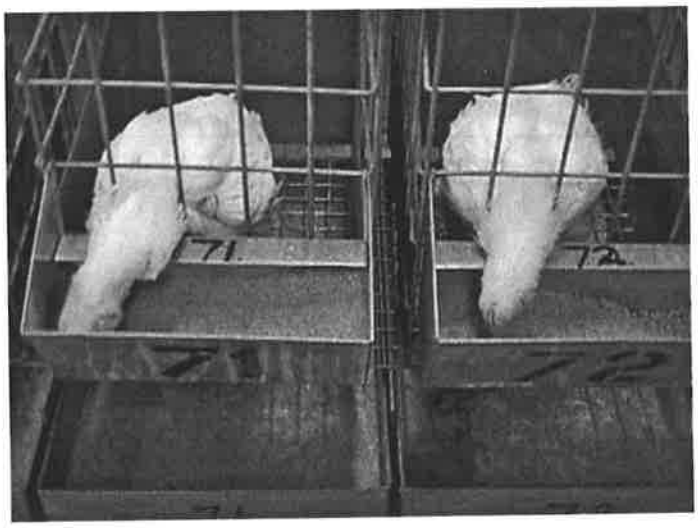


Figure 43. Single-bird cages with dimensions 26 cm in width, 40 cm in height and 36 cm in depth.

Figure 44. Multiple-bird cages with dimensions 60 cm in width, 38 cm in height and 45 cm in depth.

All cages had individual feed hoppers and drinking nipples. Each cage was shielded from others to prevent birds eating from the adjacent hopper and cross-contamination with excreta.

Appendix 3. Calculation of dry matter digestibility, apparent metabolisable energy (AME) and ileal digestible energy (DE)

$$\text{Dry matter digestibility} = \left(\text{g feed} \times \text{DM}_{\text{pellet}} - \text{g excreta}\right) / \left(\text{g feed} \times \text{DM}_{\text{pellet}}\right)$$

$$\text{AME}_{\text{diet}} = \frac{\text{g feed} \times \text{DM}_{\text{pellet}} \times \text{gross energy}_{\text{diet}} / \text{DM}_{\text{milled}} - \text{gross energy}_{\text{excreta}} \times \text{g excreta}}{\text{g feed} \times \text{DM}_{\text{pellet}}}$$

$$\text{AME}_{\text{ingredient}} = \frac{\text{AME}_{\text{diet}} - \text{AME}_{\text{casein}} \times \text{casein level}}{\text{ingredient level}}$$

Where:-

$\text{AME}_{\text{casein}}$ is assumed to be 20.1 MJ/kg dry matter basis (Annison *et al.* 1996).

$\text{DM}_{\text{pellet}}$ refers to dry matter of the pelleted feed

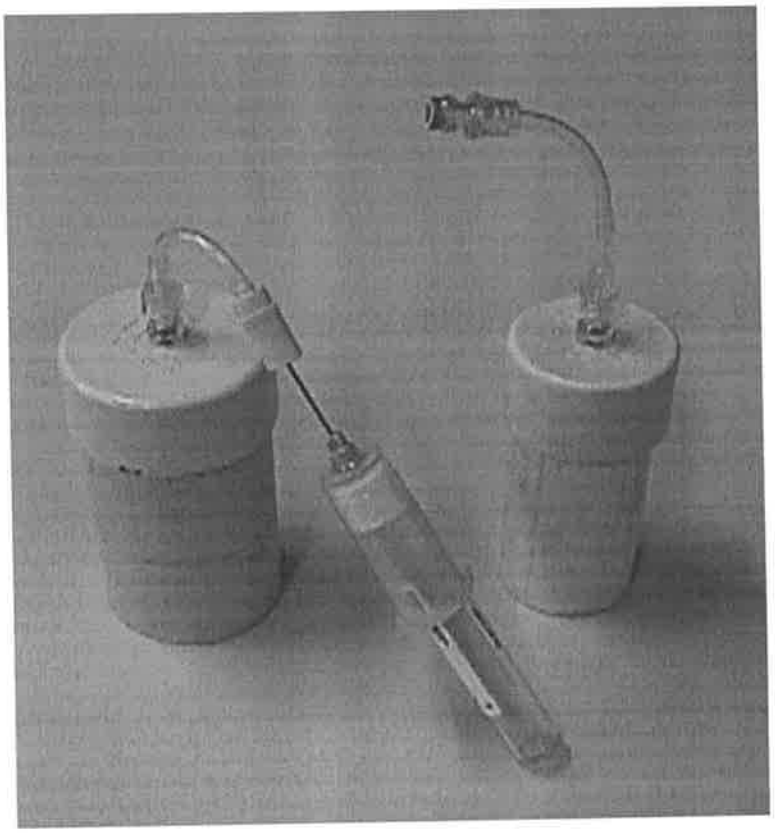
$\text{DM}_{\text{milled}}$ refers to dry matter of the milled feed

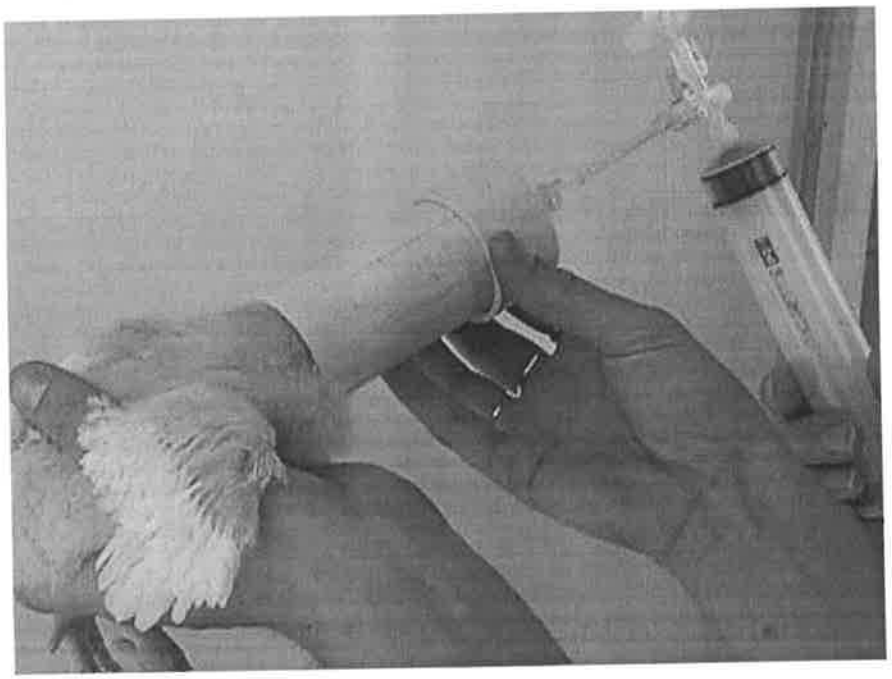
and AME_{diet} and $\text{AME}_{\text{ingredient}}$ are in MJ/kg dry matter basis

$$\text{Gross energy digestibility coefficient (GEDC)} = 1 - \frac{\text{GE}_{\text{ileal}} / (\text{DM}_{\text{ileal}} \times \text{AIA}_{\text{ileal}})}{\text{GE}_{\text{diet}} / (\text{DM}_{\text{milled}} \times \text{AIA}_{\text{diet}})}$$

$$\text{Ileal digestible energy (in MJ/kg dry matter basis)} = \frac{\text{GEDC} \times \text{GE}_{\text{diet}}}{\text{DM}_{\text{milled}}}$$

Appendix 4. Helmet and fittings for collection of samples of breath from chickens.





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