QUANTIFICATION AND ALLEVIATION OF THE ANTINUTRITIONAL EFFECTS OF PHYTATE ON POULTRY

NATALIE KARRON MORGAN

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ABSTRACT

The phosphorus (P) requirements of meat-type poultry cannot be met by plant-based diets because approximately two thirds of total phosphorus in cereals and leguminous feed materials is in the unavailable form of phytate (inositol hexaphosphate or phytic acid). Phosphorus supplements are expensive and over supplementation can increase excretion of P, with negative implications on the environment. For some time, research has focussed on the development of exogenous phytase enzymes capable of releasing phytate phosphorus for absorption, thus reducing the environmental impact of poultry production and requirements for costly dietary inorganic P supplementation. The absolute adoption of this strategy has, however, been stalled by apparent variability in rate of release of phytate by phytase enzyme. The aim of this project was to examine the availability and reactivity of phytate in diets and raw materials fed to broilers in a range of physiological environments, and to develop strategies for combating their effects.

A series of *in vitro* studies and bird trials were conducted to investigate phytate reactivity by observing the relationship between pH, protein phytate interactions and phytate susceptibility to the effects of phytase. A bird trial was undertaken comparing the current commercial level of 500 FTU/kg phytase and a superdose of 5000 FTU/kg phytase in soyabean meal and rapeseed meal based broiler diets. Feeding phytase doses above the commercial recommendation resulted in increased bird performance through phytate destruction, leading to heightened alleviation of the anti-nutritional effects of phytate. This suggests that superdosing broiler diets can improve profitability by both increasing availability of phosphorus and removing restrictions of using cheaper feed ingredients which are viewed as undesirable due to their high phytate content.

There is a common misconception that poultry lack any endogenous phytase, but a study sequentially sampling broilers from age d4 to d14 illustrated that phytase activity from intestinal mucosa and bacteria and diet is quantifiable in regulating phytate-P digestion. Endogenous phytase contributes

significantly towards degradation of phytate at bird age d4. By d14, ileal phytase activity levels were approximately 45 U/kg and the amount of total dietary phytate hydrolysed ranged from 21% to 36%.

Measurements of total phytate-P content of diets may be deceptive as they do not indicate substrate availability for phytase; measurements of phytate susceptible to the effects of phytase may be a more accurate measure of phosphorus availability to the bird. To verify this hypothesis in practice, a bird study was designed to compare diets formulated to contain high or low susceptible phytate. This study showed that at age d28, birds fed diets with high susceptible phytate content had significantly better cumulative BWG ($p=0.015$) and FCR ($p=0.003$) than birds fed diets with low susceptible phytate content. Furthermore, *in vitro* screening of raw materials revealed that phytate susceptibility varies considerably between ingredients and batches of ingredients, and total phytate content bears no relation to susceptible phytate content of an ingredient. Therefore, for optimum phytase efficacy, it may be advantageous to formulate diets and develop phytase matrix values based on the susceptible phytate content of the individual batch of ingredients being fed. Both total and susceptible phytate content can be measured using simple colorimetric assays. Examination of *in vitro* digestion models and subsequent correlation to *in vivo* studies indicate that exposing samples to conditions that mimic the gastrointestinal environment is the most accurate way to predict phytate degradation and mineral availability in diets prior to feeding.

The relationship between phytate reactivity and pH was investigated throughout this project. It was found that gastric pH is closer to the optimum for pepsin activity and phytate-complex degradation when in the presence of phytase. This is because phytate reduces protein digestibility, resulting in heightened presence of intact proteins in the tract, which instigates an increase in secretion of HCl and pepsin. More bicarbonate ions are released in response to this increased acidity, causing an increase in intestinal pH and resulting reduction in solubility of phytate-protein complexes.

To conclude, dietary phytate that is susceptible to phytase effects as opposed to total phytate content should potentially be considered when determining phytase matrix values. For optimum response to

phytase, it may be beneficial to formulate diets based on the susceptible phytate content of the individual batch of ingredients being fed. Some raw materials have high total phytate content but also high susceptible phytate content, meaning they have the potential to be used as replacements for more expensive feed ingredients if in the presence of phytase. This is particularly the case in diets supplemented with high doses of phytase (>500 FTU/kg), because high phytase doses alleviate the extra-phosphoric anti-nutritional effects of phytate as well as increase mineral availability.

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GLOSSARY

AA- Amino acids

ANOVA-Analysis of variance

BWG-Body weight gain

Ca- Calcium

DCP- Dicalcium phosphate

FCR- Feed conversion ratio

FI- Feed intake

GIT- Gastrointestinal tract

HPLC- High-performance liquid chromatography

ICP-OES- Inductively coupled plasma atomic emission spectroscopy

KS- Kolmogorov Smirnoff

MANOVA- Multiple analysis of variance

NSP- Non-starch polysaccharide

SBM- Soyabean meal

RSM- Rapeseed meal

CONTENTS

CHAPTER 1: Literature Review

1.1. Introduction

Poultry production has increased consistently over recent years; world poultry meat consumption increased from 11.1 kg/person/year in 2000 to 13.6 kg/person/year in 2009 (DEFRA, 2013). The growth in poultry production is expected to continue due in part to its relative cheapness as a meat protein source. The low production costs are largely due to genetic progress in strains for meat, heightened understanding of the fundamentals of poultry nutrition and improved disease control. Genetic growth rates and feed efficiency of broilers have increased dramatically over the past 30 years; in 1976 broiler chickens reached the market weight of 2kg at 63 days, but by 2009 this was reduced to just 35 days (DEFRA, 2013). Nutrient requirements and nutrient management have had to be altered in response to these changes to bird physiology, resulting in increased demand for cheap but high-quality feed and raw materials. Modern broiler diets must be protein and energy rich and contain sufficient minerals, particularly calcium (Ca) and phosphorus (P), required for skeletal system formation, general health, metabolic activity and acid-base balance management.

The phosphorus (P) requirements of growing broiler chickens cannot be met by plant-based broiler diets because approximately two thirds of total phosphorus in grains and oil seeds is in the unavailable form of phytate (Sandberg, 2002). Consequently, inorganic P is supplemented in broiler diets to meet the 0.45% available P requirement of broiler chicks (NRC, 1994). Phosphorus supplements are expensive and over supplementation can increase excretion of P, with negative implications on the environment. Phytase enzymes are added to poultry diets to overcome these issues, as poultry lack effective endogenous phytases. Phytases dephosphorylate phytate molecules, increasing phytate-P availability for broilers, and hence reduces the requirement for inorganic P supplementation and amount of P that is excreted by broilers.

Phytate is classed as an anti-nutrient because it reduces nutrient digestibility and increases endogenous secretion of nutrients. This is because it is negatively charged at acidic, neutral and basic

pH conditions, so it is able to bind to positively charged molecules in the diet and gastrointestinal tract (GIT) at all pH levels found in the GIT (Maenz, 2001). Phytate concentration varies substantially between feed ingredients (Selle *et al.,* 2003) which has subsequent implications on the phytate-P concentration of poultry diets, depending on inclusion level in the diet.

The anti-nutritional effects of phytate, and phytase efficacy in the conditions of the broiler digestive tract, are dictated by phytate reactivity and susceptibility to phytase degradation. It is therefore imperative when using dietary phytase supplementation to consider not only the total phytate concentration of diets, but also to predict phytate solubility. There are many factors that influence phytate reactivity, including the conformation and configuration of the phytate molecule, the pH of the tract environment, the presence of substrates that may compete with phytate for mineral binding, the source and type of phytate, the volume of phytate ingested and feed processing. This project examines the factors that influence phytate reactivity and phytase efficacy.

1.2. Poultry Production

The popularity of poultry meat continues to increase because of its low cost, lack of religious obstructions and nutritional advantages over other meats (Magdelaine *et al*., 2008). Chicken is the most popular poultry meat in the EU; 105 thousand tonnes of broiler meat was produced in February 2013, compared to just 17.0 thousand tonnes of turkey meat and 2.2 thousand tonnes of duck meat (DEFRA, 2013). As a result, there are now approximately 35,000 companies in the EU that are involved in the poultry industry (AVEC, 2013) and the UK poultry industry is valued at approximately £3.4 billion annually at retail value (Dent *et al.,* 2008). Modern broiler chickens are more efficient at converting feed to body mass; comparison between heritage line unselected since the 1950s and modern broilers at bird age d35 showed that breast muscle of the heritage line constituted 9% of the total body mass whereas in the modern line the breast muscle constitutes 18% of the total mass of the bird. Also in modern broilers the relative size of the heart decreased after d14 unlike in the heritage line, suggesting that selection for increased breast muscle has translated into relatively less weight of the

heart muscle. Additionally, in modern broilers the liver matures earlier, likely to improve nutrient utilisation as birds have shifted to more carbohydrate-rich feeds, and the jejunum and ileum is 20% longer, likely due to increased nutrient absorption (Schmidt *et al.,* 2009).

Increased input costs have resulted in increased costs of poultry meat. Since 2002 poultry meat prices have increased by 85%, and between 2010 and 2011 the price increased from £1.06/kg to £1.22/kg (FAO, 2013). This is primarily due to increased feed costs; between 2012 and 2013 feed costs increased by 7.8% and increased from £149 per tonne in March 2006 to £253 per tonne in March 2012 (DEFRA, 2013). Production has improved in the past 18 months; in 2013 the UK produced over 850 million broilers a year, fed approximately 3,300 thousand tonnes of broiler feed, producing approximately 1,400 thousand tonnes of chicken meat (DEFRA, 2014). As a result there has been a rise in prices paid to producers for poultry meat, but the continuing level of high feed costs has meant unfortunately there has been very little rise in profits. Total retail production of animal feed and raw material usage went up by 5.9% and 6.5% respectively in Britain between 2012 and 2013 (DEFRA, 2013). The outcome of this is competitive production to guarantee profitability. Phytate is thought to cost the poultry industry up to €5 per tonne of feed in lost performance, so the use of supplemental phytase could have huge implications on poultry feed costs and hence improve profitability.

1.3. Poultry Digestive Tract

Nutrient absorption occurs only in the small intestine of poultry, but mechanical, chemical and enzymatic procedures occur throughout the tract. Passage time of feed is inversely proportional to feed intake; digesta takes on average 3-4 hours to be transported through the tract (Sundu, 2009). Passage time has an impact on digestibility; feed intake may be increased if passage time is decreased, and reduced flow of digesta may cause increased microbial growth which causes reduced nutrient digestibility and weakens intestinal barrier function. Each section of the gastrointestinal tract has a specialised function, and hence has a different pH and different enzymes and secretions. Table 1 summarises digestive enzyme activity and pH in the poultry digestive tract.

Table 1: Poultry digestive enzyme activity

Location	Enzyme/Secretion	Role	pH
Mouth	Saliva	Lubricate and soften feed	7-8
Crop	Mucus	Lubricate and soften feed Bacterial Fermentation	4-7
Proventriculus and Gizzard	Pepsin	Protein > Polypeptides	1-4
	Lipase	Triglyceride > Fatty acids/ Monoglycerides (Selvan *et al.,* 2008)	
	HCl	Lower digesta pH Instigate protein digestion	
Duodenum	Amylase	Starch > Maltose > Glucose	5-7
	Trypsin/Chymotrypsin	Proteins > Peptides > Amino Acids	
	Carboxypeptidases	Peptides > Amino Acids	
	Bile	Emulsifies fats	
	Lipase	Fat > Fatty Acids/Monoglycerides	
Jejunum	Maltase / Sucrase	Maltose/ Sucrose > Glucose	5-7
	Peptidases	Peptides > Dipeptides/Amino Acids	
Caeca	Microbes	Cellulose, polysaccharides, starches, sugars > VFAs, B-vitamins, Vitamin K	5-7

1.3.1. Buccal Cavity

In chickens, the tongue is situated in the lower beak and is attached to the hyoid bone, which enables mobility down the oesophagus (Susi, 1969). The tongue is rigid and has very few intrinsic muscles (Duke, 1986). Chickens have very few taste buds, and hence inferior taste acuity; they are believed to have just 316 taste buds in their oral cavity (Roura *et al.,* 2012). This suggests that it is nutrient levels of the diet as opposed to taste that dictates feed consumption in broilers.

Drinking and feeding mechanisms in chickens involves cyclic movement patterns of the beak, tongue and larynx. The head is swung backwards to transport feed and water down the pharynx, the tongue is lifted and larynx lowered and gravity transports the feed and water to the oesophagus (Heidweiller *et al.,* 1992, Hill, 1976). Minimal food is broken down in the beak, it is mainly ingested whole.

Birds produce approximately 15-20ml of saliva a day. This saliva contains small amounts of amylase in an alkaline environment which instigates digestion of starch (Leeson and Summers, 2001). It also contains mucus which helps lubricate the oesophagus and humidify the feed. It is also likely that saliva contains anti-bacterial properties, and thus protects the oral cavity (Samar *et al.,* 2002).

1.3.2. Oesophagus and Crop

Feed passes from the mouth and through the pre-crop oesophagus to the crop. The oesophagus wall is comprised of four tissue layers; the innermost layer is the mucous membrane which produces mucus for lubrication to aid passage of food along the alimentary canal. The oesophagus has two sections; the upper cervical section and lower caudal section (Hill, 1976). The crop is located between these two sections; it is a dilation of the oesophagus found just before the oesophagus passes into the thoracic cavity. The structure of the oesophagus above and below the crop is similar, except there is less lymphoid tissue below the crop (Larbier and Leclercq, 1994). The crop is similar in structure to the oesophagus except there are no glands present. Food is also moistened and mixed with saliva whilst in the crop, but very little digestion occurs here despite presence of amylase from the saliva. The crop acts as a temporary food store; if the proventriculus and gizzard are full feed is stored in the crop. This enables digesta transit to be controlled and means the bird can eat large quantities of feed in one meal, whilst allowing gradual digestion. Emptying of the crop is dictated by how full the gizzard is, and particle size and moisture content of the feed (Hill, 1976; Larbier and Leclercq, 1994). When the gizzard is empty feed moves straight to the proventriculus, but if the gizzard is full feed moves gradually from the crop to the proventriculus when required.

1.3.3. Proventriculus

The proventriculus, often referred to as the 'glandular stomach', is located between the gizzard and lower oesophagus. It is tubular and has very thick walls made of has five layers of muscle. Glands in the proventriculus group to form lobules which join to form a cavity near the surface, and produce HCl and pepsinogen for digestion (Rossi *et al.,* 2005). The HCl maintains pH at 2-3 which allows pepsinogen to be activated and solubilises mineral salts (Duke, 1986). HCl also promotes release of secretin which enhances the release of pancreatic enzymes (Yang *et al.,* 2007). Secretion of HCl and pepsinogen is stimulated by nervous and chemical signals which are regulated by digesta presence in the gizzard and crop. Digestion does not occur in the proventriculus.

1.3.4. Gizzard

The gizzard, often referred to as the 'muscular stomach', is located immediately following the proventriculus, partly behind the liver. Its primary role is to grind feed, hence it is very muscular, and it is the first site of protein digestion. It has a biconvex structure and has an outer layer of tendinous tissue atop a powerful muscle layer. The inner layer of the gizzard has tough fibrous tissue with raised ridges which, coupled with grit, enables grinding of feed (Hetland *et al.,* 2005). The skin of this inner layer is very strong and flexible to endure the effects of grinding. Glands are positioned amongst the layers of tissue which secrete a keratinised substance to replenish the inner lining that has been worn away by grinding. Feed that has been moistened in the proventriculus and gizzard forms a paste called chyme which then enters the intestine.

1.3.5. Small Intestine

The small intestine, from the gizzard exit to the ileo-ceaco-colonic junction, consists of the duodenum, jejunum and ileum. Digesta is moved along the jejunum and ileum by peristalsis. All of the absorption of nutrients takes place in the small intestine, predominantly in the jejunum and ileum, as well as a large amount of digestion and enzyme production. Intestinal mucosa enzymes and transport mechanisms dictate nutrient digestion and absorption; pancreatic enzymes start the digestive process

but activity of enzymes at the mucosal surface determines absolute peptide and carbohydrate digestion.

The small intestine consists of five layers; an outer serous membrane layer, a layer of longitudinal muscle, a layer of circular muscle, a sub-mucosa layer and a mucus membrane. Blood vessels, lymph vessels and nerve fibres are situated between the longitudinal and circular muscles (Larbier and Leclercq, 1994). The mucous membrane contains thick muscularis mucosae, glands and lymphoid tissue.

Villi, projections of epithelial tissue covered by absorptive tissue, provide a vastly amplified surface area for more efficient nutrient absorption in the small intestine. They contain lymph vessels and capillaries. Columnar epithelium and goblet cells cover the lining of the villi, the latter of which secrete mucus which contains mucin (glycosylated proteins) and inorganic salts (Bansil *et al.,* 1995; Gendler *et al.,* 1995). They also contain cells which can undergo mitotic division and become precursors of enterocytes (absorptive cells). Enterocytes move from the crypts up to the tips of the villus and are dispersed into the gut lumen (Geyra *et al.,* 2001).

The duodenum is an elongated loop around the pancreas. The junction between the gizzard and the duodenum prevents large particles entering the duodenum (Maya and Lucy, 2000). The pancreas secretes peptidases, amylases and lipases, as well as a solution high in bicarbonate ions which neutralises chyme to produce an optimum pH of 6-7 for enzyme activity (Leeson and Summers, 2001). It also produces insulin and glucagon which are involved in carbohydrate metabolism, and promotes production of gastrin which stimulates secretion of HCl and aids gastric motility.

At the posterior end of the duodenum is a papilla where bile ducts from the gall bladder, liver and pancreatic ducts enter the small intestine (Nasrin *et al.,* 2012). This area marks the beginning of the jejunum. The liver has two bile ducts, one from the gall bladder, and one that delivers a direct connection between the liver and small intestine. The predominant role of bile is to aid pancreatic

lipase activity by emulsifying fats (Krogdahl, 1985). Very small channels collect and transport bile; they join together to form bile ducts which either go directly to the intestine, or to the gall bladder.

The jejunum and ileum are the major absorptive regions of the digestive tract and are similar in length, function and structure. The structure of the jejunum and ileum is similar to that of the duodenum, except there is less lymphoid tissue and shorter villi. The jejunum is where chemical breakdown of food chyme is completed and enzymes produced by the jejunum wall finalise the digestion process. The ileum is the final section of the small intestine and is where nutrients are absorbed, along with bile which is then returned to the liver through blood vessels in the intestinal wall. The ileum ends with a ringed valve which then branches into two caeca, the ileo-caecal junction. Peristalsis and segmentation movements propel digesta through the jejunum and ileum. It is generally recognised throughout the poultry industry that the Meckel's diverticulum, a small projection where the yolk sac was attached during embryonic development, distinguishes between the jejunum and ileum (Verdal *et al*., 2011; Rodehutscord *et al*., 2012).

1.3.6. Caeca, Colon and Cloaca

Very little digestion occurs in the large intestine. Contractions in the colon force digesta towards the caeca and cloaca. The caeca are two blind pouches, about 17cm long in adult birds, at the junction of the small and large intestine. They contain microorganisms that degrade undigested nutrients; the products of fermentation are then able to be absorbed (Gong *et al.,* 2002). Caeca consist of a serous membrane, outer longitudinal and circular muscles and inner longitudinal muscles (Majeed *et al.,* 2009). It is thought that caeca also play a role in water absorption and B vitamin production (Duke, 1986).

The structure of the cloaca is similar to that of the intestine, but there is no muscularis mucosa present near the vent. The cloaca has three separate chambers; the copradaeum which is a continuation of the colon, the urodeaeum which is the middle part in which the ureters and genital ducts open, and

the proctodeaum which opens to the vent (Gumus *et al.,* 2004). In the cloaca therefore there is a mixture of digestive and urinary system waste.

1.3.7. Development of the digestive tract

Although broilers are considered to be precocial, their digestive system is not mature at hatch, so gastrointestinal morphology, function and microflora must alter considerably post-hatch. During embryonic development the yolk is the nutrient supplier, but during incubation the remaining yolk is internalised to the abdominal cavity and transported to the small intestine (Sklan, 2001). Therefore, immediately post-hatch birds must be prepared to use exogenous nutrients that are chiefly carbohydrate based compared to the lipid based yolk (Parsons, 2004). In the first days post-hatch the morphology and function of the digestive tract alters dramatically. Crop microflora becomes more acidic and the presence of hydrophobic yolk in the tract reduces and secretions of bile acids and pancreatic and brush border enzymes increases, allowing improved digestion of exogenous nutrients. The small intestine rapidly increases in size in the first 2 days post-hatch, at a greater rate than the body mass of the whole bird (Sklan, 2001), and villus height doubles (Jin *et al.,* 1999). Villus volume in the duodenum increases continuously until d4 post-hatch, but jejunum and ileum villus continue to grow until about d10 post-hatch (Noy and Sklan, 1997). The duodenum and jejunum continue to develop for a longer amount of time than the ileum (Jin *et al.,* 1998). These changes allow the bird to attain full digestive capacity.

Pancreas and small intestine enterocytes increase in size rapidly in the first 3 days post-hatch but enterocyte density does not alter with age (Nitsan *et al.,* 1991; Uni *et al.,* 1998). At hatching, small intestine enterocytes proliferate, but this decreases rapidly; at d2 proliferation rate is approximately half of that at hatching (Sklan, 2001). Movement of enterocytes from the crypt to the villus takes approximately 3 days in 4 day old birds, and increases to 4 days in older birds (Geyra *et al.,* 2001).

Chicks are able to digest starches almost immediately post-hatch; competence at starch digestion is largely complete by d4 post-hatch and maltase and sucrose activity peaks at d1-2 post-hatch (Noy and

Sklan, 1999; and Sell *et al*. 1991). The ability of chicks to utilise fats increases with age; lipase secretion increases by a factor of 20 between d4 and d21 post-hatch (Nitsan *et al*., 1991; Noy and Sklan, 1995). Proteolytic activity in early post-hatch birds is not adequate enough for efficient breakdown of proteins; it is not until around d7 post-hatch that protein digestion reaches close to 80% (Noy and Sklan (1995);Uni *et al.* (1995). Trypsin and chymotrypsin activity increases with bird age post-hatch; trypsin activity plateaus at d14 post-hatch and chymotrypsin activity reaches maximum level at d11 post-hatch (Nitsan *et al.,* 1991; Noy and Sklan, 1995). Both have also been detected in embryos, for example trypsin has been observed in poultry embryos at 18 days incubation (Sell *et al.,* 1991). Aminopeptidase and dipeptidase activity in the intestines decreases post-hatch, reaching the lowest point at d10 post-hatch (Jin *et al.,* 1991).

1.4. Measuring digestibility using inert markers

Inert digestibility markers added to broiler diets eliminate the need to evaluate quantitative feed intake and excreta output, and enable nutrient utilisation to be examined along the gastrointestinal tract (Short *et al*., 1996). Inert markers must maintain digestive transit at the same speed as other dietary nutrients in the tract and be physiologically inactive, as well as being non-toxic, easily analysed, able to be homogenously mixed into a diet, indigestible and non-absorbed (Jagger *et al*., 1992; Titgemeyer *et al*., 2001; Sales and Janssens, 2003). Some of the markers used include chromic oxide (Cr_2O_3), titanium dioxide (TiO_2) and acid insoluble ash. TiO_2 as an inert marker is advantageous over Cr_2O_3 as reproducibility and homogeneity has been shown to be better with TiO_2 (Jagger *et al*. 1992), and TiO_2 is approved for use as a feed additive by the Food and Drug Administration, unlike Cr_2O_3 (Titgemeyer *et al*., 2001). Acid insoluble ash is another digestibility marker used, but it has been suggested that its digestive transit does not accurately reflect that of feed passage (Cheng and Coon 1990). TiO_2 as a digestibility marker has been used successfully in ruminants (Titgemeyer *et al*., 2001), pigs (Jagger *et al*., 1992) and poultry (Short *et al.,* 1996). In poultry TiO_2 has been used to determine the digestibility of Ca and P (Walk *et al*., 2012) as well as amino acid digestibility (Kluth and Rodehutscord, 2006) and energy digestibility (Woyengo *et al.,* 2010).

1.5. Phosphorus requirements of broilers

Phosphorus is vital nutritionally for development and maintenance of the skeletal system, and for fat and carbohydrate metabolism (Julian, 1997). It is used in nucleic acid and cell membrane phospholipids and phosphoproteins, along with a having a significant role in energy metabolism as a constituent of adenine triphosphate (ATP) and sugar phosphates. Low plasma P levels (< 5 mg/100 mL) and high plasma Ca levels (> 10 mg/100 mL), alongside signs of lameness, are indicators of P deficiency in young birds. Phosphorus is absorbed from the small intestine in the form of orthophosphate. There are two sources of phosphorus, plant and inorganic, which differ in their efficacy.

The term 'total phosphorus' encompasses any and all forms of phosphorus; 'available phosphorus' refers to that that is absorbed by the bird, and 'retained phosphorus' refers to phosphorus that stays in the body and can be apparent. The phosphorus requirements of poultry diets are based on averages of availability which can result in large errors, partly because biological availability is not constant. Phosphorus availability is dependent on many factors including presence and availability of other nutrients (for example calcium (Ca) and Vitamin D_3), bird physiology and health, and the type and concentration of the phosphorus (Angel, 2010). The NRC (1994) values for available phosphorus are also based on historical research (1952-1982) which may not reflect the requirements of the current commercial broiler, due to developments in genetic selection and management practice. These values state that birds require non-phytate P levels of 0.45%, 0.35% and 0.30% at d0-21, 21-42 and 42-56 respectively (NRC, 1994).

One of the primary problems nutritionists face is determining availability of phosphorus in the diet. The wide variation in utilisation of different phosphorus sources has resulted in wide margins of safety to avoid phosphorus deficiency, and hence loss of production. The availability of phosphorus in different feed ingredients ranges from none to 60% (Simons *et al.,* 1990), and can vary within batches of the same ingredients (Barrier-Guillot *et al.* 1996). The current approach is to measure non-phytate

phosphorus, which is easily analysed chemically, but this does not take into account other factors that may be influencing phosphorus availability.

1.6. Calcium requirements of broilers

Calcium is the most abundant mineral in broilers and plays a significant role in a number of processes. The increase in bird growth in recent decades has seen an increase in bone mass, and hence pressure on the calcium regulatory system to meet calcium and phosphorus requirements. Both insufficient and excessive supplies can have negative effects on bone formation and growth. Hydrolysis of proteins, polysaccharides and phospholipids requires calcium, as calcium-binding proteins assist protein-protein and protein-phospholipid binding (Brody, 1994). Calcium is also necessary for blood clotting proteins (Leeson and Summers, 2001) and for cell signalling (Chen, 2004). Calcium maintenance is largely controlled by parathyroid hormones which are increased when blood calcium is low and affect calcium circulation in three ways. Firstly the mobilisation of calcium from the skeleton is increased by stimulating osteocytes to break down bone matrix and osteoclasts to reabsorb the released calcium. The kidneys are stimulated to excrete P whilst holding onto Ca via reabsorption and calcium absorption from the digestive tract is promoted by increasing 1,25-$(OH)_2$ cholecalciferol production (Bar *et al*., 2003).

Commercial broilers are very rarely fed the calcium levels recommended by NRC 1984 and 1994; the recommended being 1% for starter diets, 0.90% for grower diets and 0.80% for finisher diets. According to Driver *et al.* (2005) the average calcium content fed to broilers in the United States are 0.82%, 0.77% and 0.72% for starter, grower and finisher respectively, suggesting the actual values being fed are about 20% below recommendation. This is largely because changes in feed use efficiency have meant modern broilers consume over 10% less than they did in 1984. Ideally, recommendations in modern broilers now need to take into consideration the use of phytases as well as bird sex, the environment and cost.

Calcium content of a diet has significant economic implications because excess Ca impedes availability of other minerals, namely phosphorus and zinc, while also reducing the dietary energy value of diets by binding to lipids and reducing their absorption. A balance must therefore be made between reducing the calcium content of a diet to improve performance without detrimental effects on leg health.

1.7. Measuring calcium and phosphorus in poultry digesta and excreta

A current method for determining mineral content of poultry digesta and excreta is analysis by induced coupled plasma- optical emission spectra (ICP-OES) (Mikulski *et al.,* 2012; Rodehutscord *et al.,* 2012; Kalmendal and Tauson, 2012). ICP-OES uses quantitative measurement of the optical emission from excited atoms to determine concentration of an element. The analyte atoms in the digestion solution are aspirated, desolvated, vaporised, and atomised by plasma. The analyte emits electromagnetic radiation as photons, which are detected and measured by a photo reactive chip. ICP-OES is able to measure almost any analyte on the periodic table, and can measure multiple elements in a single reading and quantify very low concentrations. An induced coupled plasma-mass spectrometer (ICP-MS) is another commonly used method for element analysis in the poultry industry (Jackson and Bertsch, 2001; Ghimpeţeanu *et al.,* 2012) which works in a similar way to ICP-OES except molecules are separated according to their mass or charge ratio and isotopes detected of each element. Other methods for measuring calcium and phosphorus concentration include atomic absorption spectrometry (Walk *et al*., 2012; Saunders-Blades *et al*., 2009), colorimetric analysis (Walk *et al*., 2012) and flame photometry (Mariam *et al*., 2004). ICP-OES and ICP-MS are preferential over other methods because they are more sensitive at quantitative analysis, have improved detection limits and are less time-consuming. ICP-OES and ICP-MS also enable several elements to be detected in parallel, which reduces preparation time and amount of sample required, and hence potentially the number of birds required.

The methods used for sample preparation for calcium and phosphorus analysis by ICP-OES and IPC-MS vary between studies, but the majority involve sample digestion with aqua regia (a mix of nitric acid and hydrochloric acid) based on AOAC 985.01. Other methods of sample preparation prior to ICP-OES analysis include microwave-assisted digestion with concentrated nitric acid and 30% hydrogen peroxide (Leytem *et al.,* 2008), digestion with a nitric acid and perchloric acid mix (Dilger and Adeola, 2006) and digestion with a nitric acid and sulphuric acid mix (Huber *et al.,* 2006). Digestion with aqua regia is preferential over the other methods based on cost, how hazardous the method is, time effectiveness and recovery analysis (Hseu, 2004).

1.8. Bone formation

Approximately 85% of the organic matrix of bone is collagen (Rath *et al.,* 2000). Collagen is a fibrous protein with a triple helix structure which contributes towards the tensile strength and framework of the skeletal tissues and orientates support for bone mineralisation (Riggs *et al.,* 1993). Bone strength and ability to withstand stress is dictated largely by the arrangement of the cross-linked collagen fibres (Rath *et al.,* 2000), as well as other intermolecular cross-linking processes such as fibril production and calcification. Reduced bone strength in poultry bones may be indicative of reduced levels of crosslinks in calcified tissues. Crosslink concentration has been correlated with bone strength in older birds, but this is also dependent on relative mineral content (Rath *et al*. 1999). Bone is in a continuous state of flux as calcium and phosphorus are deposited when levels in the extracellular fluid are adequate, and reabsorbed when there is demand by the body. Lipids are also present in bone, as well as non-collagenous proteins which enhance the mineral matrix and stabilisation of the collagen (Rath *et al.,* 2000).

Relative mineral content of bones also contributes towards bone strength. Around 95% of bone mineral matrix is calcium and phosphorus (Rath *et al.,* 1999). Calcium and phosphorus bind to form calcium phosphate, or hydroxyapatite crystals, which are deposited into the holes situated between the collagen fibres. These minerals then bind to the collagen, increasing the bone strength. Sodium,

magnesium, iron and fluorine are also sometimes incorporated into hydroxyapatite crystals (Frandson and Spurgeon, 1992). When calcium levels in the body are low, minerals from bones are released by homeostatic mechanisms (Guéguen and Pointillart, 2000), so calcium absorption must be high to sustain bone strength. Calcium absorption is largely affected by vitamin D, due to its impact on hormones that stimulate epithelial calcium channel synthesis and plasma membrane calcium pumps (Wasserman, 2004). Other organic components of bone include lipids and non-collagenous proteins, the latter of which contributes to the stabilisation of the collagen/mineral matrix (Termine and Gehron-Robey, 1996). Bone strength is therefore also partially dictated by dietary protein and fat source. The presence of water in bones may also contribute towards its visco-elastic properties.

1.9. Leg problems and lameness in broilers

The most significant form of economic loss to the poultry industry is from leg abnormalities in birds (Robins and Phillips, 2011). Bird growth rates have increased by approximately 300% in the past 50 years (Knowles *et al*., 2008), and this genetic selection for heavy bodyweights and rapid growth in association with small skeletal frames have been implicated in muscoskeletal disease in meat based poultry (Julian, 1998). Lameness can be due to under-mineralisation or the degeneration of the hypertrophic chondrocytes within the growth plates which then disrupt the maturation of the chondrocyte (Farquharson and Jefferies, 2000). One of the most recognisable signs of phosphorus deficiency is poor bone development as 80-85% of the phosphorus in a chicken is located in the bones (McDowell, 1992). When birds are fed adequate phosphorus in the starter phase, but less than required in the grower and finisher phases, phosphorus from the bones is used to meet the phosphorus requirements. Therefore both the extent and length of time the bird is deficient of phosphorus has an impact on bone integrity.

It is often assumed that rapid growth is entirely to blame for problems associated with bone strength in poultry, but in reality disrupted metabolic pathways and metabolic imbalances also play a significant role. The heightened requirements for specific nutrients or enzymes, the increased formation of

metabolic by-products (e.g. lactic acid) and the increased susceptibility to metabolic and toxic injuries are all contributors. The effects of antinutrients and toxins are not directly associated with rapid growth, but larger birds will consume more of them so the negative effects may be amplified.

The potential pain caused by lameness raises a major welfare issue. This is amplified by the preference to sit as a result of discomfort which increases the likelihood and severity of hock burns caused by the ammonia from litter in the floor substrate, alongside the starvation and dehydration due to inability to move to access feed and water. Lameness can be reduced through slower growth programmes, such as longer daily dark periods, and increased activity to reduce stress on immature bones; but a balance must be made between bird welfare and optimum economic output. High rates of mortality and morbidity of up to 30% result from leg problems (Pines *et al.* 2005), leading to obvious heavy economic losses. Incidences of lameness effect between 2 and 27% of broilers selected for growth due to the speed of their development, as a result of selection for specific traits in parent stock lines. Currently, the incidents of leg disorders are still greater than 25% of meat chickens, despite attempts to alleviate the problems.

Deposition of skeletal muscle should in theory place more load on bones, making them stronger, but in modern broilers genetic selection has meant there is more focus on increasing breast muscle not leg muscle (Al-Musawi *et al*., 2011). If focus was shifted onto selecting birds for leg muscle rather than breast muscle tibias and femurs would be stronger and less susceptible to breakage, but in the UK and most of the USA this is unlikely to happen because there is more consumer demand for breast meat than leg meat (Husak *et al*., 2008).

1.10 Bone analysis: Mineralisation, Ca and P content and strength

Bone mineralisation acts as an indicator of dietary mineral utilisation, vitamin D activity and bone health (Angel *et al.,* 2006; Venäläinen *et al.,* 2006). The tibia is the most widely used mineralisation marker as it is the fastest growing bone, and therefore considered to be the most sensitive to changes in mineral status (Applegate and Lilburn, 2002). Evaluation of tibia ash to determine mineralisation is

recommended by the AOAC (1990). However, Thorp (1992) states that there is greater external torsion on femurs than tibias, and Moran and Todd (1994) found that the femurs were more sensitive to a dietary reduction in phosphorus than the tibias, which suggests the femur may be a greater indicator of leg bone issues in broilers. This suggests that in order to observe dietary effects on bone development it is vital to observe effects in both the tibia and femur. Bone analysis needs to be conducted on only one leg per bird because both tibias and femurs grow and mineralise at the same rate (Shaw *et al*., 2010).

Analysis of bone mineralisation and strength both act as indicators of bone health. There is a lack of consensus in the literature as to the best method for preparing bones for determination of ash content. Hall *et al.* (2003) states that autoclaving the bone to deflesh it, based on the method of Boling-Frankenbach *et al.* (2001), is advantageous in that it is less time consuming and involves less exposure to harmful chemicals than the AOAC (1955) approved boiling/ extraction method. It has also been reported that defatting bones prior to analysis of ash content reduces variation (Huyghebaert *et al.,* 1988). Kim *et al.* (2004) found that bone preparation method had an impact on bone breaking strength but not on ash weight; fresh bones gave better bone breaking strength correlated to other bone parameters than dried bones.

The cortical bones in modern broilers are often porous and have low mineral content, resulting in high susceptibility to bone deformities and low breaking strength. Cortical bone consists of microscopic cylindrical structures orientated parallel to the long axis of the bone, and these are laid down in concentric rings called lamellae. There is increased porosity in fast growing broilers because osteoblasts are not able to completely fill the lamella (Williams *et al*., 2003). A negative impact of increased bone porosity from a commercial perspective is that it can lead to darkening of meat around the bones because bone marrow haem pigments leak through the bones (Smith and Northcutt, 2004).

1.11. Overview of phytate

Phytic acid (*myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate (IP_6)), or phytate as a salt, is the primary storage form of phosphorous in plant tissues of cereals and leguminous plants (Tamim and

Angel, 2003). **'**Phytate' and 'phytic acid' are terms used synonymously; phytate is the mixed cation salt of phytic acid. Approximately two thirds of the phosphorus in cereal grains and oilseed meals is bound in the phytic acid structure (Viveros *et al.,* 2000). Phytin is the name given to the complexed molecules of phytate with calcium, potassium and magnesium (Angel *et al.,* 2002; Lott *et al.,* 2000). Phytate bound to phosphorus is referred to as phytate-P, as opposed to non-phytate P which is phosphorus not bound to phytate. *Myo*-inositol is found mainly as a component of phytate, but is also found in monogastric diets in its free form, and as an inositol-containing phospholipid (Cowieson *et al.,* 2011).

Phytate represents 1-1.5% of the weight, and 60-80% of the total phosphorus in seeds (Lei *et al.,* 2013). The amount of phytate present in cereals varies from 0.5 to 2.0% (Coulibaly *et al.,* 2011). Phytate is the most abundant inositol phosphate in nature; it is vital for seed development and seedling growth and as a store of potassium, magnesium, calcium, iron and zinc. In developing seedlings, phytate also manages inorganic phosphate levels (Lott *et al.,* 2000). The phytate-P concentration of plant materials has a considerable effect on the total phytate-P concentration in poultry diets, depending on relative inclusion concentrations. Phytate is found in higher concentrations in cereal mill by-products and oil seed meals than in grains because in dicotyledons seeds phytate is located in the kernel, in the sub-cellular globoids, whereas in grains it is only located in the aleurone layer. This section examines the structure of phytate molecules and the impact that phytate has on the poultry industry from a welfare, environmental and economic viewpoint.

1.11.1. Chemical characteristics of phytate

Phytate is made up of six negatively charged phosphate groups bound to 12 hydrogens in an inositol ring. It is very electrostatically reactive because the 12 protons have pKa values of 1.5-10 so are unable to become fully protonated. Each of the 12 protons has two proton dissociation sites per phosphate group; a proton dissociation site is a site in which a H+ can be released leaving a negatively charged. Six of the proton dissociation sites are acidic (pKa < 1.5); three are weakly acidic (pKa 5.7, 6.8 and 7.6) and three are very weakly acidic (pKa 10) (Costello *et al.,* 1976). As a result, at pH less than 1 phytate

is largely unreactive as has a neutral charge. At acidic pH (1-2), 6 of the 12 protons dissociate and becomes negatively charged, so can react with proteins. At neutral and basic pH 3, protons dissociate. This means there is a wide pH range, spanning across the whole of the gastrointestinal tract, in which phytate is negatively charged (Maenz, 2001). These negative charged sites bind to divalent cations such as calcium, magnesium and zinc as well as protein residues and starches. In mature seeds phytate is found as these complexes. A phytate molecule can bind to at most six calcium molecules, but on average it binds five or less (Dendougui *et al*., 2004). This formation of insoluble salts results in poor mineral bioavailability and absorption, hence phytate is classed as having antinutritional properties. The reactivity of phytate is therefore dependent on both the arrangement and structure of the molecule and the pH of the environment (Maenz *et al.,* 1999). Once exposed to acidic conditions phytate becomes soluble as a moderately protonated moiety, so when it is exposed to the low pH conditions of the proximal gut it becomes soluble because the potassium and magnesium are replaced by H+ ions (Lott *et al*., 2000).

1.11.2. Impact of phytate on the poultry industry and bird health

Phytate is poorly digested by monogastric animals; Cowieson *et al*. (2006) states as low as 10% of phytate-P is digested by poultry. This is because monogastrics lack effective endogenous phytases necessary to release the phosphate from the inositol ring of phytate, and because interactions with other minerals and proteins present in the gastrointestinal tract reduce phytate-P availability. Also phytate-mineral complexes are soluble at low pH (< 3.5), which means that in the small intestine (pH 5-7) insoluble phytate-mineral complexes form and mineral bioavailability is reduced (Simon and Igbasan, 2002). Reactions between phytate and other nutrients, such as amino acids, starch and other mineral cations, also cause increased endogenous secretions of these nutrients (Bohn *et al.,* 2008). The result of this is reduced broiler performance; for example Liu *et al.* (2009) found that weight gain decreased by 2.6% in birds from hatch to age 21 days when dietary phytate content was raised from 0.785% to 1.57%, Ravindran *et al.* (2006) found that as phytate presence increased AME decreased, and Onyango and Adeola (2009) found weight gain, feed intake and tibia ash decreased in the

presence of phytate. Phytate anti-nutrient effects are estimated to cost £6 per tonne in lost performance. Awareness of the negative effect of phytate on monogastrics has been known for a while, possibly even as far back as 1855 when the role of phytate as a storage form of phosphorus in vegetables was first discovered (Hartig, 1885). It is not until recently however that the extent of the antinutritional problem has been considered.

The lack of successful hydrolysis of phytate by monogastrics means that approximately 82% of consumed phytate is excreted (Cowieson *et al*., 2004), hence inorganic sources of phosphorus must be supplemented to poultry diets to meet phosphorus requirements. The interaction between phytate and other food components in the digestive tract is dependent on several factors including: pH of the tract, presence of components that may compete with phytate for mineral binding (Thompson, 1993), feed processing, source and type of phytate, volume and type of ingested phytate and area of the tract (presence of phytase enzymes). Digestion and retention of phytate-P is dictated primarily by the form of dietary phytate, and mineral and vitamin D status of the bird (Maenz and Classen, 1998; Ravindran *et al*., 1995). Phytate mineral complexes also reduce the ability of the minerals to act as co-factors in enzymes.

1.11.3. Impact of phytate on the environment

Only approximately 4% of phytate is broken down in the gastrointestinal tract (Harland and Oberleas, 2010). Excess inorganic phosphorus and phytate-P have negative implications on the environment when excreted, such as soil run off, post soil saturation and eventual eutrophication. In areas of concentrated poultry production this poses particular environmental concern. The adverse effects of high-phosphorus in animal excreta came into public view primarily in the 1990s, when there was a heightened occurrence of fish deaths in the United States (Lei *et al.,* 2013). This led to increased interest in phytases, which has received wide acceptance largely as a result of legislation designed to reduce phosphorus pollution (Ravindran *et al.* 2006). Microbial phytases are favoured over plant phytases from an environmental perspective as they are active over a wider pH range and hence hydrolyse up to 50% of dietary phytate (Harland and Oberleas, 2010). Summers (1995) found that a

20% reduction in dietary phosphate, by lessening supplemental phosphate levels, caused a corresponding reduction in phosphate present in excreta from layers.

1.11.4. Economic effects of phytate

Small profit margins mean that there is high demand for ways to reduce the cost of poultry feed. Phosphorus, and hence phosphorus supplements for poultry diets, are very expensive. This is partly because, despite phosphorus being the eleventh most abundant element, it is always bonded with other elements or compounds, and is very rarely found in its pure form (Abelson, 1999). Furthermore, phosphorus is non-renewable and is being mined at an extensive rate to meet artificial fertiliser demands, meaning that eventually phosphorus deposits will be mined out (Lei *et al.,* 2013). Inorganic phosphates for poultry feed come primarily from phosphate rock, supplies of which are currently declining in both quantity and quality. 80% of globally used phosphates are used as chemical fertilisers, so just 20% must be divided between animal feed and special applications, such as detergents (Graham *et al*., 2003).

The phytase market did not develop very quickly post-introduction because it was viewed as having only an environmental impact, as opposed to economic benefits. However, an increase in phosphorus costs after 2007/2008, due largely to increased energy costs and phosphorus fertiliser use, saw a huge increase in phytase use. The current cost of dicalcium phosphate is approximately £770/tonne, compared to approximately £150/tonne in 2007 (Li *et al.,* 2012) which made phytase much more cost effective. The average cost of phytase at 1000 FTU/kg is approximately £1.50/ tonne of feed and supplementation at this level potentially replaces dicalcium phosphate (0.18% phosphorus) which highlights the economic benefits of phytase (Lei *et al.,* 2013). The phytase market has now reached approximately £224 million a year and increased focus on phosphate sustainability will continue to push it forward even further (Dayyani *et al*., 2013).

1.12. Overview of phytase

Phytases (myo-inositol hexaphosphate hydrolases) hydrolyse phosphate groups from molecules of

phytic acid (IP_6) resulting in lower myo- inositol phosphates being produced such as IP_1 to IP_5, in addition to inorganic phosphorus. Inositol is also produced which is a simple carbohydrate (Selle *et al*., 2007). Phytases represent a subgroup of phosphomonoesterases that can instigate stepwise dephosphorylation of phytate (Angel *et al.*, 2002). They are generally supplemented according to their activity in standard conditions (pH 5.5, 37°C, 5 mmol/L sodium phytate) (Greiner and Bedford, 2010). The ability of phytase to hydrolyse phytate is determined by the location of phytate within cells walls, the solubility of the phytate, and susceptibility of the phytate to the effects of phytase.

The positive effects of phytase on body weight gain, feed intake and feed efficiency have been widely reported; for example bird performance was improved in diets supplemented with 1000 FTU/kg of microbial phytase compared to diets supplemented with phosphate alone (Simons *et al*., 1990). Also Rama Rao *et al*. (1999) and Denbow *et al*. (1995) found that growth increased exponentially with graded amounts of supplemental phytase. Some studies have found that these positive effects reach a plateau at levels of around 500-1000FTU/kg, for example Denbow *et al*. (1995) found that bird performance improved linearly until 800 FTU/ Kg was reached. Other studies however have found optimum performance is reached at much higher levels of phytase inclusion, for example Wu *et al*. (2004) found supplementation with 2, 000 FTU/Kg significantly improved feed efficiency. As a result phytases are now used in approximately 80% of poultry diets and save animal producers approximately £1.31 billion a year. The current global phytase market is thought to account for over 60% of the total feed enzyme market (Lei *et al*., 2013). Phytase products are now manufactured in various ways; for example some are formed by solid-substrate fermentation (Rani and Ghosh, 2011), some by liquid fermentation using genetically and non-genetically manipulated organisms (Papagianni *et al*., 2001, Roland *et al*., 2003 and Costa *et al*., 2008) and some by recombinant DNA technology. This section examines the factors that affect the efficacy of phytase in combating the antinutritional effects of phytate in poultry diets, and explores how commercial phytases are classified.

1.12.1. Phytase classification

Phytases are classified based on whether they are fungal or bacterial, and on whether they are coated to protect them from high temperatures. They are also classified by the position on the phytate molecule in which hydrolysis first occurs; the 3-phytases hydrolyse the ester bond of myo- inositol hexakiphosphate at the third position, for example those derived from *Aspergillus niger,* and the 6-phytases hydrolyse the ester bond at the sixth position, for example those derived from *Escherichia coli* (Guenter, 1997, Singh, 2008 and Cowieson *et al.*, 2006). The sequential hydrolysis of phosphate groups following initial attack is in numerical order; 6-phytases attack only at position 1 following initiation at position 6, and 3- phytases attack at position 4 then 5, 6 and 1 following initiation at position 3. Additionally Greiner (2006) and Greiner and Bedford (2010) state that phytases can be classified based on their catalytic mechanism, or divided into acid or alkaline phytases based on their optimum pH range.

It has been suggested that bacterial phytases may be more efficient than fungal phytases; Auspurger *et al*. (2003) found that phytases derived from *Escherichia coli* released more phosphorus from phytate than phytases from two different types of fungal phytase, and Oyango *et al*. (2005) found that phytases from *Escherichia coli* are more active in the small intestine than phytases from *Peniophora lycii*. This may be due to resistance to pepsin and pancreatin, as these have the ability to repress, and even sometimes stop, phytase activity.

1.12.2. Phytase activity

Phytase activity is measured in phytase enzyme units (FTU). These are an *in vitro* measure to verify the activity of a product and do not necessarily equate to *in vivo* bio-activity of the enzyme. The definition of a phytase enzyme unit is the 'amount of enzyme that catalyses the release of 1 µMol of inorganic phosphorus per minute from 5.1 mM sodium phytate in pH 4.5 buffer at 60°C'. Phytase activity is determined by extracting the phytase, by solubilising the sample into an aqueous state and incubating the sample, and determining the reaction of phytase with a standard concentration of

substrate. Colour reactants with stopping solution are then used for enzyme reaction, and the level of phosphorus released in the incubation phase is determined. Approximately 0.8g of digestible phosphorus is released from an estimated 300-600 phytase activity units/kg of diet, and has the potential to replace 1.3 g/kg of phosphorus from dicalcium phosphate.

Phytases are generally unable to completely dephosphorylate phytate (Greiner and Konietzny, 2006). Also, a single phytase is unable to meet all the desired commercial properties; stable from heat activation during feed processing (resilience to 65-80°C temperatures), cost effective to produce and effective at releasing phytate phosphate in the upper digestive tract (Greiner and Bedford, 2010). This is because they each have different properties, which suggests there is need for a collection of phytases which are application-specific (Greiner and Konietzny, 2007). Different phytases may exhibit different degradation pathways and hence produce different *myo*-inositol phosphate intermediates; some of which may act as competitive inhibitors if they are not hydrolysed while some may go on to be dephosphorylated by other phytases (Bedford and Partridge, 2010). Phytases are therefore screened and developed for the most favourable properties. Wyss *et al.* (1999) states that phytases with broad substrate specificity are more advantageous in animal nutrition than those with a narrow specificity, as those with a broad range appear to readily degrade inositol phosphates with little accumulation of intermediates, whereas those with narrow specificity can result in inositol tri- and bi-phosphate accumulation.

Phytase thermostability also dictates its efficacy; microbial phytases have a wide optimum temperature range, ranging from 35-145°C (Wodzinski and Ullah, 1996) whereas plant phytases have a narrower range, from 45-60°C (Kumar *et al*., 2011). One approach to improving thermostability is the use of genetic modification to produce a thermotolerant enzyme, but the genetically modified products currently available are occasionally unsuccessful in some pelleted feeds (Bedford *et al*., 2003). Sometimes liquid phytases are sprayed onto feed post-pelleting but this is costly and accuracy and consistency of application is questionable, so this method is not widely practised. Another approach is to coat the phytase with a thermo-tolerant, or add stabilising additives (Ravindran and

Adeola, 2010), but the coating may reduce the efficacy of the enzymes (Kwakkel *et al*., 2000). This is likely because the coating may incur a delay in phytase release in the intestine, causing performance per unit of enzyme to be compromised.

1.12.3. Phytase in poultry diets

Alkaline phosphatases and endogenous phytase are present in the poultry digestive system, so birds are able to partly dephosphorylate phytate into free phosphate and inositol, but digestion of phytate is incomplete, which is why diets must be supplemented with exogenous phytases (Cowieson *et al.,* 2011). Exogenous phytase supplementation in broiler diets is however not always successful. Phytases are most active in the gastric region of the digestive tract because pH is low, so the conditions are favourable for soluble phytate. However, the environment in this section of the tract is very proteolytic, so for the phytase to be efficient it needs to be resistant to pepsin hydrolysis. *E. coli* phytases have been shown to be more efficacious at gastric pH and more resistance to pepsin attack than *Aspergillus* phytases (Igbasan *et al.,* 2002). Accumulation of phytate in the gizzard does not necessarily have a negative effect on phosphorus availability because there is the potential that partially phosphorylated inositol phosphates will be dephosphorylated further in the small intestine (Greiner and Bedford, 2010). This suggests that it may not necessarily be complete dephosphorylation of a phytate molecule that dictates how efficient phytase is. Calcium has been found to reduce the effects of endogenous phytase (Tamim *et al*., 2004), partly because calcium increases pH and consequently makes the environment less favourable. This is an issue in broilers because calcium is supplied in high levels in the diet.

Supplementing diets with phytase is the most convenient and practical way of combating the antinutritional effects of phytate, and has increased substantially in the past 10 years due to the increase in raw material prices. An alternative method is to chemically degrade feed phytate prior to feeding, but this could have a detrimental effect on quality of the feed (Pandey *et al.,* 2001). Another method is to add phytase-producing microbes to diets but there is concern over potentially contaminating the environment with the microbes, and also how to control the colonisation of the

microbes (Lei *et al.,* 2013).

1.13. Phytate reactivity

Phytate presence in the gut has a breadth of extra-phosphoric effects beyond reducing phosphorus digestibility, such as reducing energy metabolism and amino acid and vitamin utilisation (Cowieson *et al.,* 2011; Maenz, 2001; Nahm, 2009). Phytate solubility and susceptibility to the effects of phytase dictates phytase efficacy. Phytate degradation varies greatly between feedstuffs; for example Leske and Coon (1999) found that a fungal phytase increased phytate degradation by 38% in soyabean meal, but by just 15% in rice bran.

The intrinsic properties of the phytase and concentration of both phytate and phytase in the diet, in addition to bird age and diet composition, dictate phytase efficacy and phytate reactivity. Solubility and retention time in the different areas of the gastrointestinal tract are both influenced by, and have an impact on phytate reactivity. The form of phytate also effects its reactivity; for example Onyango *et al.*(2009) found that there was a difference between either free or magnesium-potassium phytate with regards to their anti-nutritional effect on amino acid and mucin loss in broilers.

Digestive enzyme secretion and activity is reduced by phytate presence due to the formation of insoluble mineral-protein-phytate complexes (especially in the small intestine) because phytate binds to positively charged enzyme co-factors, such as zinc and calcium. Liu *et al.* (2008) showed that α-amylase activity in the duodenum was decreased by 8.3% in the presence of phytate. Reduced activity of α-amylase can have negative repercussions on energy digestibility as it reduces absorption of carbohydrates. Phytate has also been shown to reduce utilisation of lipids by reducing reabsorption of fats, cholesterol and bile salts (Liu *et al.,* 2009). This section examines factors that dictate phytate reactivity, including location of phytate in ingredients fed to poultry, position of phosphates groups in phytate molecules and gastrointestinal pH.

1.13.1. Location of phytate in poultry feed ingredients

Location of phytate varies between seeds; in cereals such as wheat and barley (monocotyledonous seeds) it is found in the aleurone layer (Coulibaly *et al.,* 2011), whereas in oilseeds such as soyabean meal it is found mainly in the protein bodies (Manangi and Coon, 2006) and in corn it is found mainly in the germ (Wyatt *et al.,* 2004). Phytate is located in electron-dense areas, which are known as phytate globoids, and these are usually located in protein storage vacuoles. The size of these globoids is dependent on total phytate content of the seed (Classen *et al.,* 2010). These globoid crystals are comprised of phytate salts, mainly with potassium and magnesium (Brejnholt *et al.,* 2011). The location and hence chemical affiliation of phytate can influence nutrient availability. Protein concentrates tend to have higher levels of phytate than either cereals or corns.

1.13.2. Degradation of phytate

As stated previously, the 6 phosphate groups on the inositol ring of phytate are hydrolysed in sequential order, with 6-phytases starting at position 6, then position 1, 2, 3, 4 and 5. Degrading IP_6 to IP_4 eliminates most of the antinutritional effects of phytate, but does not generate the full phosphorus hydrolysing capacity. The rate of degradation to IP_4 is dependent on several factors, including whether the phytase is a 3 or a 6- phytase, the enzyme: substrate ratio, phytate solubility and the intestinal health of the bird. Retention time in the tract is also a factor as the greater the retention time in the gizzard the greater the IP_6 reduction. IP_4 and IP_3 form weaker mineral complexes and bind to fewer minerals than IP_5 and IP_6 (Simpson and Wise, 1990); IP_3 has only 10% the chelation capacity of IP_6. These lower esters are either absorbed in the small intestine or precipitated with cations and excreted; cation concentration and extent of dephosphorylation dictates the amount of esters that are absorbed.

Konietzny and Greiner (2002) found that the phosphate attached to the second carbon of the inositol ring is resistant to hydrolysis by phytase unless in the presence of very high concentrations of phytase at prolonged incubation. This phosphate group is attached axially, as opposed to the other phosphate

groups which are positioned equatorially (Wyss *et al.* 1999; Irvine and Schell, 2001). As a result, once the phosphate at position 1 has been removed the phytases tend to seek other substrates rather than continuing on the current inositol ring (Greiner *et al.,* 2000). With 6-phytases therefore, only the phosphates at position 6 and 1 are removed resulting in a build-up of IP_4, because phytases hydrolyse IP_6 before IP_4. Only when the concentration of IP_4 reaches a high enough level can the phytase continue to remove the remaining inositol-phosphates (Greiner *et al*., 2000). It is therefore imperative that adequate phytase is present to completely dephosphorylate phytate molecules.

6-phytases reduce a greater proportion of phytate than 3-phytases because phytase activity is determined by the amount of phosphate released, as opposed to the rate of IP_6 hydrolysis; 6-phytases release more phosphorus than 3-phytases but 3-phytases depolymerise phytate at a faster rate than 6-phytases (Wyss *et al.,* 1999). The biochemical classification of phytases does not however indicate its efficacy; it only defines the mode of action used by the phytase. Phytases should instead be selected based on ability to release phosphorus from phytate substrates, physical characteristics, the matrix values and stability under feed processing and storage conditions.

1.13.3. Gut pH

Phytase presence and activity is affected by gut pH and the optimum pH of the enzyme. Most 6-phytases have a very specific pH range; usually 4.5-5, whereas 3-phytases have a broader range; 2.5-5, except *Bacillus* phytases which work most effectively at neutral pH (Simon and Igbasan, 2002). At low pH (less than 4) phytate-mineral complexes are soluble but at neutral pH, such as in the poultry small intestine, insoluble phytate-mineral complexes are readily formed (Tamim and Angel, 2003) thus reducing mineral bioavailability and increasing excretion of minerals and amino acids. Phytate is therefore most susceptible to attack by phytases in the crop (pH 4-7), proventriculus and gizzard (pH 0.5-4) (Wyatt *et al.,* 2004).

1.13.4. Mineral presence

Phytate molecules have 12 different acid dissociation constants, two for each phosphate group, and

are negatively charged even at low pH. As pH increases along the gastrointestinal tract the negative charge increases resulting in increased probability that phytate-cation reactions will occur, particularly with divalent cations such as calcium. This results in formation of stable salts which precipitate out of solution. The order in which phytate forms complexes when presented with cations and free acid has been investigated extensively (Vohra *et al.*, 1965 and Maenz *et al.*, 1999). The ranking of minerals based on ability to inhibit phytate hydrolysis by phytase at neutral pH in order is as followed; Zn^{2+}>Fe^{2+}>Mn^{2+}>Ca^{2+}>Mg^{2+} (Maenz *et al.,* 1999). Sodium is not affected by phytate, which suggests that minerals with one valence electron may not be subjected to the anti-nutritional effects of phytate (Scheuermann *et al.,*1988). This may be due to the bond between monovalent cations and phytate being weaker than that presented between multivalent cations and phytate making them more likely to be reabsorbed.

The extent of phytate degradation is proportionate to the amount of phosphorus liberated, but the amount of calcium liberated varies and often exceeds that of phosphorous. Phytase supplementation has also been reported to enhance mineral availability beyond phosphorus, for example it has been shown to increase both magnesium and iron digestibility (Viveros *et al.,* 2002 and Ravindran *et al.,* 2006 respectively). This section examines the relationship between phytate and Ca, 1,25-dihydroxycholecalciferol, zinc and vitamin D_3.

1.13.4.1. Calcium

Calcium is important because high dietary calcium levels reduce phytate digestibility in chicks (Selle *et al.,* 2007); with Ca concentrations of less than 5g/kg able to increase ileal digestibility of phytate-P by up to 70% (Tamim and Angel, 2003; Tamim *et al.,* 2004). When calcium and phytate levels exceed a critical concentration, salt formation and precipitation occurs which reduces the amount of calcium available in the intestine for absorption. Dietary calcium should therefore be kept to a minimum to facilitate maximum phytase efficacy, without compromising skeletal development. Mohammed *et al*. (1991) found that reducing dietary inorganic phosphorus alone had no impact on phytate-phosphorus

utilisation, but reducing calcium as well increased utilisation by 65%, and that phytate-P utilisation was approximately 59% in diets with normal Ca compared to approximately 77% in diets low in calcium. Care must be taken when reducing dietary calcium levels because levels below requirements alters the Ca: P ratio and can put strain on phosphorus utilisation (Cowieson *et al.,* 2011). Diets should be formulated on available Ca rather than total Ca to optimise phytase efficacy. Levels of phytase in layers and broilers is different due to the retention time in the gizzard and differences in Ca absorbed. Limestone particle size can also affect phosphorus release by phytase in that it takes longer to solubilise larger particles.

Calcium-phytate complexes in the small intestine reduce calcium availability (Tamim *et al*., 2004; Plumstead *et al.,* 2008). As a result, dietary calcium levels have a significant effect on the response of supplemental phytase (Sebastian *et al*., 1996); with phytate digestibility and phytase efficacy increasing when diet calcium levels are reduced to 0.75-0.85% (Ballam *et al*., 1984; Perney *et al*., 1993). Phytate-P decreases in proportion to the calcium: phytate molar ratio (Rama Rao *et al*., 1999) with phytate digestibility being lower when the calcium to phytate ration is greater than 2:1 (Wise, 1983). As a result, below pH 4, calcium and phytate-P are very soluble (Grynspan and Cheryan, 1983) but as pH increases solubility reduces. At highly acidic pH calcium can hinder the extent of protein-phytate complex formation by reacting with either phytate or protein, preventing the formation of binary protein-phytate complexes (Selle *et al*., 2009). Dietary limestone concentration can therefore potentially be used as a tool to control phytate activity through manipulation of digesta pH, particularly as a rise in pH reduces the susceptibility to the effects of phytase. Moles of calcium per mole of phytate varies between feed ingredients for example in soyabean and oats there are approximately three moles of calcium per mol of phytate, whereas in rice corn extract there is approximately two and in corn semolina just one (Dendougui *et al*., 2004).

1.13.4.2. 1,25- dihydroxycholecalciferol

Intestinal absorption of calcium and phosphorus is stimulated by 1,25-dihydroxycholecalciferol; the lower the calcium and phosphorus levels the higher the plasma 1,25-dihydroxycholecalciferol levels. Dietary supplementation with 1,25-dihydroxycholecalciferol has been found to improve phytate-P digestibility and retention in broilers, and reduce the need for inorganic phosphorus supplements (Ravindran *et al.,* 1995; Mitchell and Edwards, 1996, Edwards, 1993). For example Edwards (1993) found supplementation with 1,25-dihydroxycholecalciferol caused phosphorus utilisation to increase by approximately 30%, and Mohammed *et al.* (1991) found that when inorganic phosphorus and calcium are low, increasing cholecalciferol levels can increase phytate-P utilisation by over 75%. This may be because 1,25-dihydroxycholecalciferol is a phosphate transport hormone and its presence causes an increase in the concentration of serum phosphorus, thought to be due to a stimulation of phosphate and reabsorption in the kidney and intestine (Edwards, 1993; Tanka and DeLuca, 1974).

1.13.4.3. Zinc

Zinc is secreted into the duodenum via the pancreas (Harland and Oberleas, 2010); two to four times as much zinc goes into the duodenum in a day via the pancreas as is consumed in the diet (Oberleas, 1996). Dietary phytate complexes to zinc, which is the most vulnerable essential cation. This zinc is approximately 30% dietary zinc and 70% secreted zinc (Harland and Oberleas, 2010). The strength of the complex depends on zinc concentration and pH. Complexes are most likely to be formed at pH 6-7, so the proximal small intestine is the main site of zinc-phytate complex formation. The shift of pH from the gizzard to the duodenum causes phytate to readily seek cations to complex and precipitate with, and the vulnerability of zinc means that in the presence of high volumes of phytate, zinc deficiency is likely to occur.

Zinc deficiency results in reduced growth and decreased appetite, as well as increased susceptibility to infections, as zinc acts as a cofactor for cell division and regulates over 50 enzymes (Harland and Oberleas, 2010). Zinc deficiency therefore has significant economic implications in the poultry

industry. Zinc deficiency only occurs when phytate is present; there is enough zinc in poultry feed to meet the needs of monogastric species and, in the absence of phytate, it is readily absorbed by the small intestine (O'Dell and Savage, 1960). Zinc deficiency can therefore be easily predicted based on only analysis of feed for phytate and zinc content (Oberleas and Harland, 2005). Phytase improves the bioavailability and digestibility of zinc by approximately 1g per 100 units of phytase (Yi *et al.,* 1996).

1.13.4.4. Vitamin D_3

Vitamin D_3 is essential for regulating calcium metabolism and acts as a precursor for 1,25-dihydroxycholecalciferol. Endogenous intestinal phytase and alkaline phosphatase activity has been shown to increase with supplementation of vitamin D_3; Davies *et al.* (1970) found an increase from 750 to 7500 I.C.U./kg of vitamin D_3 in phosphorus-deficient diets increased phytase and alkaline phosphatase activity and improved tibia ash content. This may be due to vitamin D_3 stimulation of phosphate transport mechanisms in the intestine, therefore vitamin D_3 deficiency has a direct impact on phosphorus metabolism (Harrison and Harrison, 1961 and Wasserman and Taylor, 1973).

1.14. Protein-phytate complexes

Protein-phytate interactions are fundamental to the negative impact of phytate on protein digestibility. 1g of phytate can bind approximately 10g of protein under ideal conditions (Cowieson *et al*., 2009). Protein-phytate complexes form when a negatively charged phytates have a strong electrostatic attraction to positively charged proteins. The strength of the attraction between phytate and protein is dependent on concentration and solubility of the phytate, the pH of the environment and the isoelectric point and structure of the protein. Most protein-phytate complexes are formed in the beginning of the gastrointestinal tract, although some may be present in the feedstuffs. The likelihood of protein binding to phytate is dependent on the abundance of amino acid residues and the precise structure of the phytate. Cowieson and Cowieson (2011) suggest that interactions between protein and phytate may not be a result of binding between protein and phytate, but rather because phytate competes with protein for water, which reduces the solubility of the protein. It is

thought that phytate has a hydration shell that causes less polar proteins to precipitate, and hence competition for water dictates activation of pepsinogen. Therefore it may be that protein solubility is reduced by phytate altering the thermodynamics of water so that aggregation of proteins is likely (Cowieson and Cowieson, 2011).

The impact of phytate on protein solubility results in increased endogenous loss of amino acids and reduced amino acid digestibility. For example, Cowieson and Ravindran (2007) found that amino acid loss was increased by 87% when phytate levels were increased from 0.85% to 1.45%, and Cowieson *et al.* (2006) found supplementing diets with synthetic phytate caused amino acid digestibility to decrease significantly. Phytate also reduces reabsorption of secreted amino acids (Lui *et al.,* 2009). Glycine, serine, threonine and proline are the amino acids most affected by phytate (Cowieson and Ravindran, 2007).

Protein-phytate complexes are often resistant to pepsin digestion and solubilisation by HCl, causing increased secretion of both. The result of this is heightened mucin secretion, and a further loss of amino acids. Consequently, when phytate is hydrolysed protein absorption is increased. However, the ability of phytase to improve amino acid availability has been widely debated (Selle and Ravindran, 2007); with some studies finding that amino acids increased significantly with dietary inclusion of microbial phytase (Rutherfurd *et al*., 2012), whereas some studies found no effect of phytase on amino acid utilisation (Liao *et al.,* 2005). This section examines the impact of protein isoelectric points and gastrointestinal pH on the prevalence and strength of protein-phytate complexes.

1.14.1. Protein isoelectric points

The interaction between phytate and protein is largely dictated by pH; tertiary phytate-protein complexes form at around neutral pH, whereas binary phytate-protein complexes form at acidic pH (Selle and Ravindran, 2007). Proteins with a high isoelectric point (5-6) are electrostatically positively charged so phytate is readily complexed to these proteins, and conversely phytate does not readily bind to proteins with low isoelectric points. At neutral pH proteins are nearly all negatively charged

because of the weakly acidic residues present in almost all proteins. Consequently, in the gizzard, phytate-protein complexes do not readily form, hence why phytate is most susceptible to the effects of phytase in this section of the gut. In sections with higher pH, such as the small intestine, phytate-protein complexes will readily form and are not as easily hydrolysed. The isoelectric point therefore determines the strength of the interactions between phytate and protein, and hence the ability of phytases to release protein from phytate-protein complexes. As poultry diets differ in their amino acid content, they will therefore have different isoelectric points. Hence phytate interferes with protein to different degrees, depending largely on the impact of the particular diet on gut pH.

1.14.2. pH profile of pepsin

Phytate-protein complexes are resistant to digestion by pepsin and solubilisation with HCl, which often results in increased secretion of pepsin and HCl (Cowieson *et al.,* 2008). The response to this is increased mucin secretion, to protect the mucosa from digestion by HCl and enzyme. Pepsin in poultry has an optimum pH of 2.5-3 and becomes denatured at pH 3.9 (Crévieu-Gabriel *et al.,* 1999); if pH is shifted away from the pH optimum, due to variance in individual bird digesta pH or presence of dietary limestone, protein solubility and utilisation is reduced.

Pepsinogen, the zymogen of pepsin, is hindered from being converted into pepsin by phytate binding to the activating peptide (Liu and Cowieson, 2010). This demonstrates another way in which phytate affects protein utilisation, and suggests that manipulation of amino acids to enhance activation of pepsinogen is a potential way of overcoming the antinutritional effects of phytate on protein.

1.15. Methods used for measuring phytate concentration

It is clear that phytate needs to be measured, but there is no commercially available quantitative method for measuring it. Over 60 methods, including electrophoresis and paper chromatography (Oberleas and Harland, 2010), have however been developed for qualitative measurement of phytate. A key hindrance in establishing this methodology is identifying the various esters after separation.

Historically both Young (1936) and Oberleas (1964) analysed phytate using a colorimetric assay whereby samples were heated with a standardised amount of $FeCl_3$ and the amount of precipitated ferric phytate was measured. This method was however non-specific, not particularly sensitive and featured no separation of other inositol phosphate esters, as it implied that ferric phytate was the only phosphate not soluble in dilute HCl (Lehrfield, 1994).

The current most widely used methods for phytate analysis involve high performance liquid chromatography (HPLC) (Cowieson *et al.,* 2006), but each analysis requires expensive and time-consuming anion-exchange purification. This method also assumes that only phytate has been purified, which may be acceptable for non-processed grains, but not for processed feeds which can contain high levels of lower myo-inositol phosphate forms which may also elute with phytate, therefore overestimating phytate content. The original HPLC methods used a reverse phase column which was quick but provided little separation or accuracy (Oberleas and Harland, 2010). As a result reverse phase columns were replaced with strong anion exchange columns which have improved specificity (Oberleas and Harland, 2007). This method involves using $NaNO_3$ to generate a gradient on a column which separates different inositol phosphates.

Another method used recently to measure phytate is nuclear magnetic resonance, which has been used to investigate phytate presence in soil (Doolette *et al*., 2010), and to ascertain the amount of phytate in ileal excreta and digesta (Leytem *et al*., 2008). Despite it being the only spectroscopic method which provides complete analysis of the whole spectrum, it is not readily used because of the large volume of sample that is required (Turner, 2004). The most sensitive method for phytate analysis is the use of metal dye detection reagents (Mayr, 1988); although this method is suitable for testing phytate in tissue cultures, it is too sensitive and expensive for use in food analyses. There is a requirement for a non-HPLC method that is sensitive enough to read between 100pmoles and 100 nmoles, and cheap enough to be used to analyse feed.

1.15.1. Megazyme™ method for determination of total phytate

The assay developed by Megazyme™ measures the total available phosphorus from feed. It involves acid extraction of inositol phosphates, treatment with phytase specific for phytic acid (IP_6) and the lower *myo*-inositol phosphates and then treatment with alkaline phosphatase to ensure complete de-phosphorylation. Total phosphate is measured colourimetrically; the amount of molybdenum blue formed is proportional to the amount of inorganic phosphorus present and is quantified as phosphorus using a calibration curve of known phosphorus standards. One aim of this project is to develop the Megazyme™ assay for measuring phytate and total phosphorus in poultry digesta samples.

1.16. Methods used for measuring phytase activity

Phytase activity assays currently measure phosphate release as opposed to disappearance of phytate, due to the difficulty surrounding measuring the inositols released from phytate degradation. Phosphate release must be linear to time in order to quantify the degrading activity of phytase so physical parameters including pH, temperature, substrate concentration and enzyme stability must be kept constant when carrying out activity assays. The ability of these assays to accurately determine phytase efficacy may be questionable based on large variation seen in the literature; despite trying to control variables there can be huge difference between experiments. This has been illustrated by Angel *et al.* (2002) in which five studies were conducted to decipher amount of phytase needed to obtain 0.1% units of phosphorus equivalency. Each of the studies used the same phosphorus and phytase source, same duration of experiment and same age and strain of broiler chick but the results ranged from 781 to 1413 FTU/Kg (Adeola and Applegate, 2010). The issue with measuring phosphate release is that all phosphate removed will be classed as derived from phytate which may not give an accurate representation of phytase activity. It is likely that most phytase act on a range of phosphorylated compounds, with only a few, such as *Aspergillus niger*, being specific to just phytate. Also substrate inhibition needs to be considered as both the phosphate produced from hydrolysis and the phytate itself can have inhibitory effects on histidine acid phytases (Konietzny and Greiner, 2002).

Methods for detecting phytase activity need to take into consideration pelleting and thermostability, and the presence of other additives and minerals in the feed (Sheehan, 2011). Phytase is either added directly to the completed feed or blended with low inclusion ingredients. Measuring phytase in these blended diets can sometimes prove problematic because mineral presence may alter pH, which has an effect on the interaction between phytate and proteins and minerals, and can hence affect the strategy needed for the extraction stage. Additionally diet manufacture has an effect on the isoelectric point due to changes in thermostable energy. Some feed companies add formaldehyde to broiler diets to combat contamination by pathogenic microorganisms which potentially affects the ability to recover enzymes from feed (Sheehan, 2011), and phytases can bind to mycotoxin binders if they are added to the feed, reducing activity (Moslehi-Jenabian *et al.,* 2010).

The limitation of assays whereby the release of inositol phosphate from sodium phytate is measured is that phytate is unlikely to exist in its sodium salt form *in vivo*, so these methods may not be providing a true representation of phytase activity in the bird. A potentially more appropriate method was developed by Trans *et al.* (2011) who demonstrated that using protein-phytate complexes may be a more relevant substrate for the evaluation of phytase efficacy, as they occur naturally in the digestive tract and feed. Additionally in conventional methods pH is maintained at 5.5, but in this assay pH is at 3, thus mimicking the environmental conditions in the gizzard (Trans *et al.,* 2011). Activity of phytase was measured based on the principle that stable protein-phytate complexes form turbid solutions, so a reduction in turbidity acts as an indicator of phytate complex hydrolysis. Decreased turbidity therefore correlates to increased inositol phosphates from phytate. Evaluation of phytases using this method showed that *E. coli* phytases are more effective at hydrolysing phytate in phytate-protein complexes than phytate in sodium phytate. This highlights that the key factors which dictate phytase efficacy are the ability to be degraded by pepsin and stability at the environmental conditions of the gizzard.

The principal of the AOAC colorimetric enzymatic assay for determination of microbial phytase in feed

(AOAC Official Method 2000.12) is that a phytase solution is incubated with a phytate substrate at pH 5 and 37°C, which liberates the inorganic phosphate from the substrate. A colour complex is produced by addition of a molybdate based solution, which is then read on a spectrophotometer at 415nm with the degree of colour development being proportional to the amount of phosphate released, and is calculated by reference to a phosphate standard curve. The amount of phosphate released indicates the enzyme activity in the sample. Engelen *et al*. (2001) ran a collaborative interlaboratory study to validate this assay which involved 27 laboratories each analysing 6 commercial feed samples that varied in phytase activity (200-400 FTU/kg), where method performance and statistical calculations were carried out according to the AOAC guidelines. They found that this assay was reliable and allowed comparison of industrial enzyme preparations between laboratories based on reported data, ranging by just 14-20.5% between different laboratories. The ISO assay (ISO 30024: 2009) for determination of phytase activity in animal feed is very similar to this assay, except the acetate buffer used differs slightly and this assay also involves use of 0.01% polysorbate 20 to stabilise the suspensions. An alternative assay, based largely on the Megazyme^{TM} assay, adds trichloroacetic acid to the incubated sample to quench it, before adding a molybdate-ascorbate solution to produce the colour complex which is read at 820nm.

The most common methods used to measure phytase activity are colorimetric, whereby the colour formation between molybdate and released inorganic orthophosphate from phytate is measured. There is however large variation and poor reproducibility using these methods because free or water-soluble P causes high background and hinders degradation of phytate added in the assay. Additionally the absorbance readings are often also unstable because the background colour of the blank is strong and thus reduces the colour contribution by phytase hydrolysis, and also the samples do not mix very well as fat and carbohydrate presence causes an oil layer to form (Kim, 2005).

1.16.1. Formulating diets for poultry with phytase

The aim for feed formulation is to achieve the nutrient levels required at the lowest cost. This is often calculated using the assumption that there is a linear relationship between nutrient supply and the material it is derived from. This is not the case with phytase as when phytase supplementation increases from 500 FTU/kg to 1000 FTU/kg the increase in available phosphorus is only 30% (ten Doeschate and Graham, 2010). Enhanced nutrient contribution through phytase supplementation may be dependent on not only phytate content, but susceptibility of the phytate to degradation by phytase, and also any interaction with other nutrients. The commercial usage of phytase is therefore based on a nutrient matrix being assigned to a specific dose of phytase. As a result of phytase effects, diet formulation from an economic perspective can focus on the price of fats and oils as opposed to phosphate.

1.17. The role of endogenous phytase: In feed ingredients

Phytase is naturally present in feed materials fed to poultry (Greiner and Egli, 2003). These phytases have the potential to degrade the phytate in that material; for example Brejnholt *et al.* (2011) found that endogenous wheat phytase was able to significantly degrade phytate in wheat. The impact of this variation is shown by Nelson (1976), in which it was found that in diets containing corn as the only grain source just 3% of the phytate-P was hydrolysed by 9 week old birds, and in diets where wheat was substituted for half of the corn just 13% was hydrolysed. Phytase concentration varies greatly between different ingredients; in maize it is believed to be below 100 FTU/kg whereas in wheat and rye it is thought to be around 1200 FTU/Kg and 5000 FTU/kg respectively (Brejnholt *et al*., 2011; Viveros *et al.,* 2000). The impact of endogenous phytase on IP_6 and IP_5 degradation however is lower than that induced by microbial phytases in the same pH range (3-5.5) (Brejnholt *et al.,* 2011). Phytase loses activity at approximately 60°C, so its efficacy is greatly reduced when exposed to 80°C steam during pelleting (Ullah and Mullaney, 1996; Cavalcanti and Behnke, 2004).

1.18. Endogenous phytase: In the bird

There is a common misconception that phytate-P is largely unavailable for utilisation because poultry lack effective endogenous phytase, when in fact phytase and phosphatase are present in the intestinal mucosa (Cowieson *et al.,* 2006), as well as in the liver and blood (Cowieson *et al.,* 2011). The effects of this endogenous phytase on phytate-P digestibility are however negligible, because of poor solubility of phytate in the small intestine, largely due to luminal cation concentration, particularly calcium (Cowieson *et al.,* 2011); if phytate-P remains soluble in the small intestine it is able to be digested. There is very little known about the contribution of endogenous brush border phytase activity to digestibility of dietary phytate-P. Phytase contribution is likely to be subject to regulation in response to the birds' dietary phosphorus status.

Maenz and Classen (1998) found endogenous brush-border phytase activity in all segments of the small intestine, with the highest activity levels found in the duodenum. Intestinal phytase activity occurs over the pH range 5 to 6.5, with optimum pH 6. In this study it was found that there was a difference in pH profile between phytase activity and non-specific phosphatase, and that the intestine brush border contained no phosphatase activity. This suggests that the intestinal border contains phytase activity that is separate from phosphatase enzyme activity; Yang *et al.,* (1991) stated that phytate is mainly hydrolysed by non-specific phosphatases, whereas Bitar and Reinhold (1972) suggested that it is hydrolysed predominantly by specific phytase activities. Intestinal phytase activity in birds fed a phosphate-deficient diet is thought to be three times that of birds fed phosphate-supplemented diets (Maenz and Classen, 1998). Davies *et al.* (1970) also found that phytase and alkaline phosphatase activities were over twice as high when no supplemental phosphorus was added to the diets, compared to when adequate calcium phosphate was added to raise the inorganic phosphorus content to approximately 0.50%.

1.19. Alkaline Phosphatase

Alkaline phosphatase, like phytase, is a phosphomonoesterase. Alkaline phosphatase catalyses hydrolysis of organic phosphate esters in an alkaline environment (pH 8-9), in the presence of

magnesium or manganese and zinc ions. When diets containing exogenous phytase are fed, the role of alkaline phosphatase is to dephosphorylate phytate molecules that have by-passed degradation in the gizzard.

Alkaline phosphatase is most commonly found in the intestinal epithelium and kidney, but is also present in yeast and animal bone. Although brush border alkaline phosphatase activity is present throughout the small intestine, it is slightly greater in the duodenum and slightly lower in the distal ileum (Perek and Snapir, 1970; Chang and Moog, 1972). High levels of dietary calcium cause alkaline phosphatase activity to reduce, partly due to influence on hormonal balance. Low levels of dietary phosphorus and vitamin D_3 increase alkaline phosphatase production (McCuaig *et al.,* 1972).

1.20. Phytate susceptibility to the effects of phytase

It is essential that P release by phytase is accurately predicted due to the implications on broiler welfare and performance. The level of phytate and susceptibility of the phytate to phytase actions dictates the catalytic capabilities of phytases as both soluble and insoluble phytate-mineral precipitates can be resistant to phytase hydrolysis (Konietzny and Greiner, 2002). The total phytate-P concentration of a diet may be misleading because feed ingredients may contribute to the total amount of phytate in the diet, but in real application this may mean little for substrate available for phytase. It is therefore important when formulating with phytase that not only the total phytate content of the diet is accounted for, but also the relative solubility, which is largely dictated by location of the phytate in the grain. Phytate is transformed from phytase resistant mineral-complexed forms to being susceptible to the effects of phytase via addition of H+ ions to the weak acid phosphate groups. Mineral chelators, such as EDTA and citrate, can aid this conversion to phytase susceptible form; soluble mineral complexes materialise in the digesta which decrease mineral binding to phytate, and can then be dissociated and rebound to intestinal brush border mineral-binding sites, or absorbed as whole complexes (Maenz *et al.,* 1999). Binding divalent cations to phytate may make a portion of

it resistant to phytase; a chelator may remove cations from binding to the phytate and hence increase levels of susceptible phytate.

1.21. Superdosing phytase

The current phytase dosage in most monogastrics is approximately 500 FTU/kg of *E. coli* phytase equivalents, but there is evidence to suggest that superdosing (quantities of above 1500 FTU/kg) may be beneficial for enhancing bird performance, bone development and digestibility. Nelson *et al.* (1971) showed early signs of the positive effect of superdosing phytase in which it was found that phytate-P disappearance was increased from 38.9% with supplementation of 950FTU/kg phytase (*Aspergillus ficuum*) to 94.4% with 7,600 FTU/kg, and that bone ash increased 59% with the higher phytase level. However, despite these early findings, interest in superdosing did not increase until the commercialisation of phytase as a feed additive in 1991 (Cowieson *et al.,* 2011). At first, the inclusion cost of phytase was relatively high which meant the levels fed were constrained to 350-500 phytase units (FTU) per kilogram, but in recent years the cost of phytase has decreased significantly meaning currently 500 FTU/kg can be fed for considerably less cost.

In the past decade there has been increased interest in superdosing; for example Cowieson *et al.* (2006b) found that doses of phytase above 1,000 FTU/kg of diet improved weight gain, toe ash percentage and nutrient utilisation, presumably through phytate degradation. Also it was found that supplementing a corn-based diet with 12,000 FTU/kg phytase increased degradation of phytate-P, from around 42% at 93 FTU/kg to 95% at 12,000 FTU/kg, as well as enhanced AME and nitrogen retention (Shirley and Edwards, 2003). Positive effects of superdosing in poultry have also been observed by Auspurger and Baker (2004) at levels of phytase ranging between 500 FTU/kg and 10,000 FTU/kg, and by Pirgozliev *et al.* (2007) at levels of phytase ranging between 500 FTU/kg and 2,500 FTU/kg.

One possible mechanism behind the observed improvements with high levels of phytase is likely to be because there is increased production of soluble lower inositol-phosphate esters, and lower levels of

IP_6 and IP_5; lower esters have a much lower capacity to chelate divalent cations, can be solubilised in the small intestine and increase mineral release. There is also more phosphate liberated and restoration of P or Ca proportionate release as well as reduced presence of residual phytate. Also low *myo*-inositol esters stimulate lipid breakdown by generating myo-inositol with lipotropic effects (Cowieson *et al.,* 2011), although this is an area that requires further investigation.

Superdosing has also been shown to reduce the likelihood of vitamin E deficiency (Karadas *et al*., 2010) and protect against fatty acid syndrome, due to their lipotropic effects (Katayama, 1999). This suggests that the role of exogenous phytases is more to prevent higher esters from leaving the gastric phase, as opposed to carrying out complete dephosphorylation of phytate into inositol and free phosphates.

The improved effects of high phytase on digestible P can only be expected to improve bird performance if P is limiting; Angel and Applegate (2002) added phytase to diets with sufficient P and found that this increased the amount of soluble P excreted, highlighting the potentially negative effects of phytase. Superdosing may stimulate, and hence increase, feed intake, particularly in P deficient diets and increases the presence of inositols which are believed to have potential growth promoting effects (Żyła *et al.,*2013). It is believed that phytase effects may be bimodal in that there is an initial improvement in digestibility followed by a second stage improvement in digestible nutrient intake and hence net energy effects. In P deficient diets P release from phytate instigates increased intake until the P requirements are met, but then continues to increase intake even further beyond this possibly because phytate is an appetite suppressant (Cowieson *et al.,* 2011).

1.22. Combining phytase with other enzymes

It is believed that enzyme cocktails as opposed to single enzyme use may potentially be advantageous due to the complex structure of feed ingredients. Combined dietary additions of phytase with other enzymes, such as proteases and non-starch polysaccharide enzymes, is widely used in monogastric production (Lei *et al.,* 2013). For example, Józefiak *et al.* (2010) found that combining

multicarbohydrase and phytase enhanced growth performance as well as liver insulin-receptor sensitivity in broilers feed full-fat rapeseed. It has been found that phytases supplemented with carbohydrases, such as xylanase, and proteases can have a positive effects on broiler performance and protein solubility (Cowieson and Adeola, 2005; Bae *et al.,* 2012). This is likely to be because phytate is located in cell walls and protein structures, so carbohydrases and proteases liberate the phytate from the cell matrix and hence expose the phytate to hydrolysis by phytase (Ravindran *et al.* 1999). The solubilisation of phytate in the presence of carbohydrases and proteases suggests that without phytases these enzymes may have a negative effect on bird performance because they cause increased production of phytate-protein complexes.

1.23. Future of phytase

Improved phytases and more diverse phytase applications are constantly being sought after and researched; for example in the past decade phytases with higher activity concentrates and greater resistance to heat inactivation during the pelleting process have been developed (Haefner *et al.,* 2005). One of the main factors driving phytase research is the need to reduce the environmental impact of excreted phosphorus (Casteel *et al*., 2011). Another main factor is that there are limited supplies of inorganic phosphorus and feed-phosphorus supplements; the supplies have become less cost effective or sustainable because countries with strong economic growth (such as India and China) have high growing demands for the supplies, and because oil prices have increased (Lei *et al*., 2013). The phytate in soil is not utilised efficiently by plants as a source of phosphorus due to the lack of phytase activity in the fluid that surrounds plant roots; improving phytate utilisation by plants would reduce the need for phosphorus in fertilisers (Wang *et al.,* 2013).

One recent development is phytase-transgenic plants seeds that contain high phytase activities, which have been produced by overexpressing the phytase genes in crops. For example, Chen *et al.* (2008) over-expressed *Aspergillus niger* phyA2 gene in maize seeds resulted in new maize hybrids with improved phosphorus availability. The main concern with this method is that the phytase may be inactivated by heat processing during pelleting. There has also been success with the development of

low-phytate soyabeans and grains (Hill *et al.,* 2009; Raboy, 2007; Mendoza*,* 2006). The main issue with development of these low-phytate seeds is that phytate is essential for seed germination, growth and disease resistance (Raboy, 2009), thus removing phytate from grains could reduce crop yield. Furthermore, pre-treating ingredients with phytase to reduce the phytate concentration has been studied by Newkirk and Classen (2001) in which it was found ileal amino acid digestibility was heightened by feeding birds dephytinised rapeseed meal in corn-soybean meal diets.

There is also current interest into the development of phytase-transgenic animals. Overexpression of the *E. coli app A* gene in the salivary glands of pigs has been investigated (Golovan *et al.,* 2001). This proved to be successful, based on a 75% reduction in the phosphorus excreted by these animals. Phytase-transgenic fish (Hostetler *et al.,* 2003) and mice (Golovan *et al.,* 2001) have also been developed, with a similar level of success. There is also the potential to produce poultry with heightened phytate digestion, for example Cho *et al.* (2006) created a chicken cell line that had overproduced multiple inositol polyphosphate phosphatase phytate-degrading enzymes. However, public view of genetically modified animals hinders the progress of this field.

The hunt for novel phytases has recently turned to searching for phytases in organisms that live in extremely hot conditions, with the belief that these phytases will be thermostable, and from organisms that live in very cold conditions, with the expectation that these phytases will have low temperature activity (Lei *et al.,* 2013). Recently phytases have been obtained from fungi; for example Zhang *et al*. (2013) have developed a highly pH and heat tolerant phytase from fresh fruiting bodies of shiitake mushrooms, and Singh and Satyanarayana (2010) isolated phytases from thermophilic fungal isolates from stored grains and animal excreta.

The search for novel phytases has also led to increased interest in protein engineering. One of the strategies involved in protein engineering is random mutagenesis, which involves screening to identify mutants that possess better characteristics, using procedures such as DNA shuffling of areas between homologous proteins and gene site saturation (Lei *et al.,* 2013; Lehmann *et al.,* 2000). Another strategy used is random design, in which preferred amino acid residue substitutions are constructed by adding

in disulphide bridges and substituting amino acid residues, and the effects on the protein function are predicted (Koonin and Galperin, 2003). An example of this is the charge of interactions of polar and ionic residues in the active site of *Aspergillus niger* phytase were altered so the activity was greatest at pH 3.5 (Kim *et al.,* 2006).

1.24. Ingredients in poultry feed

It is important to consider properties and attributes of feed as well as bird digestive tract environment and exogenous enzyme presence when assessing nutrient utilisation. The majority of protein used in poultry feed is from plant sources, predominantly oilseeds, with soyabean accounting for 75% of all protein used in compounded livestock rations worldwide (Gilbert, 2002). Annually, approximately 316 million tonnes of oilseed protein is used in the animal feed industry, compared to just 10 million tonnes from animal by-products (meat and bone meal and feather meal) and 7 million tonnes from fishmeal (FAO, 2013a). Oil is extracted from the plant material, and the remaining solid portion contains almost 50% protein (post removal of fibrous hulls). Protein sources fed to poultry vary greatly in their amino acid concentrations so synthetic amino acids have to be added to broiler diets, especially lysine (the first limiting amino acid in poultry) and methionine. It is more economical to provide these amino acids in synthetic form than to add more of a high-protein ingredient to the diet, and allows the nutritional needs of the bird to be met more precisely.

Grains are largely made up of starch and fibre so contain only around 10% protein. Grain is added to poultry feed primarily as a source of energy as starch. Maize is the most widely used grain in commercial poultry production worldwide as it is easily digested, highly palatable, high in available energy and free of anti-nutritional factors (FAO, 2013c). The metabolisable energy value of alternative grains is usually evaluated in relation to maize. In Canada and Europe however, wheat is the main energy source used with conventional broiler diets typically containing 60% or more of wheat. This section examines the main ingredients fed to poultry.

1.24.1. Soyabean Meal

Soyabean meal is the by-product of soyabean oil extraction (FAO, 2013a). Soyabeans are the most popular oilseed crop produced in the world; they represent 55% of the total global production of oilseeds, followed by rapeseed (14%), cottonseed (10%), peanut (8%), sunflower (9%), palm kernel (3%), and copra (1%) (Cromwell, 2012). Soybean meal is very high protein compared to other plant protein sources; 44% with hulls and 46-50% without hulls. It also has the highest lysine digestibility of the common protein sources fed to poultry (approximately 90%), and contains substantial methionine, cysteine and threonine. There is also less variation in digestibility in soyabean compared to other oilseeds, has no negative impact on palatability, and has low fibre content and high energy. When processed properly it also contains very little toxins or anti-nutrients (Willis, 2003; Britzman, 2005). Annually, approximately 85% of the world's soyabeans are processed into soyabean meal and oil, 98% of which are further processed into animal feed (Soyatech, 2013). Only around 3% of soyabean meal used in Europe is derived from EU supplies (Brookes, 2001); annually approximately 40 million tonnes of soyabean is imported into the EU.

Seed proteins are either enzymatic or storage proteins, with most of the protein in soyabeans being storage protein. During germination, storage proteins supply nitrogen and carbon skeletons for seed development (Wang *et al.,* 2003). Soyabean meal can have very high calcium content because limestone is sometimes added at the end of processing to prevent the warm soyabean meal clumping together.

1.24.2. Rapeseed Meal

Rapeseeds, which are a member of the mustard family, are crushed to remove the oil of which rapeseeds contain approximately 40%. The resulting rapeseed cake is then further processed by solvent extraction into rapeseed meal. Rapeseed first became popular in WWII where it was used as a source of industrial oil, and the crushed seed was fed to animals. Rapeseed is cultivated in areas with colder climates, such as Europe and Canada, which tend to be unsuitable for soyabean growth.

Rapeseed oil has become the primary feedstock for biodiesel in Europe. After crushing to remove oil, the resulting rapeseed cake is processed through a similar solvent extraction process to soya to produce rapeseed meal.

Rapeseed meal usually contains approximately 37% crude protein, 10-12% crude fibre, 1-2% lipids and around 1% phosphorus and less than 1% calcium, although composition varies widely depending on a range of factors including origin, growing conditions, the manufacturing process and degree of oil extraction (Narits, 2011). The amino acid content of rapeseed meal is commonly around 8-10% less than that of soyabean, and it contains approximately 15% less metabolisable energy due to its high fibre content (>110g/kg), although dehulling can reduce this. As a result, it is used more commonly for layers and breeders than broilers (Fenwick and Curtis, 1980; Khajali and Slominski, 2012). Generally rapeseed is a better source of Ca, Se and Zn than soyabean, but its high phytate and fibre content reduce the availability of these minerals in monogastric diets.

As well as phytate, rapeseed meal also contains high levels of other anti-nutritional factors, including glucosinolates and tannins. Glucosinolates are sulphur-containing secondary plant metabolites. They are highly goitrogenic and reduce broiler health and performance by reducing feed intake, inducing iodine deficiency and causing hypertrophy of the liver, kidney and thyroid through release of toxic breakdown products. Various processing techniques are applied to remove glucosinolates from feed ingredients, namely enzyme application and microbial fermentation at each level of processing (Tripathi and Mishra, 2007). Tannins are astringent compounds, mostly complex glycosides that bind and precipitate dietary proteins and digestive enzymes, which reduce bird protein utilisation and growth (Leeson and Summers, 2001). Heat treatment has no effect on these anti-nutritional factors, their presence can only be reduced through plant breeding; rapeseeds that have been developed to be low in glucosinolates are often referred to as ‘0-canola’, or ’00-canola’ if erucic acid has also been removed. One of the first varieties of double zero rapeseed meal was developed in Canada: Canola ‘CANadian Oilseed, Low-Acid’. The term has now been adopted as a worldwide standard covering all

double zero varieties of rapeseed meal, wherever they were produced. Virtually all rapeseed production in the European Union has shifted to rapeseed-00 but unfortunately the development of low glucosinolate varieties suited to Asian countries has been less successful. It is imperative to know the source of the rapeseed meal to ensure rate of dietary inclusion does not impede animal performance and health.

1.24.3. Other Protein Sources

Fishmeal has high digestible amino acid content, particularly lysine, as well as a high content of very long chain polyunsaturated omega-3 fatty acids which are thought to improve immune status, and high levels of calcium, phosphorus and vitamins such as biotin and choline. Fish meal is produced from either lean fish such as cod and haddock, from the remaining offal after they are filleted, or from very oily fish such as herrings and sardines; approximately 90% is manufactured from the oily fish. Processing involves cooking, pressing, drying and grinding the fish. High-quality fishmeal usually contains 60-72% crude protein (Miles and Chapman, 2006). Its use as a protein source is continually being reduced because it is unsustainable environmentally (Hardy, 2010).

Feeding meat and bone meal from cattle in animal diets was banned by the EU in 2001 due to spongiform encephalopathy (BSE) (TSER 999/2001). Prior to this it was used as a cost-effective way to enhance protein quality and mineral levels and reduce waste. The EU largely replaced the loss of meat and bone meal by using soya meal, although the ban of meat and bone meal has removed a major source of phosphorus supply.

There are numerous other potential protein sources for poultry diets.

- Maize gluten meal is rich in total protein (62-73%), has high digestible energy and methionine content (almost as high as that of soyabean meal) but is deficient in some other amino acids, particularly lysine (Chadd *et al.,* 2002). It is not very palatable to birds and contains pigments which can effect meat and egg colour (Lesson *et al.,* 2005).

- Legumes, lupins, peas and beans are also high in protein, but deficient in some amino acids and high in anti-nutritional factors. For example, peas contain 20-30% protein, but the presence of alpha-galactosides causes poor growth, so pectinase and alpha-galactosidase must be supplemented into pea-based diets. Peas also contain low levels of methionine and high levels of tannins (Igbasan *et al.,* 1997). Lupins contain approximately 44% protein but can only be successfully fed if the diets are supplemented with synthetic amino acids (Olkowski *et al.,* 2001).
- Flax seed has a high protein (26%) and oil level (41%) and is a good source of fatty acids and omega-3, but studies feeding it to poultry have shown that although it increases omega-3 content of the meat, it results in smaller body weights (Jia and Slominiski, 2010).
- The meal remaining from production of sunflower seed oil has a relatively high protein content (17-21%) so has potential to be used in poultry diets, but it has a low energy and lysine content (Slavica *et al.,* 2006).
- Algae from biofuel production has the potential to replace a third of soya protein in pig and poultry diets. It has a protein level of 20-70% (compared to 40% in soyabean meal) and it's simple single-cell structure means it can be easily degraded, unlike plant-based biofuels like corn-derived ethanol which have a complex cellulose which must be broken down, and have a high lipid content (Lei, 2012). However, there is no consistency among various algae species regarding suggested safe inclusion levels in poultry diets.
- Feather meal is produced by hydrolysing clean, undecomposed feathers using steam and pressure which breaks down the keratinous bonds in the feathers. The protein content of feather meal is usually approximately 85%, provided the feathers have been completely hydrolysed, but it is low in methionine and not very palatable (Caires *et al.,* 2010).
- Blood meal contains about 80% protein, and is a rich source of lysine, arginine, methionine and cysteine, but a poor supplier of isoleucine and glycine (Khawaja *et al.,* 2007). It is produced by spray drying blood from animal processing plants at low temperatures.

- Co-products from distillation of cereal grains for alcohol production have been shown to have no adverse effect on broiler production if added at low levels (approximately 7%). Distillers dried grains with soluble (DDGS) are a good source of protein, energy and water-soluble vitamins, but the high fibre content makes them not well suited for high inclusion levels in poultry diets (Loar *et al.,* 2010).

1.24.4. Wheat

Wheat fed to animals tends to be the wheat that has been rejected for human consumption, due to its low nutritional value, such as low weight (due to weather or disease) and presence of mycotoxins. As weight of the wheat decreases the energy level decreases and feed efficiency is reduced, requiring addition of supplementary fat or higher quality wheat to prevent reduction in performance (McCracken *et al.,* 2002). Wheat is low in fibre and high in metabolisable energy; it contains an average of 19 MJ/kg gross energy, with metabolisable energy values of approximately 14 MJ/kg.

Wheat is classified based on whether the seed coat colour is red or white, whether the kernel texture is hard or soft, and on whether it was planted in the winter or spring (Slaughter *et al.,* 1992). Kernel texture has the biggest effect on nutrient composition; hard wheats have very strong binding between the starch and protein. Hard wheats have higher lysine content than soft wheats, and red wheats contain more amino acids than white wheats. The protein level of wheat varies between 6-20% (most commonly 13-15%), depending on growth conditions and type. Wheat contains less metabolisable energy, but more protein, methionine and lysine, and available phosphorus than maize (Christopher *et al.,* 2007). However the amino acid balance in wheat is poorer than maize, which means diets formulated with wheat must be balanced on an amino acid basis rather than crude protein basis (Hoehler, 2006). The use of wheat enhances pellet durability as the proteins help to bind the ingredients during pellet processing (Fairfield, 2003).

Wheat contains around 5-8% arabinoxylans which are water soluble, highly viscous non-starch polysaccharides (NSPs). NSPs are poorly digested by poultry (approximately 12%) (Wu *et al.,* 2013)

and cause reduced energy utilisation and interference with digestibility of other feed components. The detrimental effect of NSPs is mainly associated with the viscous nature of these polysaccharides and their effect on digesta; increased viscosity results in reduced feed passage rate and hence reduced nutrient accessibility. The result of this is sticky droppings and subsequent poor quality litter, leading to burns on the hocks and breasts of the birds, as well as reduced nutrient absorption. These effects can be overcome through supplementation of non-starch polysaccharide degrading enzymes (Cheeson, 2001).

1.24.5. Maize

In the United States maize is the major energy source in poultry diets. Maize has high metabolisable energy because it has low fibre content (approximately 10%), highly digestible starch (approximately 65%) and relatively high oil (approximately 4%) (Sauvant *et al.,* 2004; Muztar and Slinger, 1981). The crude protein level of maize is however fairly low (8-11%) and has a poor balance of amino acids, being deficient in lysine and tryptophan, so diets including maize must also contain a high quality protein or synthetic amino acids (Qi *et al.,* 2002).

Most maize crops are hybrids, developed to reflect specific climates or agronomic conditions. Some have been developed to improve nutritional value; for example low phytate (Hambridge *et al.,* 2004), high lysine and high tryptophan (Huang *et al.,* 2006), or genetic modification for improved performance, such as higher yields and resistance to pests and herbicides. There is concern that genetically modifying maize could have a negative impact on insects and on non-GM plants via transfer of genomes (Dale *et al.,* 2002). Moulding and rancidity can easily occur in ground maize, so its quality must be vigorously assessed prior to feeding. Aflatoxins, a type of mycotoxin, are the biggest threat to maize quality; they reduce feed conversion and cause symptoms such as liver disorders, reduced egg production, dermatitis, vomiting and bloody diarrhoea (Pearson *et al.,* 2004).

1.24.6. Barley

Barley is a low energy grain as it has low starch and high fibre content, and high presence of non-starch polysaccharides, particularly beta-glucans. The level of beta-glucans is dictated by growing, harvesting and storage conditions (Caprita *et al.,* 2011). Barley grains characteristically contain 66% starch and 17% fibre (Aman, 2006) but barley has a higher protein and amino acid content (particularly lysine and tryptophan) than maize, although it is less easily digested. The energy content of barley is very variable (Jacob and Pescatore, 2012). The use of NSP (or carbohydrase) enzymes in barley-based diets reduces intestinal viscosity and hence improves litter quality of birds fed barley-based diets and nutrient absorption, and also reduces the variation in beta-glucans between batches of barley, which improves its feeding value and bird growth performance. The effects of these enzymes can be deleteriously affected by the presence of hull fibres.

1.24.7. Wheat Bran

Wheat bran is a by-product of dry-milling wheat into flour. It is comprised of the outer seed coat layer and a small amount of the endosperm and germ. Wheat bran embodies approximately 50% of wheat waste product and 10-20% of the kernel (Hassan *et al.,* 2008). Wheat bran contains more protein (approximately 14-20%) and minerals (approximately 6%) than whole grains as these are found predominantly in the outer layer of the grain. The oil content of wheat bran, in the form of wheat germ oil, is approximately 4%, which is higher than the whole grain (Sabry, 1993). The energy values of whole wheat are however always greater than for wheat bran due to the latter's high fibre content. This high fibre means the metabolisable energy is very low, so inclusion rates in diets are low, unless low energy pellets are being sought after, for example molt diets for layers (Murase *et al.,* 2006). Wheat bran fed at over 13% causes reduced feed intake (Mateos *et al*., 2012).

As a product there is no accepted definition of what can be sold under the name wheat bran which means there is a wide variation between wheat bran products (Laudadio *et al.,* 2009), particularly with fibre and starch content. In the milling process the grains are cleaned, then soaked to strengthen the

outer layer and soften the starchy endosperm so they can be split apart from each other. The grains are then ground; wheat bran used for feeding animals is usually a mixture of coarse bran and fine products from the final stage of grinding, fed in the form of large non-adherent flakes with a coating of flour. It is often added into mash diets due to its high palatability and can be used to lighten heavy feed mixtures (Chee *et al.,* 2005).

Wheat bran contains non-starch polysaccharides, which have an anti-nutrient effect causing poor performance and nutrient utilisation (Choct and Annison, 1990). It does however also have reasonably high endogenous phytase activity (Cavalcanti *et al*., 2004), and is a natural source of betaine which is thought to improve osmoregulation in the intestinal tract, and hence reduce the likelihood of diarrhoea and coccidiosis. Betaine is also thought to improve nutrient and fat digestibility by providing methyl groups for synthesis of metabolic substances, and decreasing the requirement for methionine and choline as methyl donors, which potentially enhances their availability (Eklund *et al.,* 2005).

1.24.8. Rice Bran

Rice bran is a good source of protein (approximately 12-18%) and fat (approximately 14-23%), and contains approximately 16% carbohydrate and 11% fibre (Muhahid *et al.,* 2003). It has been demonstrated that rice bran can be used as a replacement for cereals at levels of up to 30% with no effect on feed conversion (Tiemoko, 1992). However Vieria *et al.* (2007) suggests levels should not exceed 15% in broiler diets due to the low levels of calcium and very high levels of phytate in rice bran. Rice bran also contains other anti-nutritional factors such as trypsin and pepsin inhibitors, although these compounds are at relatively low levels and are destroyed by heat processing (Lu *et al.,* 1991). Rice bran is produced by the use of reels that remove the germ and outer layer of the rice grain, after hulling. The amount of bran meal produced is dependent on the rate of milling and type of rice, but is usually approximately 10% bran and 20% hulls (Nobakht, 2007). Rice bran contains approximately 15-20% oil (Adrizal *et al.,* 1996) which can become rancid during storage because lipolytic enzymes are able to hydrolyse glycerides in the oil resulting in formation of free fatty acids (Mujahid *et al.,* 2003)

and poor broiler performance (Taha *et al.,* 2012). Rice hulls are often added to rice bran to increase fibre content to approximately 10% (Samli *et al.,* 2006).

In order to feed rice bran to poultry enzymes such as phytase, xylanase and lipase need to be supplemented to combat the negative effects of the high phytate, fibre content and oxidative rancidity. Combinations of rice bran with other feed ingredients may increase its potential as a feed for broilers, for example addition of rumen liquor has been shown by Pujaningsih (2004) to increase phosphorus solubilisation from phytate, and hence reduce the requirement for phosphorus supplementation.

1.24.9. Other Cereal Sources

Buckwheat is the only grain that is not deficient in lysine and it has a protein content of approximately 12%, but it is considered to be of little value to the monogastric feed industry because it has a high fibre content and low nutrient digestibility. It is also high in fagopyrin which is a compound that causes increased photosensitivity, and can therefore cause increased risk of sunburn in outdoor reared birds (Gupta *et al.,* 2002). Oats have a feeding value similar to that of buckwheat; they have a high fibre content (as are made of approximately 20% hull), so provide little energy to the diet, and contain deleterious carbohydrates. Gonzalez Alvarado *et al.* (2010) however observed increased performance when 3% oat hulls were fed, although it is thought that removing the hulls from oats increases the feeding value to that similar to maize, to approximately 17% energy (Ernst *et al.,* 1994). Rye is not often fed to poultry because it is high in pentosans and other NSPs, so poultry fed rye tend to produce litter very high in moisture, and are unable to effectively utilise dietary fat and fat-soluble vitamins. This can lead to both reduced growth and increased onset of rickets. Carbohydrase enzymes are however able to counteract most of these negative effects (Lazaro, *et al.,* 2004). Sorghum has a similar composition to corn but contains tannins and has low levels of lysine, methionine and glycine, and has hence been shown to have a negative effect on bird performance and intestinal mucosa (Torres *et al.,* 2013).

1.25. *In vitro* assays of diet ingredients

In vitro assays have the potential to act as sensitive and cost effective tools for the evaluation of both phytase efficacy and mineral availability in dietary ingredients. They enable retainable and digestible phosphorus in feedstuffs and feed phosphates to be predicted, and allows the mode of action and conditions that affect efficacy of phytase to be investigated. There are however limitations to *in vitro* methodologies; it is impossible to reconstruct exactly the variability and interactions present *in situ*, so *in vitro* assays are able to measure degradability only, not digestibility.

In vitro methods vary from simple assays that test mineral solubility, to assays that mimic digestive tract conditions. In the latter, the feed stuff is usually incubated with pepsin and pancreatin to mimic the retention time and pH in the different sections of the gastrointestinal tract (Walk *et al.* 2012 and Wu *et al.* 2004b). Two-step *in vitro* methods, whereby the gizzard and small intestine are simulated, as described in Morgan *et al.* (2014), enable enzymatic dephosphorylation of phytate to be predicted based on soluble P content, and can measure the impact that phytase presence is likely to have *in situ* on the Ca:P ratio and free Ca, free phosphate and phytate levels. Wu *et al*. (2004b) developed a three-step in vitro assay which mimicked the crop, proventriculus/gizzard and duodenum.

Efficacy of phytase is predominantly analysed by release of phosphorus, as it is quicker and easier to analyse this than to measure disappearance of phytate, although is comparably less accurate. Reduced rates of hydrolysis observed *in vivo* compared to *in vitro* may be attributable to lower hydrolysis rates of partially phosphorylated inositol phosphate, and the inhibiting nature of phosphates. Accessibility of phytate may also cause this effect; namely as the active sites of all phytase molecules are unlikely to be interacting with a substrate at all times, thus it is available phytate not total phytate that is required to accurately detect phytase activity. The main issue is that pH and phytate concentrations are not constant *in vivo*; even though phytases often present optimum activity in a specific range (Applegate *et al.,* 2003), their individual pH activity profiles can differ significantly. The degrading activity of phytate is measured at standard conditions of pH 5.5, suggesting that at any other pH their

activity will differ, so if the standard pH used for all measurements were to change the ranking of phytases based on their efficacy would change.

1.26. Conclusion

Total and phytate-bound phosphorus concentrations have been measured extensively, and have been shown to vary considerably between different feed sources and between different batches of feed ingredients (Selle *et al.,* 2003; Wu *et al.,* 2009; Tahir *et al.,* 2012). It is widely accepted that these variations have substantial effects on the total phosphorus concentration of poultry diets, depending on relative inclusion concentrations. However, only very recently has it been recognised that the concept of 'total phytate concentration' is not truly indicative of substrate availability for phytase, suggesting this notion may be misleading to those using phytase. It is likely therefore that anti-nutritional effects of phytate and response of phytase, in poultry cannot be predicted by total phytate-phosphorus measurements, but rather the 'susceptibility' of the phytate present to the effects of phytase in the environment of the digestive tract. The key novel aspect of this project is that it explores phytate 'susceptibility' and the impact of the digestive tract environment, particularly pH, on phytate reactivity. This project focuses on variation in susceptible phytate content not only between feed ingredients fed to poultry, but also between batches of ingredients which has yet to be explored. Investigation into the mechanisms of endogenous phytase contribution to phytate degradation, particularly in pre-starter birds, is a further novel aspect featured in this project, as is validation and development of assays used to measure total phytate concentration in digesta samples, and susceptible phytate concentration in diets and feed ingredients fed to broilers. The implications of this project for the poultry industry are deeper understanding of phytate reactivity in the different sections of the poultry gastrointestinal tract, and the proposal that users of phytase need to consider not only total phytate content by more importantly the relative solubility and susceptibility to the effects of phytase.

1.27. Aims and Objectives

The overall aim of this project was to investigate the availability and reactivity of phytate in different raw materials and diets, and in the different environments of the broiler digestive tract. Specific objectives were to:

- Investigate factors that influence phytate reactivity and phytase efficacy
- Examine the relationship between pH and phytate reactivity
- Investigate phytate 'susceptibility' to the effects of phytase
- Examine protein phytate interactions in relation to phytate concentration and pH
- Validate assays for measuring total and susceptible phytate in both poultry feed and digesta.

CHAPTER 2: Materials and Methods

2.1. Introduction

This chapter provides an account of the general materials and methods employed throughout the studies featured in this thesis. Five bird trials were completed as detailed in table 2.1; 3 main studies and 2 pilot studies. Trial 1 observed the effects of supplemented phytase in soyabean and rapeseed meal based diets, trial 2 determined the contribution of endogenous ileal phytase activity to phytate degradation in young birds and trial 3 observed the effect of phytate susceptibility on broiler performance and mineral utilisation. Five pilot studies were run, three of which feature in the appendix as copies of published manuscripts. One of the pilot studies was run to determine the number of young birds required for digestibility studies, to assess the number of birds required for Study 2. The impact of dietary calcium on, and optimum method for determination of, gastrointestinal pH was investigated and this method was used for determining gastrointestinal pH in all the trials. The viability of using *in vitro* assays as indicators of dietary success *in vivo* was assessed; an *in vitro* assay that mimicked conditions within the gastrointestinal tract was used to analyse mineral solubility of all the diets used in the *in vivo* bird trials, and comparisons were made between the *in vitro* and *in vivo* findings. Titanium dioxide (TiO_2) was added to all the trial diets as a digestibility marker; comparisons were made between two different methods for determination of TiO_2 in each of the trial diets, and a series of other commercial diets, and digesta samples. Variation in susceptible phytate content of different feed ingredients was used to formulate the diets fed in Study 3.

Table 2.1: Description of individual trials in the study

		Chapter
Study 1	Trial 1: Effect of phytase supplementation in soyabean meal and rapeseed meal diets on poultry gastrointestinal pH and phytate-protein and phytate-phosphorus complex formation	4
Study 2	Trial 2: Evaluation of the contribution of endogenous phytase to the degradation of phytate in young broilers	5
Study 3	Trial 3: Effect of feeding broilers diets differing in susceptible phytate content	6
Pilot Study 1	Minimising the number of birds used for digestibility measures in the pre-starter period in broiler chicks	3
Pilot Study 2	Comparison between ICP-OES and UV-spectrometry assays for the determination of titanium dioxide added as an inert marker in chicken digestibility studies	3 Appendix A
Pilot Study 3	The effect of dietary calcium inclusion on broiler gastrointestinal pH: Quantification and method optimisation	3 Appendix B
Pilot Study 4	*In vitro* versus *in situ* evaluation on the effect of phytase supplementation on calcium and phosphorus solubility	3 Appendix C
Pilot Study 5	Susceptible phytic acid content of common feed ingredients fed to poultry in the UK	3

2.2. Birds and Husbandry

Institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986) were followed and all experimental procedures involving animals were approved by the School of Animal, Rural and Environmental Sciences Ethical Review Group. All bird trials used Ross 308, male broiler chicks, supplied within 24 hours of hatching by PD Hook, Cote Hatchery, Oxfordshire. Birds used in the trials were within the weight range of 38-45g and were from breeder flocks aged between 40-45 weeks. Birds were weighed using dynamic weighing which measured the average weight over a period of 3 seconds (Mettler Toledo International). The chicks were randomised by weight and housed in preheated 0.64m^2 pens in a purpose built, insulated poultry house. The birds were bedded on clean wood shavings (approximately 3cm) and fresh shavings were added into the pens as required. Birds were always allowed *ad libitum* access to the treatment diets and water for the duration of the trial. Commercial guidelines for the care and husbandry of Ross 308 broilers were followed in all studies (Aviagen, 2008).The room was thermostatically controlled to produce an initial temperature of 32°C

reduced to 21°C by day 21 using heating fans and supplementary heat lamps. The lighting regimen used was 24 hours light on d1, with darkness increasing by 1 hour a day until 6 hours of darkness was reached and this was maintained throughout the remainder of the study. Birds were checked twice daily to monitor the environmental conditions; heating and ventilation were adjusted accordingly. Any mortalities were recorded along with the date and weight of the bird and reason if culled. All birds sampled were euthanised by cervical dislocation as determined by DEFRA (DEFRA, 2007) and the Animal Scientific Procedures Act (ASPA, 1986).

2.3 Diet Formulation

All trial diets were manufactured on site and fed as mash. The particle size of each diet was uniform, consistent and typical for broiler diets, averaging at approximately 1mm. The composition and analysis of all the trial diets are detailed in the appropriate chapter. When making the diets, each ingredient was individually weighed out and mixed dry for five minutes in a ribbon mixer (Rigal Bennett, Goole, UK) before addition of oil. The diets were then mixed for a further five minutes. The mixer was brushed down at various stages throughout the mixing process to ensure oil clumps were removed. Titanium dioxide was carefully incorporated into every diet as an inert marker. It was added at 5g/kg to ensure there was sufficient titanium dioxide in the digesta samples to determine diet digestibility, and was mixed with the dry mix prior to inclusion to ensure homogeneity. For each diet the titanium dioxide level was analysed to ensure the feed was homogenous. In all studies, diets were randomly allocated to pens within the room, to eliminate any effect of room position.

2.4. Trial Period

2.4.1. Feed Intake

Each pen of chicks was fed exclusively from an individual experimental bag of diet that was pre-weighed prior to the trial. Any additional feed was weighed into the bags, and weight was recorded. Troughs were positioned horizontally to minimise spillage. On sampling days remaining feed in the

trough and bag, and any spilt feed if able to be collected, were weighed. In Trial 1 and 3 feed intake was measured on day 7,14, 21 and 28. In trial 2 feed intake was measured on d4, 6, 8, 10, 12 and 14.

2.4.2. Bird Weights

For all bird trials, chicks were weighed on arrival, and any outside the range of 38-45g were not included in the trial. Birds were distributed into pens based on average weight per pen, ensuring there were no significant differences in starting pen weight between dietary treatments. In Trial 1 and 3 birds were weighed on day 7, 14, 21 and 28. On trial 2 birds were weighed on d4, 6, 8, 10, 12 and 14. In Trial 2 and 3 individual bird weight of 2 birds per pen was measured; the birds were marked with a coloured pen and weight was recorded under the column with the corresponding colour on the data sheet. Birds were weighed using a top pan balance (Mettler Toledo, Leicester, UK).

2.4.3. Excreta Sample Collection

Excreta samples were collected in Trial 1 by placing foil-lined, wire-topped trays into the pen (under the feeding trough to ensure frequent use) for 2 to 3 days. After this period, the trays were taken out of the pens and any feed and sawdust was removed. The foils were then wrapped, leaving a small gap for air circulation, and dried at 80°C for 5 days in a forced air oven, to ensure all moisture was removed. The samples were then ground through a 1mm screen and stored in a cool place.

2.4.4. Digesta Sample Collection

On days requiring digesta sample collection and digesta pH determination, birds were sequentially fed at timed intervals, ensuring each bird had a minimum of 1 hour feeding prior to being euthanised, to ensure sufficient gut fill. Birds were euthanised in a separate room via cervical dislocation by trained persons. The gizzard was removed and sliced open, and contents were gently scraped into a pot. The area of the tract from the duodenal loop to the Meckel's Diverticulum, referred to as the jejunum (proximal small intestine), was removed and digesta was collected by gentle digital pressure along the piece of tract, whilst trying not disrupt the mucosal lining. The ileum (distal small intestine) was categorised as the piece of tract that ran from the Meckel's Diverticulum to the ileal-caecal junction.

Digesta samples were collected into labelled pots; for Trial 1 and Trial 2 digesta samples were pooled into one pot per pen/plot per section of the tract, for Trial 3 digesta was collected into separate pots per bird. The digesta samples were then weighed and immediately freeze dried (LTE Scientific, UK) for 5 days. Once the samples were dried the pot was reweighed so that digesta water content could be determined. The samples were then ground to homogenise them.

2.4.5. Tibia and Femur Collection

Tibia bones were separated at the tibiotarsal junction, where the feet were removed, and the tibiofemoral junction. Femur bones were separated at the tibiofemoral junction and the hip. Care was taken to ensure there was consistency with bone removal. For Trial 1 both the left and right tibia and femur from each bird in the trial was collected and put in labelled bags per pen per bone; the left leg was used to determine bone ash and Ca and P content, and the right leg was used to determine bone strength, length, width and weight. For Trial 3 just the right tibia and femur from 2 birds per pen was collected, put in labelled bags per bird per bone, and used to determine bone strength, length, width, weight, ash and Ca and P content.

2.5. Analytical Procedures

2.5.1. Gastrointestinal pH

Prior to measurement of gastrointestinal pH, birds were sequentially fed to ensure there was both sufficient gut fill for the analysis and no alterations in gastrointestinal pH resulting from periods of empty tract. Immediately post euthanasia the gizzard was removed intact and a digital pH meter (Mettler-Toledo, UK) with a spear tip piercing pH electrode (Sensorex, California, USA) was directly inserted into the digesta in the lumen of the proximal gizzard (proventricular opening), whilst ensuring the pH electrode did not touch the gizzard wall, and was measured and recorded in triplicate (Morgan *et al.,* 2014, Sacranie *et al.*, 2011 and Angel *et al.*, 2010). Once all three readings had been taken the probe was then rinsed with ultra-pure water (ICW 3000 water purifier for ion chromatograph, Millipore). This process was repeated in the duodenum, jejunum and ileum of the same bird; for the

duodenum the probe was inserted into the opening made by removing the duodenum from the jejunum; for the jejunum it was inserted in the opening made by removing the jejunum from the ileum at the Meckel's diverticulum and for the ileum it was inserted into the opening made from removing the jejunum from the ileo-caecal-colonic junction. The mean of the three readings per section of tract was then calculated and recorded.

2.5.2. Dry Matter Determination

Dry matter content of the diet and excreta was analysed by accurately weighing approximately 5-10g of finely ground sample into pre-weighed crucibles. The crucibles were then dried in a drying oven set at 105°C for approximately 4 days, until the weight was constant. The dried samples were then cooled in a desiccator and reweighed. Digesta dry matter content was analysed by weighing the digesta samples immediately after collection, freezing them, then freeze-drying them to a constant weight in a Lyotrap freeze drier (LTE Scientific, Oldham, UK). The samples were reweighed once dried, after approximately 5 days in the freeze drier.

2.5.3. Ash Determination

Ash content of diet, digesta, excreta and bones was analysed by accurately weighing approximately 2-5g of sample, or a whole bone, into a pre-weighed ceramic crucible. The crucibles were then placed in a muffle furnace (Nabertherm, B180) for approximately 14 hours at 650°C. The ashed samples were then cooled in a desiccator and reweighed.

2.5.4. Titanium Dioxide Determination

Titanium dioxide (TiO_2) was added into all diets as an inert marker at an inclusion rate of 5g/kg. In Trial 1 and 2 it was measured in the diets, excreta and digesta by the UV-spectrometry method developed by Short *et al.* (1996). TiO_2 standards were prepared prior to analysis by dissolving 250mg of TiO_2 in 100ml of H_2SO_4 (Fisher Scientific, UK) and bringing the solution up to a volume of 500ml with distilled water. 100ml volumetric flasks were labelled 1-10 and the corresponding 1-10ml of TiO_2 solution was added to each flask. Concentrated H_2SO_4 was then added to each flask to reach a combined volume of

10ml, followed by 10ml of 30% hydrogen peroxide (Fisher Scientific, UK). The flasks were then brought to volume with distilled water and the solutions were stored in glass vials in darkness.

0.3-0.5g of feed, excreta or freeze dried digesta was weighed into ceramic crucibles in duplicate and ashed in a muffle furnace (Nabertherm, B180) set at 650°C for approximately 14 hours. Once cooled, 10ml of 7.4M H_2SO_4 was pipetted into each crucible and they were heated on a hotplate until the sample had completely dissolved (approximately 2 hours); 5ml extra acid was added if required. Once cooled, the sample was transferred into a 125ml beaker and then filtered through Whatman 541 hardened, ashless filter papers into 100ml volumetric flasks. 10ml of 30% hydrogen peroxide was added to each volumetric flask, and the flasks were brought to volume with distilled water and mixed. Absorbance of the samples and standards was measured on a UV spectrophotometer (Unicam Helios, USA) set to 410nm. The coefficient used to determine TiO_2 concentration was derived from the regression analysis of the standard curve. The amount of TiO_2/mg in the solutions was calculated by:

$$\frac{\text{Absorbance}*100}{\text{Coefficient}*\text{sample weight (mg)}}$$

2.5.5. Apparent Metabolisable Energy

Gross energy (GE) of the feed and excreta was measured using a bomb calorimeter (Instrument 1261, Parr Instruments, Illinois, USA) (Rutherfurd *et al*., 2007 and Woyengo *et al*., 2010). Pellets of feed and excreta sample, weighing approximately 1g, were made by adding a small amount of water to the sample before pelleting it with a pellet press (Parr Instruments, USA). The pellets were dried overnight in a drying oven at 105°C, before being weighed into tin crucibles (Sartorius CP1245) and placed in the bomb. The bucket in the bomb jacket was filled with 2 litres of water. 10cm of fuse wire was threaded through the holes in the bomb which the electrodes attach, ensuring the wire touched the pellet. The bomb was then assembled, ensuring the top was tightly screwed on, and then filled with oxygen. Once filled, the bomb was put into the bucket of water, the electrodes were pushed into the bomb, and the lid of the bomb jacket was shut. Sample weight was entered and the process was started; the

calorimeter measures the energy produced (in MJ/kg) when the pellet is exploded. Apparent metabolisable energy (AME) was calculated by:

GE diet-(GE excreta*(TiO_2 in the diet/TiO_2 in excreta))

2.5.6. Tibia and Femur Ash Determination

Tibia and femur bones were wrapped individually in labelled foil and autoclaved on a metal tray for 15 minutes at 121°C. The flesh and connective tissue was then removed by hand and the bones were then put into labelled foil tins and oven dried at 110°C for approximately 4 days. The dried bones were then weighed into pre-weighed ceramic crucibles and ashed for approximately 14 hours at 650°C (Hall *et al*., 2003). The crucibles were then left to cool in a desiccator and reweighed so ash content could be derived. Bone ash was calculated as ash weight as a percentage of dry bone weight.

2.5.7. Tibia and Femur Bone Strength

Bone strength of the tibia and femur was analysed using a TA.XT plus texture analyser (Stable Microsystems, Guildford, UK) set up with a 50kg load cell and 3 point-bend fixture (Park *et al*., 2003, Taylor *et al*., 2003 and Shaw *et al*., 2010). Firstly, the bones were defleshed of muscle and tissue by hand using a scalpel. The length and width of each bone was measured using callipers and recorded. The texture analyser was set to measure force in compression. Test speed was set at 1mm/sec, and trigger force was set at 7g (0.069N). Supports of the fixture were set at 26mm for the tibia bones and 16mm for the femur bones to accommodate for length of the bones. The texture analyser was calibrated using a 5kg weight. The defleshed bone was placed on the fixtures, a test was run and the peak force in Newtons was recorded.

2.5.8. Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) determination of Calcium, Phosphorus and Titanium

Diet, digesta, excreta and tibia and femur ash were analysed for calcium (Ca) and phosphorus (P) by Inductively Coupled Plasma mass spectroscopy with Optical Emission Spectrometry (ICP-OES) (ICP-MS model PQ Excell, VG Elemental, USA). In Trial 2, Trial 3 and pilot study 2 ICP-OES was also used to analyse titanium (Ti) concentration in diet and digesta samples. Prior to the assay, all glassware was soaked in a 1% nitric acid bath for a minimum of 12 hours, rinsed with ultra-pure water and dried, to ensure there was no contamination with minerals from outside sources. Approximately 0.5g of sample was weighed in duplicate into 50ml conical flasks. The samples were then incubated for a minimum of 16 hours with 10ml of aqua regia (1 part nitric acid and 3 parts hydrochloric acid) before being boiled until dissolved (approximately 90 minutes) in a fume cupboard. If necessary, an extra 5ml of aqua regia was added and an additional 30 minutes of heating was carried out to ensure complete dissolution. One flask containing just aqua regia was prepared for each 5 sample and was measured as a blank. The samples were then cooled before the flask contents were diluted with ultra-pure water and filtered into 50ml volumetric flasks through Whatman 541 hardened, ashless filter papers. The volumetric flasks were then brought to volume with ultra-pure water, and the contents were mixed and transferred into 15ml tubes, duplicate tubes per sample. The samples were stored at 4°C. ICP-OES standards were prepared with differing levels of Ca, P and Ti (dependent on the predicted levels of the sample being analysed) using 1000ppm ICP-OES grade standards (Fisher Scientific, Loughborough, UK) diluted in ultra-pure water. The samples were analysed on the ICP-OES, set to analyse Ca at wavelength 317.933nm, P at wavelength 213.617nm and Ti at wavelength 334.940nm. The readings on the ICP-OES are presented as concentration in mg/L; to convert to g/kg:

(Ca, P or Ti in sample (mg/L))*(volume of sample (ml)/ weight of sample (g))/1000

The apparent ileal digestibility coefficient was calculated by:

[(Ca or P/TiO_2) diet - (Ca or P/TiO_2) ileum]/ (Ca or P/TiO_2) diet

The amount of dietary Ca or P digested was calculated by:

Digestibility= 1-(Ca or P digesta * 5)/ (TiO_2 digesta * Ca or P diet)

Amount of dietary Ca or P digested= digestibility *Ca or P in diet

2.5.8.1. Soluble Calcium and Phosphorus Determination

To analyse soluble Ca and P in diet and gizzard, jejunum and ileum digesta ultra-pure water was added to approximately 2-3g of sample until the final weight was 202g. The samples were then placed on a shaker (Edmund Bühler 7400 Tübingen SM25, Germany) set at 200epm for 60 minutes and centrifuged (Universal 32R Hettich Zentrifugen, Germany) at 5,200 x g for 10 minutes. The resulting supernatant was then filtered through 0.22µm syringe filters. 5 drops of concentrated HCl for each 20 ml of extract was added to acidify the samples (to prevent precipitation of calcium phosphates) (Leytem *et al*., 2008 and Self-Davis, 2000). The supernatant was then put into 15ml tubes and the samples were analysed for Ca and P content by ICP-OES. Ca and P concentration was calculated by:

(Ca or P in ICP sample (mg/L))*(volume of sample (ml)/ weight of sample (g))/1000

Mineral solubility was calculated as a percentage of the proportion of dietary mineral and converted into g/kg on a dry matter basis. Concentration on a dry matter basis in % was determined by:

((Ca or P in ICP sample (mg/L) x 0.02)/ Sample wet weight (g)) x (100 / % dry matter))

The solubility coefficient was calculated by:

Ca or P supernatant/ Ca or P diet

2.5.9. Crude Protein Determination

Samples of diet and digesta were analysed for nitrogen content using the Kjeldahl method (AOAC official method 2001.11) (Tahir *et al*., 2012, Pintar *et al.*, 2005 and Peter and Baker, 2001). Approximately 1g of sample was accurately weighed into distillation tubes (Foss Cat No. 10000155) in duplicate. Both a copper and selenium catalyst tablet (Fisher Scientific, UK) was added to each tube. 12.5ml of concentrated nitrogen-free sulphuric acid was then added to each tube, and they were

heated in a digestion unit (1007 Digester, Foss Tecator, UK) set at 450°C. for 45 minutes. Once digestion was complete, the distillation tubes were left to cool for a minimum of 20 minutes and 75ml of distilled water was added to each tube. The tubes were then distilled in a distillation unit (2100 Kjeltec, Foss Tecator, Cheshire, UK) which added 50ml of 10M sodium hydroxide to the samples, distilled them for 3 minutes, then expelled the resulting ammonia into conical flasks containing 2ml 4% boric acid with indicator, causing a colour change from orange to blue. The boric acid was then titrated back to original colour using 0.1M HCl in a burette and the volume of acid used was recorded. Starch was used as a blank. % nitrogen was calculated by:

1.4 x (V1-V2) x M / W

where:

W= Original weight of sample

V1= Volume of acid to titrate sample

V2= Volume of acid to titrate blank

M=Molarity of acid

6.25 x % Nitrogen = % crude protein

2.5.9.1. Soluble Protein Determination

To analyse feed and gizzard digesta soluble protein content 75ml of 0.2% potassium hydroxide was added to 1.5g of sample. The sample was then stirred for 20 minutes using a magnetic stirrer (Stuart BIBBY, SB 161.3) and centrifuged at 1,250 x g for 10 minutes. 15ml of the supernatant was removed and analysed for protein content using the Kjeldahl method described above (Parsons *et al*., 1991). Protein solubility was calculated as a percentage of the total crude protein in the sample.

2.5.10. Total Phytate Determination

All trial diets, feed ingredients in pilot study 5, gizzard digesta in Trial 1, and gizzard and jejunum digesta in Trial 2 and 3 were analysed for total phytic acid, using the Megazyme^{TM} K-PHYT assay (Megazyme International Ireland Ltd., UK). 20ml of 0.66M hydrochloric acid was added to 1g of feed

or freeze-dried digesta and was stirred on a magnetic stirrer for a minimum of 3 hours. 1ml of solution was then transferred into a 1.5ml microfuge and centrifuged at 9,500 x g for 10 minutes. 0.5ml of the resulting supernatant was then transferred into a fresh 1.5ml microfuge, and 0.5ml of 0.75 M sodium hydroxide was added to neutralise the sample.

To test for free phosphorus, 0.62ml of ultra-pure water, 0.2ml of an acidic buffer solution (pH 5.5 and sodium azide (0.02 % w/v) and 0.05ml of the neutralised sample was pipetted into a fresh 1.5ml microfuge. To test for total phosphorus 0.60ml of ultra-pure water, 0.2ml of the acidic buffer, 0.05ml of neutralised sample and 0.02ml of phytase was pipetted into a fresh 1.5ml microfuge. All the microfuge tubes were then mixed and incubated at 41°C for 10 minutes. 0.02ml of ultra-pure water and 0.2ml of an alkaline buffer (pH 10.4, $MgCl_2$, $ZnSO_4$ and sodium azide (0.02 % w/v) was then added to the free phosphorus samples, and 0.02ml of alkaline phosphatase and 0.2ml of the alkaline buffer was added to the total phosphorus samples. All the microfuge tubes were then mixed and incubated at 41°C for 15 minutes. 0.3ml of trichloroacetic acid (50% w/v) was then added to each tube to stop the reaction, and the microfuge tubes were centrifuged at 9,500 x g for 10 minutes. 1ml of the supernatant was then carefully pipetted into a fresh 1.5ml microfuge tube. 0.5ml of colour reagent (ammonium molybdate (5 % w/v) to ascorbic acid (10 % w/v) / sulphuric acid (1 M) in the ratio 1 part ammonium molybdate to 5 parts ascorbic acid) was added to each of the samples.

Standards were prepared by adding phosphorus standard (24 ml, 50 µg/mL) and sodium azide (0.02 % w/v) to 15ml tubes as followed: Standard 1 0ml, Standard 2 0.05ml, Standard 3 0.25ml, Standard 4 0.5ml and Standard 5 0.75ml, made to total volume of 5ml with ultra-pure water. 1ml of each of these standards and 0.5ml of colour reagent was pipetted into 1.5ml microfuge tubes in duplicate.

All the microfuges prepared for colorimetric analysis, including the standards, were incubated at 41°C for 1 hour. They were then mixed, transferred into cuvettes, and read on a UV-VIS spectrophotometer (Unicam Helios, USA) set at 655nm.

The absorbance of standard 0 was subtracted from the other standards to obtain phosphorus concentration. Mean value of phosphorus standards (µg/phosphorus) was calculated by:

$$\frac{\text{Mean STD1 + Mean STD2 + Mean STD3 + Mean STD4}}{4}$$

The absorbance of the free phosphorus samples was subtracted from the absorbance of the total phosphorus samples, thereby obtaining phosphorus concentration. The concentration of phosphorus was calculated as:

$$\text{Phosphorus} \times \frac{\text{mean value of phosphorus standards (µg/phosphorus)} \times 20 \times \text{dilution factor}}{10{,}000 \text{ (conversion µg/g to g/100g)} \times \text{sample weight (g)} \times \text{sample volume (ml)}}$$

To calculate phosphorus concentration (g/100g):

$$\text{Phosphorus} \times \frac{\text{mean value of phosphorus standards} \times 20 \times 55.6}{10{,}000 \times 1 \times 1}$$

=Mean value of phosphorus standard x 0.1112 x phosphorus

The calculation of phytic acid content assumes that the amount of phosphorus measured is exclusively released from phytic acid and that this comprises 28.2 % of phytic acid (Kumari *et al*., 2014). These calculations were simplified by using the Megazyme Mega-Calc™ downloaded from the Megazyme website.

To calculate phytic acid content:

$$\frac{\text{Phosphorus (g/100g)}}{0.282}$$

The amount of phytic acid hydrolysed in the bird was calculated by:

Digestibility= 1-(digesta phytic acid*5)/ (TiO_2 digesta* diet phytic acid)

Amount of dietary phytic acid hydrolysed =digestibility* phytic acid in diet

2.5.11. Susceptible Phytate Determination

The susceptible phytate content of the trial diets and feed ingredients was analysed by a modified version of the Megazyme K-Phyt™ assay described above. Acetate buffer was produced by adding 2.5M acetic acid to 2.5M sodium acetate until pH 4.5 was reached. This buffer was warmed to 37°C in a water bath and then 50ml of the warmed buffer was added to 10g of diet or feed sample. The samples were then incubated at 37°C for 5 minutes. 2ml of sample was then immediately transferred into a microfuge tubes and centrifuged at 9,500 x g for 10 minutes. 0.5ml of the resulting supernatant was then transferred into a fresh 1.5ml tubes and neutralised with 0.5ml 0.25M NaOH. The pH was then read using a spear tip piercing pH electrode (Sensorex, California, USA), and a 1:3 dilution with ultra-pure water was carried out on the neutralised sample. Phytic acid content was then measured using the total phytate Megazyme K-Phyt™ assay described above. Susceptible phytate content was calculated by:

$$\frac{\text{Mean phytic acid content of the sample from the susceptible phytate assay (g/100g)}}{\text{Mean total phytic acid content of sample from total phytic acid assay (g/100g)}}$$

2.5.12. Total Phytase Activity

Phosphate stock solution (50 mmol/l potassium dihydrogenphosphate dissolved in 0.25 mol/l acetate buffer (sodium acetate trihydrate dissolved in water and adjusted to pH 5.5 with 25 % mass fraction hydrochloric acid) with 0.01 % mass fraction polysorbate 20) (Fisher Scientific, Loughborough, UK) was diluted to concentrations of 25, 12.50, 6.25 and 3.125 µmol/ml with 0.25 mol/l acetate buffer containing 0.01 % mass fraction polysorbate 20. For the phosphate standards, 360µl 0.25 mol/l acetate buffer with 0.01 % mass fraction polysorbate 20 and 40µl phosphate standard solution was transferred into 2ml microfuge tubes. For the blanks, 400µl 0.25 mol/l acetate buffer with 0.01 % mass fraction polysorbate 20 was transferred into 2ml microfuges. STOP reagent was prepared by mixing together 1 volume ammonium vanadate reagent (ammonium monovanadate dissolved in dilute hydrochloric acid and water), 1 volume ammonium heptamolybdate reagent (ammonium heptamolybdate tetrahydrate dissolved in 25% mass fraction ammonia solution and water) and 2

volumes dilute nitric acid (Fisher Scientific, Loughborough, UK). 0.8ml phytate substrate solution (dodecasodium phytate with inorganic phosphorus content of 0.1 % mass fraction dissolved in acetate buffer and 25% mass fraction hydrochloric acid) (Fisher Scientific, Loughborough, UK) and 0.8ml STOP reagent was added to all the microfuges. The contents were then mixed, maintained at room temperature for 10 minutes, and centrifuged for 3 minutes at 13000g. Each standard and blank was made in triplicate, and optical density was measured using a UV-VIS spectrophotometer (Unicam Helios, USA) set to 415nm. The standard curve was formulated by plotting the optical density against the phosphate concentration (µmol/ml).

50ml of ultra-pure water and 0.5ml of 10% mass fraction polysorbate 20 was added to approximately 5g of diet or ileal digesta and mixed on a magnetic stirrer for 45 minutes. 2ml of the extract was then transferred into a microfuge tube and centrifuged for 3 minutes at 13000g. A phytase level control was included for each batch of samples; phytase stock standard solution with a known activity was diluted to a final activity of 0.15 U/ml to 0.25 U/ml, and the exact activity was determined.

For the phytase level controls, 360µl 0.25 mol/l acetate buffer with 0.01 % mass fraction polysorbate 20 and 40µl phytase level control was transferred into a 2ml microfuge. For the feed sample, 300µl 0.25 mol/l acetate buffer with 0.01 % mass fraction polysorbate 20 and 100µl of feed sample was transferred into a 2ml microfuge. The samples were mixed and pre-incubated for 5 minutes at 37°C. For the phytase level controls and feed samples, 0.8ml of phytate substrate solution was added to the sample and it was incubated at 37°C for exactly 30 minutes. 0.8ml of STOP reagent was then added; it was maintained at room temperature for 10 minutes and then centrifuged for 3 minutes at 13000g. For the blanks, immediately after the pre-incubation step, 0.8ml of STOP reagent and 0.8ml of phytate substrate was added to the sample; it was maintained at room temperature for 10 minutes and then centrifuged for 3 minutes at 13000g. Optical density of the phytase level controls, feed samples and blanks were measured at wavelength 415nm. Each phytase level control, feed sample and blank was measured in triplicate and the results were averaged. An example standard curve is presented in Figure 2.2.

Phytase activity was calculated by:

*ΔD(415)*Vd/ k*m*t*

ΔD (415)= net optical density at 415nm (ΔD (415)t-ΔD (415)b) (where ΔD (415)t is the value for the test portion and ΔD (415)b is the value for the blank)

K= slope of the standard curve

Vd= volume corrected for dilution

For phytase level control= 25000 (100ml extraction volume x 25 (dilution of stock solution) x 10 (40µl diluted stand solution + 360µl buffer))

For digesta= 2000 ml (500ml extraction volume x 4 (100µl extract + 300µl buffer))

m= mass in grams

t= incubation time

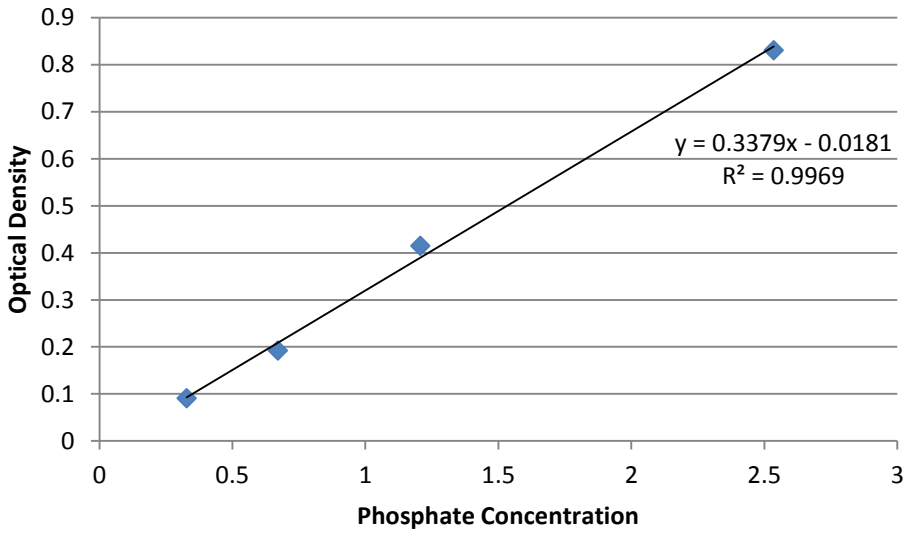

Figure 2.2: Example standard curve for phytase activity analysis

2.5.13. Supplemented Phytase Activity

Supplemented phytase was analysed by Quantiplate™ Kit for Quantum™ Phytase (EnviroLogix, Maine, USA). Wash buffer was prepared by adding phosphate buffered saline (pH 7.4 with 0.05% Tween 20) to a litre of ultra-pure water. Extraction buffer was prepared using EnviroLogix Quantum Extraction Buffer Kit Cat No ACC 061; a bottle of sodium borate, bottle of 2N sodium hydroxide and vial of 10%

Tween 20 were added to 980ml of ultra-pure water and mixed thoroughly. 80ml of extraction buffer was added to 20g or ground feed or digesta. It was shaken thoroughly for 1 minute and centrifuged at 5000 x g for 5 minutes, and the resulting supernatant was used for the analysis.

50µl of Quantum Assay Diluent was added to antibody coated wells in a plate frame. 50µl of 0, 250, 500, 1000 and 2000FTU/kg Quantum Calibrators, or 50µl of each sample extract, was then added to the respective wells, as illustrated in Figure 2.3.

Well ID	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	S1000	3	7	11	15	21	26	30	34	38	42
B	Blank	S1000	3	7	11	16	22	26	30	34	38	42
C	S0*	S2000	4	8	12	17	22	27	31	35	39	43
D	S0	S2000	4	8	13	18	23	27	31	35	39	43
E	S250	1	5	9	13	19	23	28	32	36	40	44
F	S250	1	5	9	14	20	24	28	32	36	40	44
G	S500	2	6	10	14	20	25	29	33	37	41	45
H	S500	2	6	10	15	21	25	29	33	37	41	45

*S denotes standards

Figure 2.3. Diagram of layout of wells for Quantum Phytase analysis

The contents of the wells were mixed thoroughly by moving the plate in a rapid circular motion on the bench top for 20-30 seconds. The plate was then incubated at ambient temperature for 15 minutes and the well contents were rinsed thoroughly with wash buffer. All liquid was removed with a paper towel before 100µl Quantum Enzyme Conjugate was added to each well. The contents were mixed and incubated at room temperature for 45 minutes. The wells were then rinsed again with wash buffer and dried. 100 µl of Substrate was then added to each well and it was left to incubate for 15 minutes at room temperature before 100 µl of STOP solution was added. The plate was then mixed and read at 450nm, with 630nm as the reference wavelength, on a microplate reader (Multiskan FC, Thermo Scientific, Welwyn Garden City, UK). A standard curve of absorbance at 450nm against Quantum concentration (FTU/kg) was used to calculate sample concentration. An estimate of the concentration of supplemented phytase in the samples was obtained by comparing absorbance readings to those of the calibration values.

2.5.14. Extractable Fat Determination

Samples of diets were analysed for extractable fat content by the Soxhlet method (AOAC official method 2003.05). Approximately 5g of sample was accurately weighed into an extraction thimble. A flat bottomed flask containing a small amount of anti-bumping granules was accurately weighed. 150ml of petroleum ether was added to the flask. The thimble was inserted into the bottom of the distillation unit, the distillation apparatus was connected to the condenser and the flask was attached to the apparatus and seated in the heating mantle (set to 40-60°C). The samples were left to extract for approximately 18 hours, then the remaining ether was boiled off on a hotplate and left to evaporate. The flasks were reweighed and extractable fat content was calculated by:

M2-M1 / M0 x 100 = % extractable fat

where:

M0=Original weight of sample

M1= Flask plus anti-bumping granules

M2=Flask plus fat and anti-bumping granules

2.5.15. Pepsin Activity

Pepsin activity in gizzard digesta from Trial 3 was determined using 2% bovine haemoglobin as the substrate, based on the method presented by Liu and Cowieson (2011). 2.5% haemoglobin stock solution was prepared by mixing 25 mg/ml solution of haemoglobin from bovine blood (Sigma- Aldrich, Dorset, UK) in ultra-pure water. To make 2% haemoglobin solution, 80ml of the stock solution was adjusted to pH 2.0 at 37°C using 5M HCl and was made to a final volume of 100ml with ultra-pure water. Pepsin solution was prepared by dissolving 1 mg/ml stock pepsin powder (Sigma Aldrich, Dorset, UK) in cold (2–8 °C) 10 mM HCl and then diluting it to 0.01-0.05mg/l with cold 10 mM HCl.

5ml of 2% haemoglobin solution was added to approximately 1g digesta sample and the solution was incubated at 37°C for 10 minutes. 1ml of pepsin solution was then added to the solution and it was incubated for a further 10 minutes at 37°C. The reaction was stopped by addition of 10ml of 5%

trichloroactetic acid followed by incubation for 5 minutes at 37°C and filtration through a 0.45 µm syringe filter. The optical density of the solution was then read on a UV spectrophotometer (Unicam Helios, USA) set at 280nm. For the blanks, the above steps were carried out except pepsin solution was not added after the first incubation step, and the 10ml 5% trichloroacetic acid and 1ml pepsin solution were added after the second incubation step. Each sample and blank was measured in triplicate.

Pepsin activity was calculated by:

$$\frac{\Delta D\ (280) \times \text{Dilution Factor}}{10 \times 1 \times 0.001}$$

ΔD (280)= net optical density at 280nm (ΔD (280)t-ΔD (280)b) (where ΔD (280)t is the value for the test portion and ΔD (280)b is the value for the blank)

10= Incubation time in minutes

1.0 = Volume of enzyme solution (ml)

0.001 = ΔD 280 per unit of pepsin per unit definition

2.5.16. *In Vitro* Evaluation of Calcium and Phosphorus Solubility

A 2-step *in vitro* assay procedure, based on that of Walk *et al.* (2012), was used to investigate solubility of Ca and P in the gastric and small intestine phases of digestion in all the Trial 1 and Trial 2 diets. Each feed ingredient or diet was ground through a 1 mm screen and 2.5 g was weighed into pre-weighed tubes. For each phase, gastric and small intestine, a minimum of 9 sub-samples were analysed for each diet and feed ingredient. To mimic the gastric phase, 4.5 ml of 0.13 N HCl, with 2,000 U pepsin/ml (Sigma-Aldrich, Dorset, UK) was added to the pre-weighed samples before incubation at 41°C for 20 minutes. Sample pH was then measured in triplicate using a spear tip piercing pH electrode (Sensorex, California, USA) to ensure samples were within the target range of pH 3.5-4.5. Samples were then

diluted to 20 ml with 0.1 M HCl and centrifuged at 5,200 x g at 4°C for 1 minute before the tubes were weighed. Post centrifugation, the supernatant was collected into a separate pre-weighed tube. The sample was again diluted to 20 ml with 0.1 M HCl, re-centrifuged, and the supernatant collected. The pooled supernatant was filtered through a 0.22 µm filter (Fisher Scientific, Loughborough, UK) and stored at -20°C until further analysis. The filtered supernatant was diluted 1:10 with ultra-pure water and analysed for soluble Ca and P using ICP-OES set at 213.617 nm for P and 317.933 nm for Ca as previously described. The percent gastric Ca or P solubility was then calculated according to the following equation:

(Soluble Ca or P in the gastric phase/ Total Ca or P in the diet) * 100

For the intestinal phase, the samples were weighed and incubated for 20 minutes at 41°C with HCl and pepsin as for the gastric phase. Immediately after this initial incubation, 1.5 ml of $NaHCO_3$ containing 2 mg of pancreatin/ml (Sigma-Aldrich, Dorset, UK) was added to each sample before mixing and incubating the samples at 41°C for an additional 60 minutes. The contents of each tube was then made up to 45 ml with 0.32 M $HClO_4$ to stop the enzymatic reaction and weighed before centrifugation at 4˚C at 4,000 x g for 1 minute. The supernatant was immediately filtered through Whatman 541 hardened, ashless filter papers filter papers and diluted 1:10 with 1 M HNO_3. The diluted samples were then analysed for Ca and P content using ICP-OES as described previously. The percent of soluble Ca or P in the SI was calculated according to the following equation:

(Soluble Ca or P in the SI/ Total Ca or P in the diet) * 100

2.6. Data Analysis

All data was analysed using the SPSS software version 19, 20 or 21 for Windows (IBM Statistics, 2013). After KS testing to confirm normality, statistical analysis was carried out using Univariate analysis to determine interactions between the analysed factors. One-way ANOVA was used to test the equality of the means and multiple ANOVA was used to test 2, 3 and 4-way interactions. Treatment means were separated using Duncan post hoc test, chosen because it selects protection level for error rate

based on the collection of tests as opposed to the error rate for the individual tests. Paired T-Test were used to compare and differentiate the values of means. In cases where the effect of phytase inclusion level read to be significant, a linear and quadratic contrast was conducted. Correlations were analysed by bivariate correlation using Pearson correlation, chosen because it computes based on true values and depicts linear relationships. Multiple linear regressions were used to determine the unique contribution and relatedness of factors. Interpretations of the strength between relationships was based on those of Cohen (1988): small r = 0.1-0.29, medium r = 0.30-0.39 and large r = 0.50 to 1.0. Statistical significance was declared at $p<0.05$.

CHAPTER 3: Method Development

This chapter presents a collection of pilot studies designed to develop and validate some methods and theories employed elsewhere in this thesis. In section 3.1. and 3.2. digestibility measures in broilers were examined; section 3.1. presents findings about how many pre-starter birds are required for analysis of digestibility and section 3.2. presents findings about whether an ICP-OES assay can be used as an alternative to the commonly used UV-spectroscopy assay for determination of TiO_2 as a digestibility marker. Results from these studies were used to establish the number of birds used in Trial 2 and the method used for analysing TiO_2 as a digestibility marker was implemented in Trial 2 and Trial 3. In section 3.3. the optimum sampling method for the determination of broiler digesta pH was evaluated. The method shown to provide pH readings that were most representative of the gastrointestinal environment was then used in Trials 1, 2 and 3. In section 3.4. the 2-step *in vitro* assay featured in Chapter 2 Section 2.5.16. was validated by comparing *in vitro* and *in situ* quantification of Ca and P solubility in the Trial 1, 2 and 3 diets. In section 3.5. the amount of variation that exists in susceptible phytate content between different batches of feed ingredients in the UK was investigated. The assay used in this study, featured in Chapter 2 Section 2.5.11., was also used to analyse all the trial diets and was validated by observing if the results transpired *in situ*, particularly in Trial 3. Trial 3 diets were also formulated based on measurements of phytate susceptibility in the individual feed ingredients being fed.

3.1. Minimising the number of birds used for digestibility measures in the pre-starter period in broiler chicks

3.1.1. Introduction

Immediately after hatching the morphology and function of the intestine changes dramatically as the bird makes the transition from utilising endogenous nutrients supplied by the yolk to exogenous nutrients from feed through the intestine (Sklan, 2001). In the first 48 hours post-hatch the digestive tract grows from3.8% to 8.9% of bodyweight (Noy *et al*. 2001). There is increased interest in the pre-starter stage, particularly into defining the optimum nutrition for this crucial period (Ullah *et al*. 2012),

as strong correlations have been found between bird finishing weight and growth in the first 7 days of life (Nir *et al*., 1993). Digesta samples are required to analyse and evaluate enzyme activity, digestibility and dietary affects in the gastrointestinal tract of pre-starter birds. This is problematic in young birds (<d7) as the number of birds required to achieve sufficient digesta for laboratory analysis of nutrients can be large. It is imperative that a balance is made between obtaining sufficient digesta sample for the research and justifying the number of birds used in a study. It is therefore important to specifically determine the quantity of digesta which can be collected from chicks in this pre-starter period to reduce the culling of unnecessary birds. Findings from this study were used to assess how many pre-starter birds to use in Trial 2 (Chapter 5).

Aim

- To determine the quantity of gizzard, jejunum, and ileal digesta present in broilers aged 2, 4 and 7 days post-hatch, to eliminate the use of excess birds in future studies.

3.1.2. Procedure

Two day old (n=9), four day old (n=8) and seven day old (n=8) Ross 308 chicks, from a breeder flock age of 45 weeks, were obtained from a local poultry farm in Nottinghamshire. Birds were euthanised on site and transported to the laboratory at Nottingham Trent University. Gizzard, jejunal and ileal digesta contents were weighed into pre-weighed pots, one pot per bird. The samples were then frozen at -20°C prior to freeze drying (LTE Scientific, UK) for 5 days to uniform weight. They were then freeze-dried for an additional 24 hours and re-weighed to ensure all moisture had been removed from the samples. Mean weight of the freeze-dried digesta across all the samples for each section of the tract was calculated.

3.1.3. Results

Table 3.1. shows that approximately 13 birds per replicate are required to obtain 1g of jejunal or ileal digesta from a 2 day-old bird. To obtain 1g of gizzard digesta from 2 day old birds, however, just 3

birds are required. On day 4 post-hatch 3 birds are required to provide 1g of jejunal and ileal digesta, and 2 birds to provide 1g of gizzard digesta. By day 7 each individual bird contains almost 1g or more of digesta per section of tract.

Table 3.1. Weight of freeze-dried digesta from the gizzard, jejunum and ileum of 2, 4 and 7 day old broilers

	Gizzard			Jejunum			Ileum		
Age (post-hatch)	d2	d4	d7	d2	d4	d7	d2	d4	d7
Mean freeze-dried digesta (g)	0.40	0.64	1.74	0.09	0.33	0.93	0.08	0.37	1.03
SEM	0.04	0.07	0.08	0.03	0.04	0.07	0.02	0.04	0.06

3.1.4. Conclusion

The growing interest in pre-starter digestibility in broilers requires a rapid determination of the number of birds required for these measures. The specific values produced from this study suggests that the number of birds required at 2 days old may be prohibitive due to the large numbers required to obtain sufficient digesta sample. The results of this study for 4 and 7 days post-hatch enable researchers to select the appropriate number of birds for analysis of the pre-starter phase. There were some limitations to this study, particularly that all the birds used were sourced from one farm and hatchery, so were exposed to the same environmental conditions, were fed the same diet and were euthanised at the same time each sampling day. Further investigation is needed to determine the impact of these factors on digesta content of young birds. Growth rate and feed efficiency, and hence gut development and feed intake, is influenced by bird strain and by parent flock age, as this dictates embryonic metabolism during incubation (Hamidu *et al*. 2007). In this study there was variation in parent flock age between the birds and all the birds were from the same strain, suggesting that further investigation is also needed into the impact of parent flock age and bird strain on digesta content of birds in the pre-starter phase. Nonetheless, the data derived from this study provides a valuable insight into the number of birds required for digesta analysis.

3.2. Comparison between ICP-OES and UV-spectrometry assays for the determination of titanium dioxide added as an inert marker in chicken digestibility studies

3.2.1. Introduction

Inert digestibility markers added to broiler diets eliminate the need to evaluate quantitative feed intake and excreta output, and enable nutrient utilisation to be examined along the gastrointestinal tract. The method most widely used to determine TiO_2 concentration is UV-spectroscopy, primarily based around the method of Short *et al.* (1996). This method involves the initial hydrolysis of the sample with sulphuric acid followed by a colour reaction. An intense orange/yellow colour results from the addition of hydrogen peroxide to an acidic titanium solution, and the colour intensity can be quantified by UV-spectrometry. In poultry research TiO_2 as a dietary marker has been used successfully to determine calcium and phosphorus utilisation (Walk *et al.*, 2012). Mineral digestibility and utilisation in poultry is frequently analysed by induced coupled plasma optical emission spectrophotometer (ICP-OES) in preference to UV methods (Leytem and Kwanyuen, 2008) as the ICP-OES assay can be used to analyse many elements in one preparation. Titanium concentration can be detected by ICP-OES, which suggests that there is potential for TiO_2 measurement to be made concurrently with mineral content, thus reducing analysis time and resource use. Findings from this study were used to assess if digestibility could be measured in Trial 3 (Chapter 6) using ICP-OES as opposed to UV-spectroscopy. Results from this study feature in Appendix A as a published article.

Aim

- To investigate consistency of TiO_2 recovery from an ICP-OES and a UV-spectroscopy assay, and evaluate if the ICP-OES assay can be used as an alternative to the UV-spectroscopy assay for the determination of TiO_2 as a marker in poultry digestibility studies.

3.2.2. Procedure

Ross 308, male broilers (n=452) were involved in a series of digestion studies to determine ileal digesta recovery of TiO_2 either by UV-spectroscopy by the method of Short *et al*. (1996), or by an ICP-OES

assay. Birds were fed one of 19 experimental diets in mash form, each with TiO_2 added at 5g/kg; 6 semi-synthetic starch dextrose based diets, and 13 more commercial style diets based on cereals including wheat, rapeseed, maize and rye and soya bean meal. All 19 diets were analysed for TiO_2 concentration. Each diet was fed to a minimum of 20 birds. All birds were from breeder flocks aged 42-45 weeks old and were obtained from a commercial hatchery at day of hatch. Chicks were randomised by weight and placed in 0.64 m^2 floor pens in groups of four, bedded on clean wood shavings. Birds were allowed ad libitum access to the treatment diets and water for the duration of the trials; which spanned between two and four weeks. The room was thermostatically controlled to produce an initial temperature of 32°C and reduced to 21°C by day 21. The lighting regimen used was 24 hours light on day 1, with darkness increasing by 1 hour per day until 6 hours of darkness was reached and this was maintained throughout the remainder of the study. All birds sampled were euthanised by cervical dislocation. This occurred at the same time each sampling day; after at least 6 hours of light, to ensure maximal gut fill. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the University College of Science ethical review committee. Digesta content was removed from the intestinal section distal to the Meckel's diverticulum and proximal to the ileo-ceco-colonic junction of each bird. The digesta samples were then freeze-dried and ground through a 1mm screen.

All feed and digesta samples were analysed for TiO_2 concentration by both UV-spectroscopy and ICP-OES assays. Details about these methods are featured in Chapter 2 Section 2.5.4 and 2.5.8 respectively. Briefly, 250mg titanium dioxide was added to 100ml of sulphuric acid (H_2SO_4) and diluted to 500ml with distilled water to produce a standard titanium solution of 0.5mg/ml. This standard solution was used to prepare the calibration curve for both the UV-spectroscopy and ICP-OES assays. For the ICP-OES assay, the TiO_2 standard solution was diluted with ultra-pure water in varying increments to produce standards between 0 and 10ppm. These standards were measured on an ICP-OES (Optima 2100 DV ICP-OES, model PQ Excell VG Elemental, Perkin-Elmer, USA) set to detect Ti at wavelength 334.936, and a calibration curve was derived from the readings.

For the UV-spectroscopy assay, graded volumes of TiO_2 standard solution was pipetted into individual 100ml volumetric flasks and made up to 10ml with 7.4M H_2SO_4. 10ml 30% hydrogen peroxide (H_2O_2) was then added to the solutions and the contents were made up to 100ml with distilled water before measurement on a spectrophotometer (Unicam Helios, Berkshire, UK) set at 410nm. For the UV-spectroscopy assay, triplicate aliquots (approximately 0.3g) of each digesta sample and 5 replicates of each of the feed samples were ashed for 16 hours at 650°C. Once cooled, 10ml H_2SO_4 (7.4 M) was added to each crucible and the samples were heated for approximately 1 hour until completely dissolved. The contents were then transferred quantitatively into 100ml volumetric flasks via filter papers (Whatman 541) using distilled water. 10ml of 30% H_2O_2 was then added to each flask and the flasks made to volume with distilled water. Solutions were thoroughly mixed prior to reading on a spectrophotometer set at 410nm.

For the ICP-OES assay, 10ml of aqua regia (hydrochloric acid (HCl) and nitric acid (HNO_3) at a ratio of 3:1) was added to triplicate aliquots (approximately 0.2g) of digesta sample and 5 replicates of each feed sample, and left for a minimum of 12 hours. The samples were then boiled until completely dissolved, for approximately 1 hour. The contents were then filtered through Whatman 541 filter papers into 50ml volumetric flasks and made to volume with ultra-pure water, before transferral into 15ml tubes. The samples were measured on the ICP-OES set to detect Ti at wavelength 334.936.

All data was analysed using IBM SPSS statistics version 21. T-Tests were conducted to differentiate between means. The relatedness of the readings from each assay was investigated using Pearson product-moment correlation coefficient and interpretations of the strength of the relationship between the two methods was based on guidelines by Cohen (1988); weak relationship r = 0.10 to 0.29, medium relationship r = 0.30 to 0.49 and strong relationship r = 0.50 to 1.0. Linear regressions were calculated using the true and measured titanium concentrations. Significance was accepted at $P < 0.05$.

3.2.3. Results

Table 3.2 and 3.3. shows that there were no significant differences between any readings measured by the UV-spectroscopy assay and the ICP-OES assay. There were consistently strong relationships and no significant differences between the two methods for analysis of TiO_2 concentration in the diets and ileal digesta.

Table 3.2. Relatedness of an ICP-OES assay and UV-spectroscopy assay for determination of TiO_2 concentration in broiler diets[1]

	Method of Ti Determination (g/kg)		
Diet	ICP-OES	UV-spectroscopy	Relatedness[2]
Semi-synthetic starch dextrose	6.03	6.29	0.684
Wheat Soyabean	5.93	5.69	0.794
Wheat Soyabean 0FTU/kg phytase	5.85	5.97	0.778
Wheat Soyabean 500FTU/kg phytase	5.71	6.08	0.759
Wheat Soyabean 5000FTU/kg phytase	6.64	6.97	0.708
Wheat Rapeseed 0FTU/kg phytase	6.11	6.53	0.886
Wheat Rapeseed 500FTU/kg phytase	4.90	5.08	0.866
Wheat Rapeseed 5000FTU/kg phytase	6.49	6.53	0.963
Maize Rapeseed	6.87	6.98	0.995
Maize Soyabean	4.99	4.88	0.956
Maize, Rye, Wheat, Soyabean	4.87	5.16	0.758
Maize, Rye, Soyabean	5.75	5.47	0.689
SEM	0.14	0.23	

[1]Represent the average of a minimum of 5 replicates per diet.
[2]Strength of the relationship between the ICP-OES and UV-Spectroscopy method for Ti measured in each diet where confidence in the result is $P<0.05$.

Table 3.3. Relatedness of an ICP-OES assay and UV-spectroscopy assay for determination of TiO_2 concentration in broiler ileal digesta[1]

	Method of Ti Determination (g/kg)		
Diet	ICP-OES	UV-spectroscopy	Relatedness[2]
Semi-synthetic starch dextrose	13.58	13.40	0.776
Wheat Soyabean	13.99	13.53	0.550
Wheat Soyabean 0FTU/kg phytase	13.43	13.65	0.512
Wheat Soyabean 500FTU/kg phytase	15.63	15.87	0.822
Wheat Soyabean 5000FTU/kg phytase	13.32	12.42	0.887
Wheat Rapeseed 0FTU/kg phytase	13.16	12.48	0.529
Wheat Rapeseed 500FTU/kg phytase	14.19	14.95	0.613
Wheat Rapeseed 5000FTU/kg phytase	12.92	12.71	0.858
Maize Rapeseed	12.23	12.01	0.584
Maize Soyabean	12.49	12.99	0.726
Maize, Rye, Wheat, Soyabean	12.33	12.04	0.563
Maize, Rye, Soyabean	12.19	12.06	0.646
SEM	0.20	0.26	

[1] Represent the average response of a minimum of 20 birds per diet, 452 birds in total, with digesta samples collected at age 14, 21 or 28 days post-hatch. Analysis was replicated a minimum of 3 times per digesta sample.
[2]Strength of the relationship between the ICP-OES and UV-Spectroscopy method for Ti measured in each digesta sample where confidence in the result is $P<0.05$.

3.2.4. Conclusion

Findings from this study suggest that the ICP-OES assay used in this study was successful at identifying diet and ileal digesta TiO_2 concentration, and hence has the potential to replace the widely used UV-spectroscopy assay. The main advantage of the ICP-OES assay when compared to the UV-spectroscopy is that the former has been shown to be more sensitive at quantitative analysis with improved detection limits. The ICP-OES assay is also less time-consuming, and the ICP-OES enables several elements to be detected in parallel which reduces preparation time and the amount of sample, and hence potentially the number of birds, required. There are however some advantages to the UV-spectroscopy assay compared with the ICP-OES assay. The ICP-OES assay is more expensive due to the cost to run the ICP-OES and to maintain the argon gas supplies, although this is mitigated by the potential for concurrent mineral analysis. The ICP-OES assay is also more hazardous as involves the use of aqua regia which is moderately more corrosive than sulphuric acid.

3.3. Optimum sampling method for the determination of broiler digesta pH in birds fed differing levels of dietary calcium

3.3.1. Introduction

Digesta pH is one of the major gastrointestinal tract (GIT) factors which influence nutrient bioavailability and the intestinal microbiota. Small changes outside the normal pH range can have significant negative implications on digestion and mineral absorption. Accurate determination of digesta pH in broiler chickens could therefore act as a tool to indicate potential for optimum gut health and for maximum nutrient absorption. The majority of methods for measuring GIT pH use a pH meter with a hand held probe, but sample handling prior to pH testing varies between studies; in particular whether the measurement is determined *in situ* or *ex situ*. As a result, this study explores the impact of removing the digesta from the tract, with the view that exposing the digesta to air may reduce its temperature and cause carbonate from dietary limestone, blood buffering capacity and pancreatic secretions to dissociate to CO_2 and water, resulting in removal of hydrogen ions from the milieu (Zhang and Coon, 1997; Guinotte *et al.,* 1995). The sampling methods assessed in this study were the effect on pH of removing the digesta from the gut, subjecting the digesta to prolonged air exposure and altering temperature of the digesta pH assay. The determined optimum method was then used in Trial 1, 2, and 3 (Chapter 4, 5 and 6 respectively) for analysis of GIT pH. Results and diet formulations from this study, as well as investigations into the effect of digesta water content and dietary limestone inclusion on digesta pH, feature in Appendix B as a published article.

Aim

- To establish the optimum sampling method for the determination of broiler digesta pH that is most representative of the gastrointestinal environment.

3.3.2. Procedure

Ross 308 male broilers (n=24) were used to assess the effect of removing the digesta from the GIT on measuring digesta pH. Birds were from a 42-week-old breeder flock and were obtained from a

commercial hatchery at day of hatch. Chicks were randomised by weight and placed in 0.64 m^2 floor pens in groups of six, bedded on clean wood shavings. Birds were allowed ad libitum access to the treatment diets and water for the duration of the trial. The room was thermostatically controlled to produce an initial temperature of 32°C reduced to 21°C by day 21. The lighting regimen used was 24 hours light on d 1, with darkness increasing by 1 hour a day until 6 hours of darkness was reached, and this was maintained throughout the remainder of the study. All birds sampled were euthanised by cervical dislocation. This occurred at the same time each sampling day; after at least 6 hours of light, to ensure maximal gut fill. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the University College of Science ethical review committee. Sampling was carried out on 8 birds per sampling day on d 7, 14 and 28 post-hatch.

Immediately post euthanasia, *in situ* gizzard and duodenal pH was determined for every bird on each sampling day by removing the gizzard intact and inserting a spear tip piercing pH electrode (Sensorex S175CD, California, USA) with digital pH meter (Mettler-Toledo, UK) directly into the digesta in the lumen of the proximal gizzard (proventricular opening), whilst ensuring the pH electrode did not touch the gizzard wall, and recording the pH. This was repeated six times, putting the probe in different areas of the gizzard each time. The probe was rinsed with ultra-pure water once all six readings had been taken. The process was then repeated in the duodenal loop of the same bird. Readings were taken at the distal end of the duodenum; based on average length of the duodenum across the bird ages the duodenum was cut at a point 30 cm from the gizzard (Yadav *et al.,* 2010) and the pH electrode was inserted directly into this opening. Again, measurements were repeated six times.

For half the birds the digesta was removed, immediately after *in situ* pH had been determined, and was put into centrifuge tubes that had been maintained at room temperature (14.4°C +/- 0.15 SEM). A stop watch was started the instant the digesta had been put into the centrifuge tubes, and pH was recorded every 15 seconds for three minutes using a spear-tip electrode and digital pH meter. This

entire process was carried out on the other half of the birds, except the digesta was put into centrifuge tubes that had been previously warmed to 41°C in a water bath.

All data was analysed using IBM SPSS statistics version 21. Multiple linear regressions, with individual bird number as a covariate, were used to determine the unique contribution and relatedness of time exposed to air (log time (seconds)) and digesta temperature on variance in gizzard and duodenal pH. Interpretations of the strength between the relationships were based on those of Cohen (1988): small r = 0.1-0.29, medium r = 0.30-0.39 and large r = 0.50 to 1.0. T-tests were conducted to make statistical comparisons between *in situ* pH and pH at the exponential time point where digesta pH ceased to fluctuate post-removal from the tract. Two-, 3- and 4-way interactions between time exposed to air, digesta temperature, and bird age were determined by multiple ANOVA. Significance was accepted at $P < 0.05$.

3.3.3. Results

Table 3.4. shows there was an interaction between temperature of the digesta and bird age on gizzard pH; on d7 and d28 gizzard pH was significantly higher when measured at room temperature than when measured at 41°C, but temperature had no effect on gizzard digesta pH on d14. Maintaining samples at room temperature after removal from the tract led to gizzard and duodenum pH readings being consistently higher *ex situ* than *in situ*, but when the digesta pH was measured *ex situ* in samples maintained at 41°C this was not always the case. Duodenum pH was numerically higher when measured at room temperature than when measured at 41°C and *in situ*. At d28 gizzard and duodenum pH measured *ex situ* at 41°C was lower than that measured *in situ*.

Table 3.4. Influence of bird age, method[2] and digesta temperature on gizzard and digesta pH of broilers measured *ex situ* over a 3 minute time period[1]

	In situ	*Ex situ*	
		14°C	41°C
Gizzard			
d7	2.38^{f}	2.61^{e}	2.39^{f}
d14	2.57^{d}	2.77^{d}	2.69^{d}
d28	2.22^{g}	2.29^{f}	2.20^{g}
SEM		0.079	
Temp x Age		0.001	
Temp x Method x Age		0.003	
Duodenum			
d7	5.85^{c}	6.05^{c}	5.97^{c}
d14	6.12^{b}	6.25^{a}	6.16^{b}
d28	5.82^{c}	5.86^{c}	5.78^{c}
SEM		0.100	
Temp x Age		0.048	
Method		0.033	

[1] Means represent the average of 8 birds per day, 24 birds total.

[2] pH measured *in situ* or at 75 s (the highest exponential point) after the digesta had been removed from the tract.

[a-e] Means with no common superscript are different ($P < 0.05$).

The time of digesta exposure to air had no significant effect on gizzard or duodenum digesta pH (data not shown), but initial removal of digesta from the tract lead to a numerical rise in pH before the readings plateaued. Table 3.5. shows that digesta temperature made the strongest unique contribution to duodenum pH, and second strongest contribution to gizzard pH, when the effects of diet and time exposed to air were controlled for, and there were correlations between digesta temperature and pH.

Table 3.5. Correlations and relative contributions of the effect of time exposed to air, temperature, and dietary limestone on digesta pH and dietary limestone effect on gizzard and duodenal digesta pH of broilers [1]

	Gizzard pH			Duodenum pH		
Age, day	d 7	d 14	d 28	d 7	d 14	d 28
Relative contributions						
R-square	0.63	0.95	0.93	0.80	0.24	0.49
Beta[2]						
Time[3]	0.02	0.07	0.05	0.11	0.33	0.10
Temperature[4]	0.81	1.56	0.63	2.39	0.65	0.57
Unique Contribution to R^2 (%)						
Time	0.00	0.65	0.02	1.23	10.62	0.00
Temperature	6.30	55.46	28.83	54.46	9.30	23.43
Correlations[5]						
Time	0.02	0.07	0.00	0.02	0.33	-0.05
Temperature	0.39	0.08	-0.21	0.65	0.33	-0.26

[1] Represent the average response of 8 birds per age, 24 birds in total.
[2] Coefficient to indicate statistically significant unique contribution of the factor.
[3] Log time of seconds digesta was exposed to air post removal from the tract (15 to 180 s).
[4] Digesta measured at either room temperature (14.4°C) or at 41°C.
[5] Correlations between factor and pH readings.

3.3.4. Conclusion

This study suggests that measuring pH of digesta that has been removed from the tract may not be providing a true representation of any dietary effects on the GIT environment. The generally observed increase in pH when measured *ex situ* compared to *in situ* in both the gizzard and duodenum is potentially attributable to CO_2 release from carbonate buffering pH on exposure to air by altering the equilibrium of carbonic acid dissociation towards water and CO_2. The observed plateau in *ex situ* pH illustrates the point at which no further CO_2 remains to be released from the carbonate in the digesta. The associated temperature reduction caused by removing digesta from the tract can be partially mitigated through use of a water bath to maintain bird body temperature, but this approach is not recommended as the buffering effect upon removal cannot be overcome. The method that gives the most accurate representation of broiler GIT environment when determining digesta pH is to insert a pH probe directly *in situ* into the gut lumen immediately post euthanasia.

3.4. *In vitro* versus *in situ* evaluation on the effect of phytase supplementation on calcium and phosphorus solubility

3.4.1. Introduction

In vitro assays have the potential to act as sensitive and cost effective tools for the evaluation of Ca and P availability in dietary ingredients. They could therefore potentially be used as an alternative to expensive and time-consuming animal trials. There are however limitations to *in vitro* methodologies, namely that it is impossible to reconstruct exactly the variability and interactions present *in situ*, so *in vitro* assays are able to measure degradability only and not digestibility. This study examines the relatedness between an *in vitro* assay and *in situ* analysis of Ca and P solubility in the diets fed to birds in Trial 1, 2 and 3 (Chapters 4, 5 and 6). Results from *in vitro* and *in situ* evaluation of the diets fed in Trial 1 (see Chapter 4) feature in Appendix C as a published article.

Aim

- To evaluate a 2-step *in vitro* assay by comparing Ca and P solubility determined *in vitro* to Ca and P solubility measured *in situ* in both the gastric and SI phase in a range of diets.

3.4.2. Procedure

Details about the individual trials (Trial 1, 2 and 3), including the composition and calculated and analysed content of the diets and bird care, are featured in Chapters 4, 5 and 6. Details about measuring Ca and P solubility and the 2-step *in vitro* assay are featured in Chapter 2 Section 2.5.8.1. and 2.5.16 respectively. Briefly, for *in situ* analysis of Ca and P solubility digesta was collected from the gizzard and jejunum (the intestinal section distal to the duodenal loop and proximal to the Meckel's diverticulum) and frozen at -20°C prior to freeze drying (LTE Scientific, UK) for 5 days to uniform weight. In Trial 1 and 2 digesta was pooled per pen but in Trial 3 digesta was analysed on an individual bird basis. 2g of dried digesta sample was then weighed into pre-weighed bottle and 200 ml ultra-pure water (ICW 3000 water purifier for ion chromatograph, Millipore) was added. The sample was placed on a shaker set at 200 epm for 60 minutes prior to being centrifuged at 5,200 x g for 10 minutes. The

supernatant was then filtered through Whatman #541 filter papers before measurement by ICP-OES, with wavelengths for Ca and P set at 317.933 nm and 213.617 nm respectively. Six replicates were analysed for each sample. ICP standards were made by diluting 1,000 ppm standard (Fisher Scientific, UK) and ultra-pure water. Percentage Ca or P solubility was calculated according to the following equation:

(Soluble Ca or P in digesta supernatant/ Total Ca or P in the diet) * 100

For the *in vitro* analysis, to mimic the gastric phase (indicating proventriculus and gizzard) 4.5 ml of 0.13 N HCl, with 2,000 U pepsin/ml (Sigma-Aldrich, UK) was added to the pre-weighed samples before incubating at 41°C for 20 minutes. Sample pH was then obtained in triplicate using a spear tip piercing pH electrode (Sensorex, California, USA) to ensure samples were within the target range of pH 3.5-4.5. Samples were then diluted to 20 ml with 0.1 N HCl before centrifugation at 4000 x g at 4°C for 1 minute and the tubes weighed. Post centrifugation, the supernatant was collected into a separate pre-weighed tube. The sample was again diluted to 20 ml with 0.1 N HCl, re-centrifuged, and the supernatant collected. The pooled supernatant was filtered through a 0.22 µm filter (Fisher Scientific, UK) and stored at -20°C until further analysis. The filtered supernatant was diluted at 1:10 with water and analysed for soluble Ca and P using ICP-OES set at 213.617 nm for P and 317.933 nm. The percent gastric Ca or P solubility was then calculated according to the following equation:

(Soluble Ca or P in the gastric phase/ Total Ca or P in the diet) * 100

For the intestinal phase (indicating the duodenum and jejunum), the samples were weighed and incubated with the HCl/pepsin as for the gastric phase and incubated for 20 minutes at 41°C. Immediately after incubation 1.5 ml of $NaHCO_3$ containing 2 mg of pancreatin/ml (Sigma-Aldrich, UK) was added to each sample. The samples were mixed and incubated at 41°C for an additional 60 minutes. The tubes were then made up to 45 ml with 0.32 M $HClO_4$ to stop the enzymatic reaction and weighed before centrifugation at 4˚C at 4,000 x g for 1 minute. The supernatant was immediately

filtered through Whatman 541 filter paper and diluted 1:10 with HNO_3. The diluted samples were then analysed for Ca and P using ICP-OES as described previously. The percent of soluble Ca or P in the SI was calculated according to the following equation:

(Soluble Ca or P in the SI/ Total Ca or P in the diet) * 100

All data was analysed using IBM SPSS statistics version 19. Independent sample t-tests were used to directly compare the findings from the *in vitro* and *in situ* methodologies. The relatedness of the methodologies was investigated using Pearson product-moment correlation coefficient and interpretations of the strength of the relationship between the two methods was based on guidelines by Cohen (1988); weak relationship r = 0.10 to 0.29, medium relationship r = 0.30 to 0.49 and strong relationship r = 0.50 to 1.0. Significance was accepted at $P < 0.05$.

3.4.3. Results

There were both consistencies and differences between the *in vitro* and *in situ* methods featured in this study. Table 3.6. shows there were predominantly strong and medium relationships between the two methods in the gastric phase. Table 3.7. shows in the SI phase there was a higher proportion of weak relationships between the methods compared to the gastric phase.

Table 3.6. Comparison between diet effects on Ca and P solubility from diets subjected to the gastric phase of a 2-step *in vitro* assay procedure and in the gizzard of broilers

	Ca			P		
Diet	*In situ*	*In vitro*[1]	Relatedness[2]	*In situ*	*In vitro*[1]	Relatedness[2]
Trial 1[3]						
A	0.68	0.42	0.398	0.63	0.48	0.988
B	0.52	0.34	0.893	0.67	0.49	0.965
C	0.68	0.43	0.862	0.71	0.54	0.563
D	0.78	0.55	0.497	0.45	0.39	0.544
E	0.65	0.40	0.467	0.52	0.42	0.722
F	0.81	0.56	0.580	0.66	0.45	0.159
SEM	0.02	0.03		0.02	0.01	
Trial 2[4]						
A	0.66	0.70	0.969	0.42	0.47	0.858
B	0.64	0.73	0.591	0.42	0.48	0.812
C	0.64	0.71	0.658	0.43	0.45	0.906
D	0.67	0.73	0.826	0.47	0.48	0.722
SEM	0.01	0.02		0.01	0.02	
Trial 3[5]						
A	0.69	0.65	0.663	0.37	0.36	0.388
B	0.74	0.70	0.782	0.43	0.42	0.428
C	0.65	0.62	0.532	0.36	0.38	0.230
D	0.74	0.69	0.576	0.42	0.43	0.190
SEM	0.18	0.02		0.17	0.02	

[1]*In vitro* means represent the average of a minimum of 10 replicates per diet.

[2]Strength of the relationship between the *in vitro* and *in situ* readings for each mineral solubility measured in each diet where confidence in the result is $P < 0.05$

[3] *In situ* results represent the average response of 32 28 day-old birds per diet, 8 pens of 4 birds per pen, and 192 birds in total. Means represent the average of 6 replicates per pen.

[4] *In situ* results represent the average response of 18 14 day-old birds per diet, 6 plots per diet, 3 birds per plot, 72 birds in total. Means represent the average of 6 replicates per pen.

[5] *In situ* results represent the average response of 24 28-day old birds per diet, 12 pens of 2 birds per pen, 96 birds in total. Means represent the average of 6 replicates per pen.

[a-b] Means within the same column with no common superscript differ significantly ($P < 0.05$).

Table 3.7. Comparison between diet effects on Ca and P solubility from diets subjected to the small intestine phase of a 2-step *in vitro* assay procedure and in the jejunum of broilers

	Ca			P		
Diet	*In situ*	*In vitro*[1]	Relatedness[2]	*In situ*	*In vitro*[1]	Relatedness[2]
Trial 1[3]						
A	0.26	0.21	0.002	0.28	0.24	0.284
B	0.24	0.17	0.996	0.29	0.25	0.115
C	0.28	0.24	0.156	0.29	0.29	0.604
D	0.30	0.22	0.883	0.26	0.22	0.363
E	0.29	0.19	0.186	0.27	0.23	0.567
F	0.31	0.25	0.136	0.28	0.25	0.998
SEM	0.014	0.011		0.002	0.009	
Trial 2[4]						
A	0.32	0.28	0.584	0.31	0.34	0.781
B	0.37	0.30	0.930	0.33	0.35	0.905
C	0.37	0.29	0.655	0.35	0.36	0.861
D	0.38	0.30	0.870	0.31	0.35	0.910
SEM	0.01	0.02		0.01	0.01	
Trial 3[5]						
A	0.39	0.39	0.136	0.29	0.28	0.162
B	0.43	0.42	0.373	0.32	0.30	0.082
C	0.39	0.40	0.303	0.28	0.32	0.355
D	0.41	0.41	0.299	0.31	0.33	0.739
SEM	0.08	0.01		0.08	0.01	

[1]*In vitro* means represent the average of a minimum of 10 replicates per diet.
[2]Strength of the relationship between the *in vitro* and *in situ* readings for each mineral solubility measured in each diet where confidence in the result is $P < 0.05$
[3] *In situ* results represent the average response of 32 birds per diet, 8 pens of 4 birds per pen, and 192 birds in total. Means represent the average of 6 replicates per pen.
[4] *In situ* results represent the average response of 18 birds per diet, 6 plots per diet, 3 birds per plot, 72 birds in total. Means represent the average of 6 replicates per pen.
[5] *In situ* results represent the average response of 24 birds per diet, 12 pens of 2 birds per pen, 96 birds in total. Means represent the average of 6 replicates per pen.
[a-b] Means within the same column with no common superscript differ significantly ($P < 0.05$).

3.4.4. Conclusion

The 2-step *in vitro* assay in this study was successful at identifying the effect of phytase supplementation on Ca and P solubility in the gastric and SI phase, signifying it can be used as a tool to indicate dietary effects on the GIT environment. The *in vitro* assay is however unable to measure digestibility as is a closed system so cannot completely mimic bird GIT conditions, suggesting *in situ* analysis is still required to fully distinguish phytase effects on mineral solubility. The *in vitro* assay was more comparable to *in situ* measures for mineral solubility in the gastric phase, rather than the SI phase, most likely due to the lack of absorption and secretion in the SI phase *in vitro* compared to *in*

situ. Dietary levels of Ca, P and phytate dictate precipitation of soluble Ca and P and hence availability of these minerals, therefore calcium phosphate precipitation occurs if the Ca: P ratio is not balanced and if the pH is high. The effects of phytase on dietary free Ca and P and phytate are positive *in situ*, but potentially cause increased calcium phosphate precipitation *in vitro*, which reduces the reliability of this assay.

3.5. Susceptible phytic acid content of common feed ingredients fed to poultry in the UK

3.5.1. Introduction

The organic P component of feed ingredients fed to poultry exist in enzyme-susceptible and enzyme-resistant forms, due to binding of divalent cations to phytic acid causing a portion of it to become resistant to phytase hydrolysis. Measurements of total phytate-P content of diets may therefore be deceptive as, although different feed ingredients contribute to the total phytate content of the diet, this does not indicate substrate availability for phytase (Dayyani *et al.,* 2013). Consequently, phytase matrix values should potentially be developed based on reactive phytate content as opposed to total phytate content. This study explores how much variation there is in percentage of total phytate that is receptive to hydrolysis by phytase under GIT conditions. Diets fed in Trial 3 (Chapter 6) were formulated based on measuring the susceptible phytate content of the individual batches being fed using this method.

Aim

- To determine how much variation exists in susceptible phytate content between batches of feed ingredients fed to poultry in the UK.

3.5.2. Procedure

25 samples of wheat, 17 samples of soyabean meal (SBM), 16 samples of rapeseed meal (RSM), 10 samples of barley, 14 samples of maize and 10 samples of wheatfeed were collected from various feed manufacturers and mills from different geographical regions throughout the UK. The susceptible phytate content of each sample was analysed in triplicate. Details about the susceptible phytate

method are featured in Chapter 2 Section 2.5.11. Briefly, 50ml warmed acetate buffer (pH 4.5, 37°C) was added to 10g of feed sample which were then incubated at 37°C for 5 minutes. 2ml of sample was then transferred into a microfuge tube, centrifuged at 9,500 x g for 10 minutes and 0.5ml of the resulting supernatant was transferred into a fresh 1.5ml tubes and neutralised with 0.5ml 0.25M NaOH. The sample was then diluted 1:3 with ultra-pure water and phytic acid was measured using the Megazyme K-Phyt™ assay (described in Chapter 2 Section 2.5.10.). Susceptible phytate content was calculated according to the following equation:

$$\frac{\text{Mean phytic acid content of the sample from the susceptible phytate assay (g/100g)}}{\text{Mean total phytic acid content of sample from total phytic acid assay (g/100g)}}$$

3.5.3. Results

Table 3.8 and 3.9 show that both total and susceptible phytic acid content varied considerably between batches of ingredients. There was approximately 15% difference in percentage susceptible phytate content between different batches of wheat, approximately 10% difference between batches of SBM, RSM, barley and maize and approximately 7% difference between batches of wheatfeed. There were no correlations between total phytic acid content and phytate susceptibility in any ingredient.

Table 3.8. Total phytic acid content of feed ingredients fed to poultry in the UK

Ingredient	Total Phytic Acid (g/100g)	SEM	Range in Total Phytic Acid (g/100g)
Wheat	0.426	0.013	0.351-0.552
SBM	0.707	0.009	0.631-0.762
RSM	1.644	0.029	1.422-1.825
Barley	0.778	0.006	0.755-0.791
Maize	0.872	0.018	0.742-0.947
Wheatfeed	0.673	0.018	0.601-0.779

Table 3.9. Susceptible phytic acid content of feed ingredients fed to poultry in the UK

Ingredient	Free Phosphorus (g/100g)	SEM	Phytic Acid Phosphorus (g/100g)	SEM	Total Phosphorus (g/100g)	SEM	Phytic Acid (g/100g)	SEM	Susceptible Phytic Acid (%)	SEM (%)	Range in Susceptible Phytic Acid (%)
Wheat	0.043	0.002	0.069	0.002	0.111	0.003	0.243	0.009	57.45	0.85	49.51-63.45
SBM	0.029	0.002	0.096	0.002	0.124	0.003	0.339	0.007	48.55	0.69	43.62-53.34
RSM	0.095	0.004	0.235	0.005	0.330	0.007	0.833	0.017	50.81	0.76	46.84-56.16
Barley	0.092	0.005	0.122	0.002	0.214	0.004	0.434	0.006	55.82	0.87	52.68-61.45
Maize	0.050	0.004	0.141	0.003	0.192	0.005	0.501	0.011	57.44	0.63	51.82-60.78
Wheatfeed	0.104	0.003	0.134	0.004	0.238	0.003	0.475	0.015	70.55	0.82	66.34-73.36

3.5.4. Conclusion

It is imperative to understand how potentially limiting the phytate is when relying upon phytase supplementation to improve P utilisation, through measuring both the total phytate and relative solubility of the phytate when formulating with phytase. The importance of measuring the individual feed ingredient being fed is highlighted in this study by the large variation between individual batches of ingredients. Phytate susceptibility is dictated by conformation and configuration of the phytate molecule, pH of the environment and location of the phytate in the grain. Minerals and divalent cations bind to the weak acid phosphate groups of phytate, making that portion of the phytate resistant to phytase. Phytase susceptibility can therefore be improved by the presence of mineral chelators which bind to minerals and increase the formation of soluble mineral complexes, and by addition of H+ ions which compete with minerals for the binding sites of phytate (Maenz *et al.,* 1999). Consequently, measuring phytate susceptibility of feed ingredients means diets can be formulated, particularly with regards to presence of mineral chelators and impact on GIT pH, so they are as responsive to phytase as possible.

Measuring the solubility of phytate-mineral precipitates alone does not indicate degradation by phytase which is why in this study samples were exposed to conditions that mimicked the GIT and then phytic acid degradation was determined. This method enables the response by the bird to phytase supplementation to be predicted and hence improves the accuracy of phytase matrix values. The assay is also cheap, not labour intensive and takes only a little more time than the assay used for measuring total phytate.

3.6. Implications

Overall, these studies have greatly contributed to rigour and confidence with which the rest of the project was undertaken. The number of pilot studies required to clarify investigations in this project indicates lack of consensus over many methods used in poultry nutrition studies, despite the long

history of research in this field. The value placed on establishing method consensus is demonstrated by the level of scientific interest each publication generated.

CHAPTER 4: Trial 1: Effect of soyabean meal and rapeseed meal on the efficacy of phytase

4.1. Introduction

Failure to predict the amount of P released by phytase can have substantial negative implications on animal productivity and welfare. Evaluating the anti-nutritional effect of phytate is however difficult as any alterations to diets to reduce phytate concentration will result in other modifications in diet composition which may also interfere with animal performance. Phytase is supplemented into poultry diets to overcome this issue. The rationale for protein responses to supplemented phytase still remains largely speculative. This is because the response of phytase is a multifaceted area; it may be attributed to prevention of formation of protein-phytate complexes in the gut, the release of protein from protein-phytate complexes and reduction of the negative impact of phytate on the activities of proteolytic enzymes, such as pepsin and trypsin (Selle *et al.,* 2000). Solubility of phytate salts and protein, and their influence on degree of phytate-protein complex formation, potentially gives a more relevant indication of the anti-nutritional effects of phytate than observing total phytic acid (Ravindran *et al.,* 1999). It has been stated in numerous scientific papers that interactions between phytate and protein are ionic and hence pH dependent (Cowieson *et al.,* 2006; Selle *et al.,* 2012). As pH comes closer to isoelectric point, the charge on the protein becomes more neutral, and the phytate becomes more soluble. The presence of cations, such as calcium and zinc, mean that, in its soluble state, phytate binds with proteins, because the cations act as a link between the phytate and the negative charged protein carboxyl groups (Anderson, 1985). Protein solubility is thus reduced due to the formation of phytate-protein-mineral complexes, which has negative implications on the function of proteins, particularly those functions that are reliant on protein hydration.

Phytase presence is believed to have a positive effect on protein utilisation, although this has yet to be fully quantified to enable for dietary inclusion in the most cost effective way. The extent of the effect of phytase is dependent on both phytic acid concentration and protein digestibility. Phytase efficacy varies between feedstuffs, and there has been conflicting results reported regarding its

success, and physiological effects on bird GIT environment. In this study soyabean meal and rapeseed meal based diets with varying levels of phytase supplementation were used as tools to identify the effect of phytase on protein-phytate complexes and protein precipitation and determine the consequence of phytate-protein hydrolysis by phytase on bird health and gut pH.

Aim

- To compare the efficacy of phytase inclusion in soyabean and rapeseed meal based diets
- To examine the relationship between pH and phytate reactivity
- To observe the effect of 'superdosing' phytase on mineral and protein absorption and utilisation

4.2. Trial Procedure

192 Ross 308 broiler chicks, from a breeder flock age of 42 weeks, were housed from day old for 28 days in 80x80cm floor pens with wood shavings as litter substrate. Feed was provided *ad libitum* via troughs attached to the front of the pen, and water by bell drinkers. The lighting and temperature was controlled as described in Chapter 2, Section 2.2. Temperatures at 2 positions in the room, health records (including any mortalities) and any observations were recorded twice daily. Presence and absence of dirty vent was also recorded. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the School of Animal, Rural and Environmental Sciences Ethical Review Group.

Birds were fed one of six wheat based diets; three containing soyabean meal as the main protein source and three containing rapeseed meal. Diets were supplemented with 0 FTU/kg, 500 FTU/kg or 5000 FTU/kg of phytase (Table 4.1). Diets were formulated by ABVista, mixed in house using a ribbon mixer, one batch per diet, and fed as mash. The diets were tested for nitrogen content immediately after manufacture, and reanalysed after 5 days, before they were fed, to check protein content did not change over time. They were also analysed for crude energy, total calcium and phosphorus,

available phosphorus, dry matter, ash, supplemented and total phytase activity, total phytic acid, susceptible phytate-P and fat content using the methods described in Chapter 2 (Table 4.2). The diets contained titanium dioxide at a rate of 0.5% to act as an inert marker so digestibility could be measured. Diets were stored at room temperature. Each treatment was fed to 8 pens each containing 4 chicks; one pen was considered to be one replicate. Birds were fed their allocated diet from arrival, and feed troughs were kept horizontal and no more than half full to avoid excessive feed wastage.

Table 4.1. Composition of soyabean meal (SBM) and rapeseed meal (RSM) basal diet (g/kg)

	SBM Diet	RSM Diet
Wheat - Feed	605.90	605.20
Fishmeal 72 protein	30.00	30.00
Rapeseed Extruded		250.00
Soyabean meal 46	180.00	
Soya Hulls	70.00	
Soya oil	55.90	54.40
Salt	3.60	3.60
Valine	2.10	2.20
DL Methionine	4.10	2.90
Lysine HCl	5.00	6.50
Threonine	2.40	2.40
L-Tryptophan	0.10	0.20
Glycine	3.20	5.10
L-Arginine HCl	3.60	5.50
Isoleucine	1.90	2.80
Limestone	10.00	8.50
Dicalcium Phosphate	13.00	11.40
Coccidiostat	0.20	0.20
Vitamin premix*	4.00	4.00
Titanium Dioxide	5.00	5.00

* Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.2 mg; retinol, 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 µg; folic acid, 1.5 mg; and biotin 125 mg.

Table 4.2. Calculated and analysed content of diets

	SBM[1] 0 FTU/kg		SBM 500 FTU/kg		SBM 5000 FTU/kg		RSM[2] 0 FTU/kg		RSM 500 FTU/kg		RSM 5000 FTU/kg	
	Calculated	**Analysed**	**Calculated**	**Analysed**	**Calculated**	**Analysed**	**Calculated**	**Analysed**	**Calculated**	**Analysed**	**Calculated**	**Analysed**
GE Content (MJ/kg DM)		19.17		20.16		19.83		20.61		19.45		19.60
ME Content (MJ/kg)	12.81		12.81		12.81		12.81		12.81		12.81	
Total P Content (g/kg DM)	6.80	7.30	6.80	7.40	6.80	7.20	7.70	7.80	7.70	7.40	7.70	7.20
Total Ca Content (g/kg DM)	10.00	11.10	10.00	11.80	10.00	11.40	10.00	11.90	10.00	11.90	10.00	12.40
DM Content (g/kg)		871.13		867.12		873.46		870.16		852.09		885.87
Ash (g/kg)		6.26		6.30		6.27		6.25		6.22		6.27
Protein Content (g/kg DM)	201.10	241.62	201.10	236.24	201.10	238.20	198.60	233.37	198.60	236.15	198.60	224.60
Available Phosphorus (g/kg)	4.50	6.18	4.50	5.46	4.50	5.51	4.50	6.42	4.50	6.23	4.50	6.25
Supplemented Phytase Activity (FTU/kg)	0	<50	500	471	5000	4893	0	<50	500	613	5000	5343
Total Phytase Activity (FTU/kg)[3]		369		834		6632		493		967		6714
Phytic Acid Content (g/kg)	5.40	5.29	5.40	5.04	5.40	5.92	6.40	6.06	6.40	7.29	6.40	6.09
Susceptible Phytate-P (g/kg)[4]		4.08		3.12		2.63		5.17		4.18		3.64
Fat (g/kg)	70.90	70.72	70.90	70.68	70.90	77.40	87.30	87.05	87.30	87.11	87.30	87.09
Fibre (g/kg)	45.50		45.50		45.50		42.80		42.80		42.80	

[1] SBM: Soyabean Meal

[2] RSM: Rapeseed Meal

[3] Dietary cereal phytase activity and supplemented phytase activity combined, analysed by ISO 30024

[4] Phytate-P susceptible to hydrolysis by phytase, see Chapter 2 Section 2.5.11 for method of analysis

On arrival birds were individually weighed and allocated to a pen. Pen allocation was randomised across the room. Total pen weight and mean chick weight per pen was calculated, and diet allocation was arranged to ensure there was no significant difference in body weights by pen across diets. Each pen was individually fed from a pre-weighed labelled bag containing 2.5kg of appropriate diet per week. All birds in a pen were weighed on day 7, 14, 21 and 28. Feed intake was also measured on these days; feed remaining in the trough was poured into the individual feed bags for each pen and the bag was weighed. Feed conversion ratio of birds aged 0-14 days and 0-28 days was calculated based on feed intake divided by body weight gain, taking into account any mortalities. On day 25 foil lined excreta collection trays with wire mesh overlay were placed into each pen below the feeders, allowing for 3 days excreta collection. Excreta was dried and homogenised as described in Chapter 2, Section 2.4.3. Birds were euthanised by cervical dislocation on day 28.

4.3 Determined Parameters

4.3.1. Digesta sample collection and pH determination

Immediately post euthanasia, gastrointestinal pH was determined by inserting a spear tip piercing pH electrode (Sensorex, California, USA) directly into the gut lumen of the gizzard, duodenum, jejunum and ileum as soon as they had been excised, as described in Chapter 2, Section 2.5.1. Readings were repeated three times per section of gut per bird (making sure the probe did not touch the gut wall) and average pH was calculated. Immediately after measurement of gastrointestinal pH, digesta content of the gizzard, jejunum and ileum was then squeezed into individual pre-weighed pots per pen; the digesta from each bird within a pen was pooled. The pots were then weighed, stored at -20°C and then freeze-dried to determine digesta sample dry matter content, as described in Chapter 2, Section 2.5.2. Dry matter content of the diets was analysed by the oven-drying technique described in Chapter 2, Section 2.5.2.

4.3.2. Total and Soluble Calcium and Phosphorus Determination

For each pen, total and soluble Ca and P content was determined in the freeze-dried gizzard, jejunum and ileum digesta based on the average of 6 readings per sampling site per pen. Diets were also analysed for total and soluble Ca and P; 6 repeats per diet were carried out. Total Ca and P of the feed and freeze-dried digesta was determined by acid digestion with aqua regia, followed by filtration through Whatman #541 filter papers and analysis by Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) set at phosphorus wavelength 213.167 and calcium wavelength 317.933, as described in Chapter 2, Section 2.5.8. Soluble Ca and P content of the freeze-dried digesta, and soluble P content of the feed, was determined by adding ultra-pure water to the samples, shaking and centrifuging them, and then measuring Ca and P content of the supernatant on an ICP-OES set at phosphorus wavelength 213.167 and calcium wavelength 317.933, as described in Chapter 2, Section 2.5.8.1.

4.3.3. Protein and Soluble Protein Determination

Total protein content of the freeze-dried gizzard, jejunum and ileum digesta and feed was analysed by Kjeldahl analysis of nitrogen content. The percentage nitrogen value was multiplied by 6.25 to determine percentage crude protein, as described in Chapter 2, Section 2.5.9. Soluble protein content of the freeze-dried gizzard digesta samples was determined by adding 0.2% KOH to the samples, mixing and centrifuging them, and then measuring protein content of the supernatant using the Kjeldahl assay, as described in Chapter 2, Section 2.5.9.1.

4.3.4. Apparent Metabolisable Energy

Gross energy of the feed and excreta was determined by bomb calorimetry, as described in Chapter 2, Section 2.5.5. Diet digestibility was analysed by determination of titanium dioxide in the diet, excreta and jejunum and ileum digesta using a UV-Spectroscopy assay, as described in Chapter 2, Section 2.5.4.

4.3.5. Total and Susceptible Phytate

Total phytic acid content of the gizzard digesta samples and diet was analysed by the Megazyme K-Phyt™ assay, as described in Chapter 2, Section 2.5.10. Susceptible phytate content of the diet was analysed by adding warmed acetate buffer to the sample, incubating and centrifuging it, neutralising the supernatant with 0.25M NaOH, and subsequently using the Megazyme K-Phyt™ assay to analyse phytic acid content, as described in Chapter 2, Section 2.5.11.

4.3.6. Phytase Activity

Supplemented phytase activity of the diet and ileal digesta was determined using a Quantiplate™ Kit for Quantum phytase, as described in Chapter 2, Section 2.5.13. Total phytase activity of the diet and ileal digesta was determined by adding acetate buffer to the samples, incubating it at 37°C, adding a phytate substrate solution and molybdate/vanadate/nitric acid solution to the samples before centrifuging them and measuring the optical density at 415nm, as described in Chapter 2, Section 2.5.12.

4.3.7. Tibia and Femur Mineralisation and Ca and P content

Post euthanasia both the left and right legs (tibia and femur) were removed, put in labelled bags and stored at -20°C. The left tibia and femur of each bird were wrapped separately in labelled foil, autoclaved, defleshed, oven-dried and weighed into pre-weighed crucibles. They were then put into a muffle furnace until completely ashed, left to cool, and reweighed so mineral content could be determined, as described in Chapter 2, Section 2.5.3.

The ashed bone samples were acid-digested with aqua regia, filtered, diluted and analysed for Ca and P content by ICP-OES set at phosphorus wavelength 213.167 and calcium wavelength 317.933, as described in Chapter 2, Section 2.5.8.

4.3.8. Tibia and Femur Bone Strength

The right tibia and femur of each bird was used to determine bone strength. The tibias and femurs were defleshed of muscle and tissue by hand using a scalpel. The length and width of each bone was measured using callipers. A TA.XT plus texture analyser was set up with a 50kg load cell and 3 point-bend fixture, and test speed was set at 1mm/sec and trigger force at 7g (0.069N). The bone was placed on the fixture, and peak force to break it was recorded, as described in Chapter 2, Section 2.5.7.

4.3.9. Data Analysis

All data was analysed using the SPSS software version 21 for Windows (IBM Statistics, 2013). After KS testing to confirm normality, statistical analysis was carried out using Univariate analysis to determine interactions between the analysed factors, and one-way ANOVA to test the equality of the means. Treatment means were separated using Duncan post hoc test. In cases where the effect of phytase inclusion level read to be significant, a linear and quadratic contrast was conducted. Statistical significance was declared at $p<0.05$. Correlations were analysed by bivariate correlation using Pearson correlation.

4.4. Results

4.4.1. Bird Performance

Table 4.3. shows that there was a significant interaction between bird age and diet on feed intake (FI) and body weight gain (BWG); at bird age d14-21 and d21-28 FI and BWG were lower in birds fed RSM 0 FTU/kg phytase than those fed any other diet, but at bird age d0-7 and d7-14 FI and BWG was the lowest in birds fed RSM 500 FTU/kg phytase. At bird age d14-21 and d21-28 FI was significantly higher in birds fed SBM compared to those fed RSM, but this difference was not observed in the younger birds. There was no significant interaction between bird age and diet on the feed conversion ratio (FCR). Feed conversion was significantly higher at d0-7 and d7-14 compared to the other bird ages, and was significantly lower at bird age d21-28 than any other bird age.

Table 4.3. Effect of bird age and diet (soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase) on bird performance

Age (days)[a]	Diet	Individual FI (g)	Individual BWG (g)	FCR
0-7	SBM 0 FTU	105.69^{ab}	88.63^{ab}	1.21
	SBM 500 FTU	112.59^{a}	100.56^{a}	1.13
	SBM 5000 FTU	111.81^{a}	101.03^{a}	1.11
	RSM 0 FTU	95.97^{ab}	78.53^{b}	1.23
	RSM 500 FTU	91.94^{b}	77.13^{b}	1.20
	RSM 5000 FTU	103.75^{ab}	89.59^{ab}	1.17
	SEM	3.10	3.83	0.02
7-14	SBM 0 FTU	327.44^{ab}	268.13^{a}	1.22
	SBM 500 FTU	338.72^{a}	275.22^{a}	1.23
	SBM 5000 FTU	350.70^{a}	294.76^{a}	1.19
	RSM 0 FTU	278.09^{c}	221.93^{b}	1.25
	RSM 500 FTU	275.22^{c}	220.94^{b}	1.25
	RSM 5000 FTU	293.69^{bc}	238.34^{b}	1.23
	SEM	12.11	11.39	0.01
14-21	SBM 0 FTU	670.28^{a}	472.06^{a}	1.42
	SBM 500 FTU	695.19^{a}	490.03^{a}	1.42
	SBM 5000 FTU	722.90^{a}	510.47^{a}	1.42
	RSM 0 FTU	544.69^{b}	407.78^{b}	1.37
	RSM 500 FTU	553.72^{b}	369.53^{b}	1.50
	RSM 5000 FTU	575.53^{b}	409.56^{b}	1.40
	SEM	29.11	20.66	0.02
21-28	SBM 0 FTU	959.84^{a}	653.75^{ab}	1.46
	SBM 500 FTU	987.19^{a}	669.81^{ab}	1.48
	SBM 5000 FTU	1017.04^{a}	683.29^{a}	1.49
	RSM 0 FTU	826.28^{b}	537.31^{c}	1.64
	RSM 500 FTU	836.34^{b}	545.03^{c}	1.56
	RSM 5000 FTU	863.94^{b}	590.94^{bc}	1.49
	SEM	30.87	23.96	0.03
Age				
0-7		103.63	89.24	1.17^{c}
7-14		310.64	253.05	1.23^{c}
14-21		627.05	443.24	1.42^{b}
21-28		915.11	612.50	1.52^{a}
SEM		154.30	98.42	0.07
P-values				
Age		<0.001	<0.001	<0.001
Diet		<0.001	<0.001	0.306
Diet x Age		<0.001	0.012	0.896

[a,b] Means within the same column, within the same age group, with no common subscript differ significantly (P<0.05)

Table 4.4. shows that there were no significant interactions between protein source and phytase supplementation level on FI, BWG or FCR from d0-28. FI and BWG were significantly greater in birds fed SBM than those fed RSM. BWG was significantly greater in birds fed diets supplemented with 5000 FTU/kg than in birds fed diets supplemented with 0 FTU/kg or 500 FTU/kg phytase.

Table 4.4. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on bird performance from d0-d28

Diet	Individual FI (g)	Individual BWG (g)	FCR
SBM 0 FTU	2063.31	1482.57	1.39
SBM 500 FTU	2133.72	1535.63	1.39
SBM 5000 FTU	2202.51	1589.66	1.39
RSM 0 FTU	1745.02	1244.67	1.40
RSM 500 FTU	1757.20	1213.62	1.45
RSM 5000 FTU	1836.91	1328.41	1.38
SEM	74.91	58.96	0.01
Protein Source			
SBM	2133.12[a]	1535.91[a]	1.39
RSM	1779.72[b]	1262.20[b]	1.41
SEM	124.94	96.77	0.01
Phytase			
0 FTU	1904.14	1363.57[b]	1.39
500 FTU	1945.50	1374.62[b]	1.42
5000 FTU	2019.68	1458.99[a]	1.38
SEM	27.60	24.61	0.01
P-values			
Protein Source	<0.001	<0.001	0.294
Phytase	0.057	0.020	0.423
Protein Source x Phytase	0.696	0.410	0.494

[a,b] Means within the same column with no common subscript differ significantly (P<0.05)

4.4.2. Apparent Metabolisable Energy

Table 4.5. shows there were no significant interactions between protein source and phytase supplementation level on apparent metabolisable energy (AME). Phytase supplementation level had no significant effect on AME, but AME was significantly higher in birds fed SBM than those fed RSM.

Table 4.5. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on apparent metabolisable energy (AME) from d0-d28

Diet	AME (MJ/kg)
SBM 0 FTU	12.71
SBM 500 FTU	12.28
SBM 5000 FTU	13.52
RSM 0 FTU	11.86
RSM 500 FTU	11.78
RSM 5000 FTU	11.15
SEM	0.31
Protein Source	
SBM	12.84[a]
RSM	11.66[b]
SEM	0.42
P-values	
Protein Source	0.011
Phytase	0.840
Protein Source x Phytase	0.245

[a,b] Means within the same column with no common subscript differ significantly (P<0.05)

4.4.3. Gastrointestinal pH

Table 4.6. shows there were no significant interactions between, or effect of, protein source and phytase inclusion level on pH in any section of the tract measured.

Table 4.6. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on gastrointestinal pH at d28

Diet	Gizzard	Duodenum	Jejunum	Ileum
SBM 0 FTU	2.85	6.00	6.03	7.03
SBM 500 FTU	2.99	6.05	6.14	6.94
SBM 5000 FTU	2.88	6.00	6.08	6.97
RSM 0 FTU	2.43	6.02	5.91	7.06
RSM 500 FTU	2.45	6.16	6.00	6.89
RSM 5000 FTU	2.34	6.11	5.99	7.01
SEM	0.04	0.02	0.03	0.02
P-values				
Protein Source	0.052	0.059	0.074	0.887
Phytase	0.734	0.184	0.060	0.514
Protein Source x Phytase	0.872	0.604	0.984	0.546

[a,b] Means within the same column with no common subscript differ significantly (P<0.05)

4.4.4. Total and Soluble Ca and P

Table 4.7. shows there were no significant interactions between protein source and phytase inclusion level on Ca or P concentration or solubility in the gizzard. Protein source and phytase inclusion level had no significant effect on total Ca and P concentration or Ca solubility in the gizzard. Both protein source and phytase inclusion level however had a significant effect on P solubility in the gizzard; as phytase level increased P solubility increased, and P solubility was greater in birds fed SBM than those fed RSM.

Table 4.7. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on Ca and P solubility in the gizzard

	Total (g/kg)		Soluble (g/kg)		Solubility Coefficient	
Diet	Ca	P	Ca	P	Ca	P
SBM 0 FTU	8.80	5.07	4.66	3.50	0.42	0.48
SBM 500 FTU	8.47	5.16	4.21	3.63	0.36	0.49
SBM 5000 FTU	9.46	5.30	4.90	3.94	0.43	0.55
RSM 0 FTU	8.08	5.24	6.55	3.04	0.55	0.39
RSM 500 FTU	7.76	5.54	4.76	3.11	0.40	0.42
RSM 5000 FTU	9.04	5.92	6.94	3.24	0.56	0.45
SEM	0.23	0.12	0.43	0.13	0.03	0.02
Protein Source						
SBM	8.91	5.18	4.52	3.69[a]	0.40	0.51[a]
RSM	8.29	5.57	6.08	3.13[b]	0.50	0.42[b]
SEM	0.22	0.14	0.55	0.20	0.02	0.02
Phytase						
0 FTU	8.28	5.16	5.61	3.27[c]	0.49	0.43[c]
500 FTU	8.28	5.35	4.49	3.37[b]	0.38	0.46[b]
5000 FTU	9.25	5.61	5.92	3.59[a]	0.49	0.50[a]
SEM	0.26	0.11	0.22	0.08	0.02	0.01
P-values						
Protein Source	0.738	0.111	0.307	0.047	0.014	0.017
Phytase	0.129	0.522	0.373	0.044	0.500	0.033
Protein Source x Phytase	0.969	0.188	0.373	0.268	0.992	0.512

[a-c] Means within the same column with no common subscript differ significantly (P<0.05)

Table 4.8. shows there were no significant interactions between protein source and phytase inclusion level on Ca or P concentration or solubility in the jejunum. Phytase inclusion level had a significant effect on total Ca and P concentration and Ca and P solubility; as phytase level increased total and soluble Ca and P presence increased in a dose dependent manner.

Table 4.8. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on Ca and P solubility in the jejunum

	Total (g/kg)		Soluble (g/kg)		Solubility Coefficient	
Diet	Ca	P	Ca	P	Ca	P
SBM 0 FTU	6.20	3.17	2.33	1.75	0.21	0.24
SBM 500 FTU	5.54	3.33	2.01	1.85	0.17	0.25
SBM 5000 FTU	6.76	3.74	2.74	2.09	0.24	0.29
RSM 0 FTU	7.32	4.31	2.62	1.72	0.22	0.22
RSM 500 FTU	6.97	4.63	2.26	1.70	0.18	0.23
RSM 5000 FTU	7.92	4.72	3.10	1.85	0.25	0.26
SEM	0.31	0.25	0.15	0.05	0.06	0.01
Protein Source						
SBM	6.17^{c}	3.41^{b}	2.36^{b}	1.90	0.21	0.26^{a}
RSM	7.40^{a}	4.55^{a}	2.66^{a}	1.76	0.22	0.24^{b}
SEM	0.44	0.40	0.11	0.05	0.01	0.01
Phytase						
0 FTU	6.76^{b}	3.74^{c}	2.48^{b}	1.74^{b}	0.22^{b}	0.23^{b}
500 FTU	6.26^{c}	3.98^{b}	2.14^{c}	1.78^{b}	0.18^{c}	0.24^{b}
5000 FTU	7.34^{a}	4.23^{a}	2.92^{a}	1.97^{a}	0.25^{a}	0.27^{a}
SEM	0.26	0.12	0.19	0.06	0.02	0.01
P-values						
Protein Source	0.004	<0.001	<0.001	0.105	0.101	<0.001
Phytase	<0.001	0.048	0.008	0.002	<0.001	<0.001
Protein Source x Phytase	0.076	0.719	0.187	0.887	0.181	0.338

$^{a-d}$ Means within the same column with no common subscript differ significantly (P<0.05)

Table 4.9. shows there was a significant interaction between protein source and phytase inclusion level of Ca and P solubility coefficients and soluble P presence in the ileum. The Ca solubility coefficient was significantly lower in birds fed diets supplemented with 500 FTU/kg compared to the diets supplemented with 0 or 5000 FTU/kg phytase, and significantly higher in birds fed diets supplemented with 5000 FTU/kg compared to those fed diets supplemented with 0 or 500 FTU/kg. Soluble P presence and P solubility coefficient increased significantly in both diets as phytase supplementation level increased in a dose dependent manner. P solubility in birds fed SBM diets supplemented with 5000 FTU/kg was not significantly different to P solubility in birds fed RSM diets supplemented with 500 FTU/kg, but was significantly higher in birds fed RSM supplemented with 5000 FTU/kg than those fed any of the SBM based diets, or RSM based diet with 500 FTU/kg.

Table 4.9. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on Ca and P solubility in the ileum

	Total (g/kg)		Soluble (g/kg)		Solubility Coefficient	
Diet	Ca	P	Ca	P	Ca	P
SBM 0 FTU	4.73	1.81	1.85	0.58^{d}	0.16^{b}	0.08^{d}
SBM 500 FTU	4.39	2.23	1.51	0.98^{c}	0.13^{c}	0.13^{c}
SBM 5000 FTU	4.95	2.31	2.18	1.15^{b}	0.19^{a}	0.16^{b}
RSM 0 FTU	5.08	2.46	1.99	0.87^{c}	0.17^{b}	0.11^{c}
RSM 500 FTU	4.89	2.60	1.60	1.18^{b}	0.13^{c}	0.16^{b}
RSM 5000 FTU	5.28	2.71	2.38	1.29^{a}	0.19^{a}	0.18^{a}
SEM	0.11	0.12	0.12	0.10	0.01	0.01
Protein Source						
SBM	4.69^{c}	2.12^{b}	1.85^{c}	0.90	0.16	0.12
RSM	5.08^{a}	2.59^{a}	1.99^{b}	1.11	0.16	0.15
SEM	0.14	0.17	0.05	0.07	0.00	0.01
Phytase						
0 FTU	4.91^{b}	2.14^{c}	1.92^{b}	0.73	0.17	0.10
500 FTU	4.64^{c}	2.42^{b}	1.56^{c}	1.08	0.13	0.15
5000 FTU	5.12^{a}	2.51^{a}	2.28^{a}	1.22	0.19	0.17
SEM	0.11	0.09	0.17	0.12	0.01	0.02
P-values						
Protein Source	0.035	<0.001	0.038	<0.001	0.567	<0.001
Phytase	0.038	0.033	<0.001	<0.001	<0.001	0.001
Protein Source x Phytase	0.451	0.173	0.316	0.001	0.029	0.013

$^{a-d}$ Means within the same column with no common subscript differ significantly (P<0.05)

Table 4.10. shows there was a significant interaction between protein source and phytase inclusion level on the amount of Ca absorbed by the jejunum and ileum; in birds fed the RSM based diets there was a significant increase in Ca absorbed between the control diet and diet supplemented with 500 FTU/kg phytase, but this was not the case in birds fed the SBM based diets. Also, the amount of Ca absorbed was higher in birds fed the SBM based diets than the RSM based diets at phytase level 0 FTU/kg and 500 FTU/kg, but at phytase level 5000 FTU/kg Ca absorption was higher in birds fed the RSM based diets than SBM based diets. Protein source and phytase inclusion level had a significant effect on P absorbed by the jejunum and ileum; more P was absorbed by birds fed the RSM based diets than those fed the SBM based diets, and as phytase level increased P absorption significantly increased. Phytase inclusion level also had a significant effect on the amount of Ca and P excreted; as phytase level increased the amount of Ca and P excreted reduced significantly.

Table 4.10. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on absorption of Ca and P by determination at the jejunum and ileum and in the excreta

	Jejunum (g/kg diet)		Ileum (g/kg diet)		Excreta (g/kg diet)	
Diet	Ca	P	Ca	P	Ca	P
SBM 0 FTU	8.03[b]	2.71	9.27[b]	4.68	2.53	1.57
SBM 500 FTU	9.00[b]	3.77	10.24[b]	5.48	2.03	0.96
SBM 5000 FTU	10.18[a]	4.04	11.08[a]	6.32	1.42	0.75
RSM 0 FTU	6.09[d]	4.89	6.15[d]	4.02	2.56	0.89
RSM 500 FTU	8.11[c]	5.39	8.71[c]	4.95	1.84	0.57
RSM 5000 FTU	10.57[a]	6.13	11.12[a]	5.69	1.62	0.50
SEM	0.61	0.46	0.70	0.30	0.18	0.14
Protein Source						
SBM	9.07	3.51[c]	10.19	5.49[a]	1.99	1.09
RSM	8.26	5.47[a]	8.66	4.89[b]	2.01	0.65
SEM	0.38	0.69	0.54	0.21	0.00	0.16
Phytase						
0 FTU	7.06	3.80[c]	7.71	4.35[c]	2.54[a]	1.23[a]
500 FTU	8.55	4.58[b]	9.47	5.21[b]	1.93[b]	0.76[b]
5000 FTU	10.38	5.09[a]	11.10	6.00[a]	1.52[c]	0.66[c]
SEM	0.78	0.30	0.80	0.39	0.24	0.14
P-values						
Protein Source	<0.001	<0.001	<0.001	<0.001	0.747	0.735
Phytase	<0.001	<0.001	<0.001	<0.001	0.033	<0.001
Protein Source x Phytase	0.043	0.063	<0.001	0.199	0.943	0.975

[a-d] Means within the same column with no common subscript differ significantly (P<0.05)

4.4.5. Ileal Ca and P digestibility

Table 4.11. shows there were no significant interactions between protein source and phytase inclusion level on apparent ileal digestibility of Ca and P. Ileal Ca digestibility was significantly greater in birds fed diets supplemented with 5000 FTU/kg than those with 500 FTU/kg phytase, and P digestibility increased significantly as phytase inclusion level increased in a dose dependent manner. The apparent ileal Ca and P digestibility values presented in Table 4.11. are higher than would be expected based on comparison with similar published studies.

Table 4.11. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on apparent ileal Ca and P digestibility

	Apparent ileal digestibility coefficient	
Diet	Ca	P
SBM 0 FTU	0.82	0.84
SBM 500 FTU	0.78	0.86
SBM 5000 FTU	0.84	0.90
RSM 0 FTU	0.81	0.83
RSM 500 FTU	0.80	0.85
RSM 5000 FTU	0.81	0.85
SEM	0.01	0.01
Phytase		
0 FTU	0.82^{ab}	0.83^{c}
500 FTU	0.79^{b}	0.86^{b}
5000 FTU	0.83^{a}	0.88^{a}
SEM	0.01	0.01
P-values		
Protein Source	0.612	0.058
Phytase	0.048	0.017
Protein Source x Phytase	0.340	0.502

$^{a-c}$ Means within the same column with no common subscript differ significantly ($P<0.05$).

4.4.7. Total and Soluble Protein

Table 4.12. shows there were no significant interactions between protein source and phytase inclusion level on protein solubility in any section of the gastrointestinal tract. Total and soluble protein presence in the jejunum and ileum was higher in birds fed the SBM based diets than those fed the RSM based diets. As phytase inclusion level increased soluble protein presence measured in the jejunum and ileum, and total protein in the ileum increased significantly in a dose dependent manner.

Table 4.12. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on protein solubility in the gizzard, jejunum and ileum

	Gizzard		Jejunum		Ileum	
	Total (g/kg)	Soluble (g/kg)	Total (g/kg)	Soluble (g/kg)	Total (g/kg)	Soluble (g/kg)
SBM 0 FTU	171.01	119.20	93.62	84.16	70.09	63.00
SBM 500 FTU	188.62	131.59	133.48	101.42	102.75	86.64
SBM 5000 FTU	191.61	135.87	138.77	123.52	106.35	93.52
RSM 0 FTU	165.17	102.71	74.98	68.65	43.94	39.41
RSM 500 FTU	175.38	121.75	80.25	74.55	51.29	42.13
RSM 5000 FTU	183.59	123.33	92.82	87.53	70.35	62.53
SEM	3.87	4.30	10.14	7.43	9.61	8.28
Protein Source						
SBM	183.75	128.88	121.96[a]	103.03[a]	93.06[a]	81.06[a]
RSM	174.71	115.93	82.68[b]	76.91[c]	55.19[b]	48.03[b]
SEM	3.19	4.58	13.88	9.24	13.39	11.68
Phytase						
0 FTU	168.09	110.95	84.30	76.41[c]	57.02[c]	51.21[b]
500 FTU	182.00	126.67	106.86	94.48[b]	77.02[b]	74.59[a]
5000 FTU	187.60	129.60	115.80	105.53[a]	88.35[a]	78.03[a]
SEM	2.61	4.73	7.65	6.93	6.53	6.88
P-values						
Protein Source	0.467	0.179	<0.001	<0.001	<0.001	<0.001
Phytase	0.426	0.800	0.054	<0.001	0.028	<0.001
Protein Source x Phytase	0.969	0.915	0.310	0.161	0.394	0.177

[a-c] Means within the same column with no common subscript differ significantly (P<0.05)

Table 4.13. shows there were no significant interactions between protein source and phytase inclusion level on absorption of protein by the jejunum and ileum. Phytase inclusion level had no significant effect on protein absorption, but significantly more protein was absorbed by birds fed the SBM based diets than those fed the RSM based diets.

Table 4.13. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on absorption of protein by determination at the jejunum and ileum

	Jejunum (g/kg diet)	Ileum (g/kg diet)
SBM 0 FTU	138.40	179.78
SBM 500 FTU	169.11	181.50
SBM 5000 FTU	172.23	184.05
RSM 0 FTU	103.06	162.74
RSM 500 FTU	108.38	172.51
RSM 5000 FTU	114.27	178.90
SEM	11.45	2.90
Protein Source		
SBM	159.92[a]	181.78[a]
RSM	108.57[b]	171.39[b]
SEM	18.15	3.67
Phytase		
0 FTU	120.73	171.26
500 FTU	138.75	177.01
5000 FTU	143.25	181.48
SEM	5.62	2.41
P-values		
Protein Source	<0.001	0.018
Phytase	0.138	0.102
Protein Source x Phytase	0.567	0.857

[a-c] Means within the same column with no common subscript differ significantly (P<0.05)

4.4.8. Digesta and Excreta water content

Table 4.14. shows there were no significant interactions between protein source and phytase inclusion level on the dry matter content of the gizzard, jejunum or ileum digesta. There was a significant interaction between protein source and phytase inclusion level on excreta dry matter content; excreta dry matter content of birds fed the SBM based diets supplemented with 500 FTU/kg was not significantly different from birds fed the RSM based diets supplemented with either 500 or 5000 FTU/kg phytase. The dry matter content of the ileal digesta from birds fed the SBM based diets was significantly lower than that of birds fed the RSM based diets.

Table 4.14. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on dry matter content of digesta and excreta

	Gizzard (g/kg)	Jejunum (g/kg)	Ileum (g/kg)	Excreta (g/kg)
SBM 0 FTU	329.99	233.56	169.68	311.75[d]
SBM 500 FTU	339.35	228.85	144.98	383.85[b]
SBM 5000 FTU	317.88	225.32	154.60	399.49[a]
RSM 0 FTU	323.25	257.24	172.82	334.59[c]
RSM 500 FTU	334.72	255.68	185.64	373.95[b]
RSM 5000 FTU	373.47	247.84	188.91	378.18[b]
SEM	7.35	5.20	6.39	12.41
Protein Source				
SBM	329.07	229.24	156.42[b]	365.03
RSM	343.81	253.59	182.46[a]	362.24
SEM	3.01	4.97	5.31	0.57
Phytase				
0 FTU	326.62	245.40	171.25	323.17
500 FTU	337.04	242.27	165.31	378.90
5000 FTU	345.68	236.58	171.76	388.84
SEM	3.18	1.49	1.19	11.80
P-values				
Protein Source	0.818	0.178	0.019	0.900
Phytase	0.740	0.919	0.862	0.261
Protein Source x Phytase	0.909	0.995	0.320	0.031

[a-c] Means within the same column with no common subscript differ significantly ($P<0.05$)

4.4.9. Gizzard Phytic Acid

Table 4.15. shows there was no significant interaction between protein source and phytase inclusion level on the amount of phytic acid hydrolysed in the gizzard. Phytase inclusion level had a significant effect on phytic acid hydrolysis; as phytase level increased phytic acid hydrolysis increased in a dose dependent manner.

Table 4.15. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on amount of phytic acid hydrolysed in the gizzard

	Phytic Acid Hydrolysed (g/kg diet)
SBM 0 FTU	2.79
SBM 500 FTU	2.86
SBM 5000 FTU	4.58
RSM 0 FTU	3.55
RSM 500 FTU	4.69
RSM 5000 FTU	4.12
SEM	0.31
Protein Source	
SBM	3.41
RSM	4.12
SEM	0.25
Phytase	
0 FTU	3.17[c]
500 FTU	3.78[b]
5000 FTU	4.35[a]
SEM	0.28
P-values	
Protein Source	0.815
Phytase	0.003
Protein Source x Phytase	0.107

[a-c] Means within the same column with no common subscript differ significantly (P<0.05)

4.4.10. Ileal Phytase Activity

Table 4.16. shows there were no significant interactions between protein source and phytase inclusion level on supplemented phytase and total phytase activity determined in the ileum. Total phytase is defined as the combined phytase activity from the exogenous supplemented phytase and endogenous phytase from intestinal mucosa and bacteria and from dietary cereals. Phytase inclusion level had a significant effect on both supplemented and total phytase activity; as phytase inclusion level increased phytase activity increased in a dose dependent manner. Total phytase activity was significantly higher in birds fed the RSM based diets than those fed the SBM based diets.

Table 4.16. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on supplemented and total phytase activity in the ileum

Diet	Supplemented Phytase (FTU/kg)	Total Phytase (FTU/kg)
SBM 0 FTU	4.20	58.03
SBM 500 FTU	354.81	482.43
SBM 5000 FTU	1343.79	3624.78
RSM 0 FTU	4.08	76.21
RSM 500 FTU	393.17	645.38
RSM 5000 FTU	1348.78	3765.85
SEM	231.13	656.19
Protein Source		
SBM	567.60	1388.41[c]
RSM	582.01	1495.81[b]
SEM	5.09	37.97
Phytase		
0 FTU	4.14[c]	67.12[e]
500 FTU	373.99[b]	563.90[d]
5000 FTU	1346.29[a]	3695.32[a]
SEM	326.80	927.29
P-values		
Protein Source	0.439	<0.001
Phytase	<0.001	0.003
Protein Source x Phytase	0.655	0.505

[a-e] Means within the same column with no common subscript differ significantly (P<0.05)

4.4.11. Tibia and Femur Mineralisation

Table 4.17. shows there was a significant interaction between protein source and phytase activity on femur mineralisation; in birds fed the RSM based diets there was a significant increase in femur ash content between the control diet and diet supplemented with 500 FTU/kg, but this was not seen in birds fed the SBM based diets. Phytase inclusion level had a significant effect on tibia ash content; as phytase inclusion level increased ash content increased in a dose dependent manner.

Table 4.17. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets supplemented with 0, 500 or 5000 FTU/kg phytase on tibia and femur mineralisation

Diet	Tibia Ash (%)	Femur Ash (%)
SBM 0 FTU	45.44	42.40[bc]
SBM 500 FTU	46.74	44.77[b]
SBM 5000 FTU	49.32	47.06[a]
RSM 0 FTU	43.42	41.65[c]
RSM 500 FTU	47.46	44.71[b]
RSM 5000 FTU	50.18	47.05[a]
SEM	0.93	0.84
Protein Source		
SBM	47.17	44.74
RSM	47.02	44.47
SEM	0.05	0.10
Phytase		
0 FTU	44.43[c]	42.03
500 FTU	47.10[b]	44.74
5000 FTU	49.75[a]	47.06
SEM	1.25	1.19
P-values		
Protein Source	0.224	0.544
Phytase	<0.001	<0.001
Protein Source x Phytase	0.676	<0.001

[a-e] Means within the same column with no common subscript differ significantly (P<0.05)

3.4.12. Tibia and Femur Ca and P content

Table 4.18. shows there were no significant interactions between protein source and phytase inclusion level on tibia and femur Ca and P content. Protein source had a significant effect on tibia Ca content; there was more Ca present in the tibias of birds fed the RSM based diets than those fed the SBM based diets. Phytase inclusion level had a significant effect on tibia Ca content; there was significantly more Ca in the tibias of birds fed diets supplemented with 5000 FTU/kg phytase compared to those fed the control diet, but not between the diets supplemented with 500 FTU/kg and diets supplemented with either 0 or 5000 FTU/kg phytase. Phytase inclusion level also had a significant effect on Ca content on the femur and P content of both the tibia and femur; as phytase level increased Ca and P content increased in a dose dependent manner.

Table 4.18. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets, supplemented with 0, 500 or 5000 FTU/kg phytase on tibia and femur Ca and P

	Tibia (% of tibia ash)		Femur (% of femur ash)	
Diet	Ca	P	Ca	P
SBM 0 FTU	30.39	12.96	30.59	14.56
SBM 500 FTU	31.12	13.87	34.06	15.28
SBM 5000 FTU	34.09	15.38	35.91	16.94
RSM 0 FTU	33.27	12.84	31.57	16.61
RSM 500 FTU	34.60	14.39	35.90	17.70
RSM 5000 FTU	40.88	15.06	37.00	18.85
SEM	1.39	0.39	0.24	0.14
Protein Source				
SBM	31.87^{b}	14.07	33.52	15.59
RSM	36.25^{a}	14.10	34.83	17.72
SEM	1.55	0.01	0.46	0.75
Phytase				
0 FTU	31.83^{b}	12.90^{c}	31.08^{c}	15.59^{c}
500 FTU	32.86^{ab}	14.13^{b}	34.98^{b}	16.49^{b}
5000 FTU	37.49^{a}	15.22^{a}	36.46^{a}	17.89^{a}
SEM	1.42	0.55	1.31	0.55
P-values				
Protein Source	0.023	0.957	0.345	0.092
Phytase	0.039	0.002	0.007	<0.001
Protein Source x Phytase	0.654	0.764	0.961	0.841

$^{a-c}$ Means within the same column with no common subscript differ significantly (P<0.05)

4.4.12. Tibia and Femur Strength

Table 4.19. shows there was no significant interaction between protein source and phytase inclusion level on tibia length, width, weight or strength. Phytase inclusion level had a significant effect on bone weight and strength; as phytase level increased tibia strength increased in a dose dependent manner, and tibia weight was significantly higher in birds fed diets supplemented with 5000 FTU/kg than in birds fed diets supplemented with either 0 or 5000 FTU/kg phytase. Protein source had a significant effect on tibia length, width, weight and strength; the tibias were longer and stronger in birds fed the SBM based diets compared to those fed the RSM based diets, but were wider and heavier in birds fed the RSM based diets.

Table 4.19. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets, supplemented with 0, 500 or 5000 FTU/kg phytase on tibia length, width, weight and bone breaking strength

Diet	Length (mm)	Width (mm)	Weight (g)	Strength (N)
SBM 0 FTU	88.79	6.46	12.06	298.66
SBM 500 FTU	89.44	6.45	11.68	345.05
SBM 5000 FTU	91.03	6.61	12.98	373.91
RSM 0 FTU	86.81	6.74	13.29	257.88
RSM 500 FTU	85.23	7.04	13.62	270.24
RSM 5000 FTU	84.91	7.20	14.64	298.97
SEM	0.91	0.12	0.40	16.52
Protein Source				
SBM	89.75[a]	6.51[b]	12.24[b]	339.21[a]
RSM	85.65[b]	6.99[a]	13.85[a]	275.70[c]
SEM	1.45	0.17	0.57	22.45
Phytase				
0 FTU	87.80	6.60	12.68[b]	278.27[c]
500 FTU	87.34	6.75	12.65[b]	307.65[b]
5000 FTU	87.97	6.91	13.81[a]	336.44[a]
SEM	0.15	0.07	0.31	13.71
P-values				
Protein Source	<0.001	0.004	<0.001	<0.001
Phytase	0.897	0.163	0.023	0.001
Protein Source x Phytase	0.348	0.764	0.735	0.320

[a-c] Means within the same column with no common subscript differ significantly (P<0.05)

Table 4.20. shows that there were no significant interactions between protein source and phytase inclusion level on femur length, width, weight or strength. Phytase inclusion level had a significant effect on femur strength; femur strength was greater in birds fed diets supplemented with 5000 FTU/kg phytase compared to those fed the control diet, but there were no significant differences between birds fed the diets supplemented with 500 FTU/kg and the diets with either 0 or 5000 FTU/kg phytase. Protein source had a significant effect on femur length, width, weight and strength; femur length, width and weight was greater in birds fed the RSM based diets than those fed the SBM based diets, but femur strength was greater in birds fed the SBM based diets.

Table 4.20. Effect of soyabean meal (SBM) and rapeseed meal (RSM) based diets, supplemented with 0, 500 or 5000 FTU/kg phytase on femur length, width, weight and strength

Diet	Length (mm)	Width (mm)	Weight (g)	Strength (N)
SBM 0 FTU	60.81	7.43	8.53	237.11
SBM 500 FTU	60.00	7.37	8.51	248.21
SBM 5000 FTU	61.75	7.54	9.17	274.46
RSM 0 FTU	62.47	7.75	9.49	205.36
RSM 500 FTU	63.23	7.86	9.49	215.36
RSM 5000 FTU	63.51	7.90	9.32	230.63
SEM	0.51	0.08	0.17	9.16
Protein Source				
SBM	60.85[b]	7.45[b]	8.74[b]	253.26[a]
RSM	63.07[a]	7.84[a]	9.43[a]	217.11[b]
SEM	0.78	0.14	0.25	12.78
Phytase				
0 FTU	61.64	7.59	9.01	221.23[b]
500 FTU	61.62	7.62	9.00	231.79[ab]
5000 FTU	62.63	7.72	9.25	252.54[a]
SEM	0.27	0.03	0.07	7.51
P-values				
Protein Source	<0.001	0.003	0.004	<0.001
Phytase	0.172	0.657	0.611	0.023
Protein Source x Phytase	0.353	0.843	0.240	0.781

[a-c] Means within the same column with no common subscript differ significantly (P<0.05)

4.5. Discussion

Insoluble phytate-mineral precipitates and soluble mineral-phytate complexes may be resistant to hydrolysis by phytase, which is consistent with incomplete hydrolysis of phytate-P and negative effects on mineral digestibility. In this thesis 'susceptibility' is defined as the availability and reactivity of the phytate to phytase effects and is a concept explored throughout. The 'susceptibility' of the phytate in a diet is dependent on the ingredients used and the solubility of the phytate, which is influenced by gastrointestinal pH and mineral and protein presence.

4.5.1. Performance

Body weight gain was greater in birds fed the SBM based diets compared to those fed the RSM based diets because feed intake was lower in the latter. This is likely to be due to fundamental nutritional differences between the diets due to their differing ingredient combinations. It is widely acknowledged that reducing dietary phosphorus availability reduces feed intake (Beiki *et al.,* 2013; Martins *et al.,* 2013), suggesting the higher total phytate levels in the RSM based diets may have partly

contributed towards this reduced feed intake. RSM also has a higher presence of other antinutritional factors compared to SBM, such as glucosinolates (Mawson *et al.*, 1993) and tannins (Yapar and Clandinin, 1972), which form complexes with proteins. These protein complexes are precipitated which results in inhibited trypsin and amylase activity, damage to the gut wall and ultimately reduced feed intake. The trial diets were formulated to contain adequate P so it is probable that the better feed conversion observed in birds aged 0-7 days when the diets were supplemented with phytase may be because at this age the birds were more vulnerable to the anti-nutritive effects of phytate and hence responded more to the effects of phytase, as opposed to an increase in P availability. Improved amino acid digestion could also potentially have contributed to improved growth. Levels of phytase from intestinal bacteria and mucosa and from dietary cereals are very low in young birds and increase with age (Sebastian *et al.* 1998). The addition of supplemental phytase enables access to amino acids from protein-phytate complexes that would otherwise have remained complexed. In the older birds however, the effect of exogenous phytase on feed conversion is possibly less pronounced because intestinal and cereal phytase levels are higher in older birds.

4.5.2. Apparent Metabolisable Energy

Energy requirements for maintenance of the bird are increased by the anti-nutritional effects of phytate on both endogenous enzyme and protein secretion and the digestibility of minerals and amino acids. The calculated metabolisable and analysed crude energy of the SBM based and RSM based diets were similar, and the SBM based diets had a higher crude fibre content, yet AME was significantly higher in birds fed the SBM based diets than those fed the RSM based diets. This may be due to a number of reasons but may be partly because the RSM based diets had higher total phytate levels which may have caused increased HCl and pepsin secretion.

4.5.3. Gastrointestinal pH

Gizzard pH was more alkaline in birds fed the SBM based diets compared to those fed the RSM based diets. This may be because RSM contains more P in the monobasic form, as opposed to the dibasic

form, than SBM; P in the monobasic form is an extremely strong acidogenic anion. RSM also contains more phytate than SBM and phytate has an acidogenic effect. Therefore it is likely that in birds fed the RSM based diets there was increased secretion of bicarbonate ions into the gizzard in response to the increased acidity caused by the presence of phytate and monobasic P.

Gizzard pH was sufficiently low in all diets to allow phytic acid hydrolysis to occur, evidenced by the lack of correlation between gizzard pH and phytic acid hydrolysis in the gizzard ($r=0.074$, $p=0.086$). Phytate-mineral complexes are most soluble at pH less than 3 and least soluble at pH 4-7. Precipitated phytate-mineral complexes that have not been hydrolysed in the gizzard are not accessible for breakdown or absorption further down the intestine. Therefore, the lack of protein source effect on pH observed further along the gastrointestinal tract is probably because phytase activity is diminished upon reaching the posterior jejunum, so no further degradation of Ca-phytate complexes occurs.

Duodenal pH is dictated by secretions from earlier sections of the GIT; upon entering the duodenum HCl from the proventriculus and peptides from initial HCl denaturation of proteins in the gizzard are promptly neutralised by bicarbonate released from the centroacinar cells and intercalated ducts in the pancreas (Kadhim *et al.,* 2010). Neither protein source or phytase inclusion level had an effect on duodenum pH because digesta is neutralised accordingly to the optimum pH as it enters the duodenum.

4.5.4. Mineral Solubility and Absorption

The analysed P values in the SBM based diets were noticeably higher than the formulated values. Ca solubility in the gizzard and jejunum was lower in the diets supplemented with 500 FTU/kg phytase compared to the control diet and diet supplemented with 5000 FTU/kg. This effect was greater in the gastric phase than SI phase, and may be associated with the molar Ca concentration of the diets and the Ca to P ratio. Also, increased production of inert Ca-phytate complexes, particularly due to non-parallel release of Ca and P from phytate, may have promoted free phosphate or phytate precipitation of Ca. At low phytase doses more Ca is released than P, but when high doses of phytase are supplemented more P than Ca is released, as there is near complete phytate destruction. This results

in a balanced digestible Ca to P ratio because the amount of P released from phytate is balanced with the amount of Ca available for absorption.

The phytase used in this study starts phytate degradation at the 6-phosphate position of the phytate molecule and continues round the inositol ring, hydrolysing each of the inositol phosphates in numerical order. At 5000 FTU/kg, the enzyme was present at a sufficiently high concentration to complete phosphate hydrolysis of each molecule, leaving a phytate free diet and increasing free phosphate to a ratio above that of the free Ca, increasing mineral solubility and absorption. In the control diets the majority of the Ca may have been complexed to phytate. At 500 FTU/kg however there may have been only partial hydrolysis of phytate, suggesting that at this phytase supplementation level there may have been a high presence of IP_4 and IP_3, and consequently lower concentration of IP_6 for the phytase to act on, resulting in only partial release of Ca. The ability of IP esters to aggregate decreases from IP_6 to IP_5, and becomes negligible at IP_4-IP_1 (Yu *et al*., 2012), and phytase will only work on IP_4 when levels are high. It is therefore possible that Ca released from the diets supplemented with 500 FTU/kg promoted Ca-phosphate and Ca-phytate precipitation, hence why ileal digestibility of Ca was lower at this phytase level. Additionally, phytases tend to have a 1:1 relationship between Ca and P, but it may be that when 500 FTU/kg was supplemented the Ca to P ratio was closer to 2:1 than 1:1. This may mean that when higher phytase doses were fed the balance was restored, causing P release to become linear and performance to improve. The mechanism of continuing improvement in P digestibility with inclusion of high doses of phytase may be because the phytate can be degraded at a faster rate or to a greater extent, the phytase is able to find the phytate substrate more quickly or because the active phytase continues working in the small intestine after leaving the gizzard (Kies *et al*., 2006; Zeng *et al*., 2014).

The observed increase in Ca absorption between the control diet and diet supplemented with 500 FTU/kg in the RSM based diets, but not the SBM based diets, may be because the phytate in RSM is more susceptible to degradation by phytase than the phytate in SBM. Solubility in water however is not the only factor that dictates effective mineral utilisation; for example presence of oligosaccharides

and interactions between minerals have an impact.

Dietary Ca levels should be kept to a minimum in phytase supplemented diets (Selle *et al.*, 2009) without compromising performance or bone mineralisation. Ca ion concentration in the small intestine is thought to dictate the rate of hydrolysis of phytate-P by endogenous intestinal and cereal phytases. For example, when high levels of dietary Ca are present, phytate-P hydrolysis by endogenous phytase reduces between 23-46% at the pH of the jejunum (Tamim and Angel, 2003). In this study, mineral-phytate complexes were degraded more readily in the jejunum of birds fed RSM than SBM, possibly because the phytate in RSM is reactive to the effects of phytase than the phytate in SBM. There were therefore comparatively more free minerals present that could bind to H+ ions and influence pH in the gastrointestinal tract of birds fed the RSM based diets.

4.5.5. Protein Utilisation

Protein solubility is reduced in the presence of phytate. This is because feed proteins, particularly those with high concentrations of basic amino acids, become positively charged at pH below their isoelectric point, resulting in attraction between protein and phytate (which is negatively charged at low pH) and production of phytate-protein complexes. Another possible way in which phytate reduces protein solubility is that at low pH phytate has a hydration shell around it which competes with other compounds for water (Cowieson *et al*., 2011). This hydration shell is able to change water conformation and reduce protein solubility by moving water molecules closer to phytate and further from the protein (Barać *et al*., 2004). The reduced amount of water around the protein molecule results in reduced protein solubility. Presence of protein in the GIT stimulates gastrin and cholecystokinin secretion which stimulates HCl and pepsinogen secretion in the stomach. Thus reduced protein solubility caused by phytate presence results in increased pepsin, bile, sodium bicarbonate and HCl production in the stomach (Cowieson *et al*., 2011), resulting in more undigested protein reaching the duodenum. Additionally, HCl and pepsinogen production has an irritant effect on gut mucosa, compensated by increased mucus production, which results in increased endogenous loss of amino acids. Heightened sodium bicarbonate production, secreted in response to the reduced

stomach pH, also has a direct negative impact on protein utilisation because excess sodium can compromise amino acid absorption. Additionally, binary protein-phytate complexes form at pH less than the isoelectric point of proteins and these complexed proteins are resistant to pepsin degradation. These binary complexes, as well as tertiary ones, hinder amino acid absorption by the small intestine.

Phytate reduces pepsin and trypsin activity, namely through binding to cofactors (such as zinc and calcium) that activate enzymes, which leads to endogenous secretions of protein and mucin (Selle *et al.,* 2012). Endogenous protein turnover and mucin loss account for 13% of the total maintenance energy requirements of the bird. The higher total phytic acid content of RSM compared to SBM may have resulted in comparatively greater endogenous amino acid flow and hence reduced amino acid utilisation. Also, the proteins present in RSM are less soluble than those present in SBM (Fernandez *et al.,* 1993). This study suggests that the ability of phytase to degrade phytate so that protein is soluble and available for absorption is heavily influenced by its interaction with Ca, and may be dependent on the influence that the hydration shell of phytate has on water structure and hence protein solubility.

4.5.6. Bone Strength and Mineralisation

Phytase supplementation level had a direct impact on bone development and strength; as phytase level increased tibia and femur mineralisation, strength and Ca and P content increased, due to heightened hydrolysis of phytate-bound Ca and P. This is illustrated by Figures 4.1. and 4.2. which show correlations between Ca and P absorbed and bone Ca and P content, and Figures 4.3. and 4.4. which shows correlations between Ca and P absorbed and bone strength. Statistical analysis however revealed that these correlations were significant between Ca and P absorbed and tibia Ca and P ($p=0.048$ and $p=0.044$ respectively) and tibia strength ($p<0.001$ and $p=0.036$), but not between Ca and P absorbed and femur Ca and P ($p=0.675$ and $p=0.271$ respectively) and femur strength ($p=0.193$ and $p=0.187$ respectively). This suggests that the tibia is more sensitive to the effects of phytase supplementation than the femur at this bird age. This is in agreement with the work of Onyango *et al.*

(2003) among other literatures, and is likely to be because cellular sensitivity is greater in the tibia than in the femur, especially at the epiphyseal end (Applegate and Lilburn, 2002).

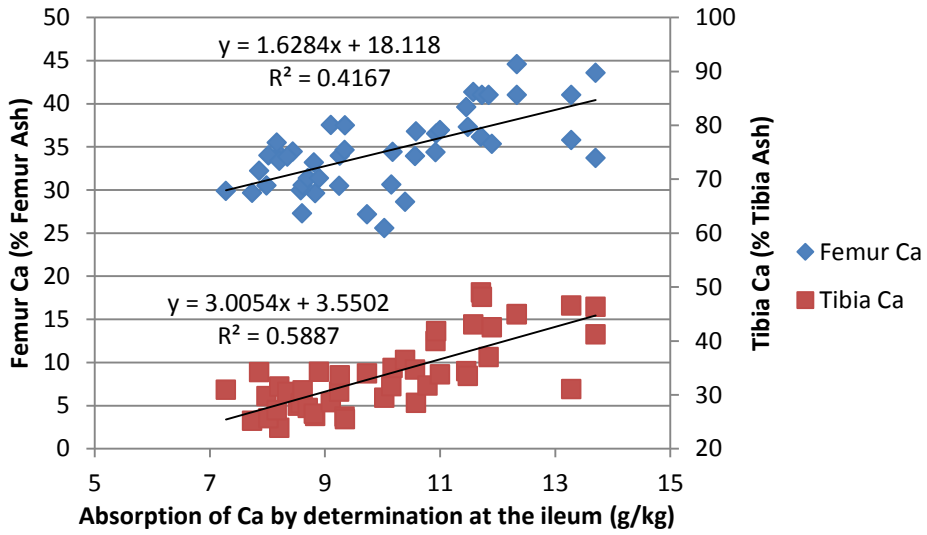

Figure 4.1. Correlation between Ca absorbed and Ca in the tibia and femur

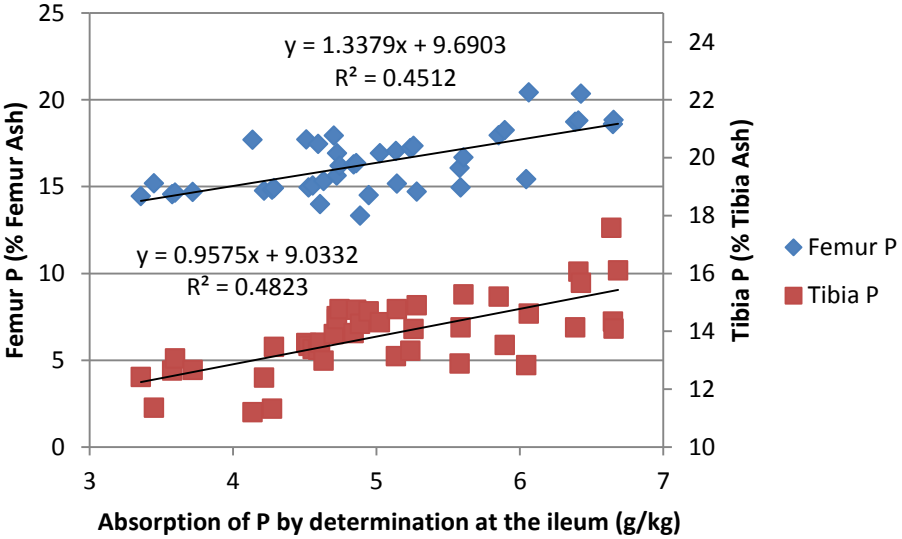

Figure 4.2. Correlation between P absorbed and P in the tibia and femur

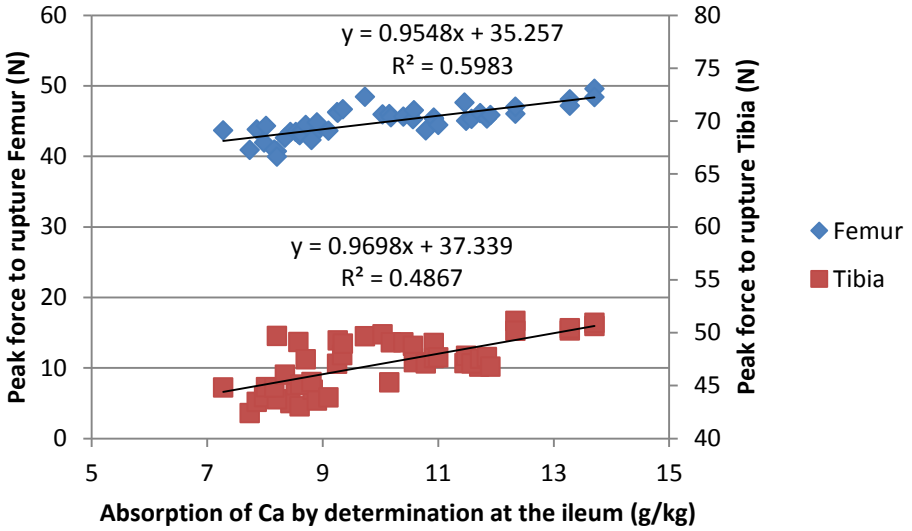

Figure 4.3. Correlation between Ca absorbed and tibia and femur strength

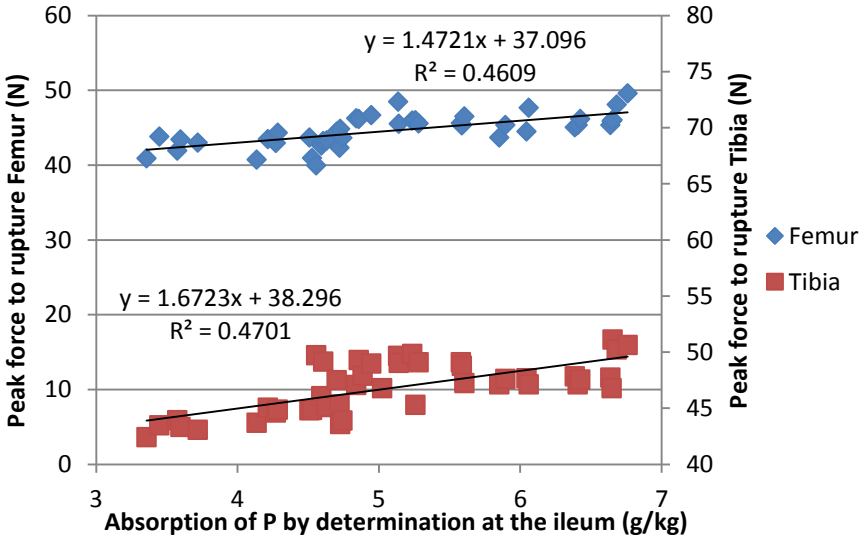

Figure 4.4. Correlation between P absorbed and tibia and femur strength

The measured parameters for bone development did not show a uniform trend in their response to protein source; whilst strength, mineralisation and tibial length were greater in birds SBM, all other bone morphometrics were greater in birds fed RSM. Phytase had a greater impact on bone strength in birds fed the RSM based diets possibly because the susceptibility of the phytate in the RSM based diets to the effects of phytase was greater than that in the SBM based diets.

A significant correlation was observed between tibia length and BWG (P=0.018) and length and strength (P=0.023), but not between femur length and BWG (P=0.125) or length and strength (P=0.088). This suggests that Ca and P distribution to leg bones may have prioritised developing length of the bones and hence development of the tibias first, particularly in birds fed the SBM based diets, to maintain centre of gravity and distance from the floor substrate (Reiter and Bessei, 1997; Applegate and Lilburn, 2002).

The femurs in birds fed the RSM based diets were longer than those fed the SBM based diets possibly because birds fed SBM had greater body weights. There is a strong relationship between bird walking ability and body weight (Talty *et al.,* 2010) which suggests that the greater body weights in birds fed the SBM based diets may have promoted compensatory gait adaptations (Corr *et al.,* 2003); longer leg length means a longer stride so minimised energy expenditure. The morphology of broilers pushes their legs further apart which means they position their centre of mass laterally (Schmidt *et al.,*2009), suggesting that the greater tibia length in the heavier birds may be a mechanism to maintain weight distribution and reduce joint pressure. In birds fed RSM however body weight was lower so there was less focus on bird stability and keeping up with fast growth rate, and more on development of strong bones and stride symmetry. Ca and P was therefore evenly distributed between the femurs and tibias in birds fed the RSM based diets, hence why femur development was better in birds fed these diets. It should however be noted that increased bone strength does not necessarily directly result in reduced lameness (Waldenstedt, 2005). Another contributor may be that RSM contains more dietary lipids which may influence collagen cross-linking (Wradale and Duance, 1996), so the bones of birds

fed the RSM based diets may have had greater tensile strength, although this would require further investigation.

4.6. Conclusion

The current commercial phytase recommendation of 500FTU/kg is adequate for allowing reduced levels of inorganic P to be supplemented into broiler diets without compromising performance, while improving P availability and digestibility. However, this inclusion level of phytase results in destruction of only around 60% of the phytate present, whereas this study suggests that higher doses could result in phytate destruction beyond this, leading to further improvements in performance. Alleviating the anti-nutritional effects of phytate using high phytase levels also enables feed ingredients which may be viewed as undesirable, due to their high phytate content, to be used successfully in poultry diets. The capability of phytase to completely degrade phytate cannot be predicted based on enzyme activity or ability of the enzyme to release P, but is instead may be dependent on the 'susceptibility' of the phytate present to the effects of phytase in the environment of the digestive tract, and relative solubility of the phytate. Further investigation is needed to quantify the varying response of phytate to phytase in different feed ingredients to determine optimum phytase dosage of poultry diets.

4.7. Implications

The use of high doses of phytase can increase profitability by reducing formulation costs by removing the restrictions of using high phytate ingredients, such as RSM, and significantly improving performance through phytate destruction. The potential significance of the findings from this study is that it suggests it may be possible to make poor quality, cheap protein sources produced in the UK provide similar nutritional value as more expensive imported unsustainable protein sources.

CHAPTER 5: Contribution of intestinal and cereal derived phytase activity to phytate degradation in young broilers

5.1. Introduction

It has been well documented that phytate-P is largely unavailable for utilisation due to a lack of effective phytase from intestinal bacteria and mucosa or from dietary cereals themselves, commonly referred to as endogenous phytase (Cowieson *et al*., 2006). However, there is very little information throughout the literature regarding the actual contribution of endogenous phytase to phytate hydrolysis. Therefore estimation of total phytase activity in the gastrointestinal tract of poultry may be flawed when based on exogenous supplemental phytase alone, as such measures do not account for background interference caused by presence of endogenous phytase (Yu *et al.,* 2004).

Endogenous phytase activity has been detected in all sections of the broiler small intestine but predominantly in the duodenum (Maenz and Classen, 1998). Its presence and activity has been shown to increase with bird age (Maronek *et al*., 2010), potentially due to increased maturity and small intestinal mucosal surface area. There is, however, no data currently available stating the age at which birds first begin to produce intestinal phytase. There is also little consensus among studies as to the exact capability of poultry to utilise dietary phytate without supplemental phytase. For example, Mohammed *et al*. (1991) found that phytate digestibility ranged from 32-54%, Edwards (1993) found it ranged from 56-63% and Applegate *et al.* (2003), Leske and Coon (2002) and Plumstead *et al.* (2008) identified apparent ileal phytate hydrolysis of approximately 40%, 32% and 20% respectively. There is also variation amongst literature with regards to how much phytase is present in cereals fed to broilers; for example for wheat, Marounek *et al.* (2011) and Eeckhout and De Paepe (1994) found similar levels of 1137U/kg and 1193U/kg respectively, but Chalova *et al.* (2012) and Viveros *et al.* (2000) found higher levels of 1759U/kg and 1637U/kg. This study examined the contribution of intestinal and cereal phytases to phytate degradation in young broilers.

Aim

- To investigate the impact of phytase activity from the intestine and diet on degradation of phytate in birds aged 0-14 days post hatch
- To determine bird age at which intestinal phytase contributes towards phytate degradation.

5.2. Trial Procedure

720 Ross 308 broiler chicks, from a breeder flock aged 43 weeks, were obtained on day of hatch and housed in 80 x 80cm floor pens in an environmentally controlled room with wood shavings as litter until d14. Birds were distributed into 48 pens with 15 birds per pen. Two pens were considered as one replicate (classified as a 'plot'); therefore there were 24 plots with 30 birds per plot at the beginning of the study. 27 birds were sampled in the study, with three spare birds per plot. Feed was provided *ad libitum* via troughs attached to the front of the pen and water by nipple drinkers. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the School of Animal Rural and Environmental Sciences Ethical Review Group.

Birds were fed one of 4 diets; diets were formulated to have similar total phytate levels of 10 g/kg, phytase level of 460U/kg and dietary calcium and available phosphorus levels of 11 g/kg and 4 g/kg, respectively. Each diet had a different combination of raw materials; treatment A and B contained rapeseed meal and rice bran at differing inclusion levels, treatment C contained rye and wheat and treatment D contained rye and rice bran (Table 5.1). Diets were formulated by ABVista, mixed in house using a ribbon mixer, one batch per diet, and fed as mash. The diets were tested for nitrogen content immediately after manufacture, and reanalysed after 5 days, before they were fed, to check protein content did not change over time. They were also analysed for crude energy, total calcium and phosphorus, available phosphorus, dry matter, ash, phytase activity, total phytic acid, susceptible phytate-P and fat content using the methods described in Chapter 2 (Table 5.2). The diets contained

titanium dioxide at a rate of 0.5% to act as an inert marker so digestibility could be measured. Diets were stored at room temperature.

Table 5.1. Composition and nutrient content of experimental diets (g/kg)

	A	B	C	D
Maize	513.10	531.30	265.10	536.90
Rye			50.00	75.00
Wheat - Feed			300.00	
Rapeseed Extruded	200.00	50.00		
Soybean meal 46	177.00	263.40	294.50	298.70
Rice Bran	15.00	80.00	0.00	10.00
Soy oil	44.90	26.20	37.10	25.70
Salt	4.60	4.60	4.40	4.60
Valine	1.00	1.00	1.00	1.00
DL Methionine	2.60	3.30	3.60	3.60
Lysine HCl	3.80	3.50	3.60	3.60
Threonine	0.90	1.10	1.40	1.30
L-Arginine HCl	1.80	0.90	1.10	1.10
Isoleucine	1.10	0.80	0.80	0.80
Limestone	7.30	6.30	10.70	9.40
Dicalcium Phos	17.80	18.40	17.40	19.10
Coccidiostat (Coban - monensin)	0.20	0.20	0.20	0.20
Vitamin premix*	4.00	4.00	4.00	4.00
Titanium Dioxide	5.00	5.00	5.00	5.00

* Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.2 mg; retinol, 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 µg; folic acid, 1.5 mg; and biotin 125 mg.

Table 5.2. Calculated and analysed content of diets

	A		B		C		D	
	Calculated	Analysed	Calculated	Analysed	Calculated	Analysed	Calculated	Analysed
GE Content (MJ/kg DM)		18.99		19.34		19.52		19.18
ME Content (MJ/kg DM)	12.81		12.81		12.81		12.81	
Total P Content (g/kg DM)	8.40	8.03	8.80	8.09	7.40	7.54	7.70	7.22
Total Ca Content (g/kg DM)	10.00	11.97	10.00	11.82	10.00	11.45	10.00	10.23
DM Content (g/kg)		861.52		861.34		832.32		854.43
Ash Content (g/kg)		6.12		5.90		6.24		5.79
Protein Content (g/kg DM)	207.00	199.50	206.50	216.40	208.00	214.20	204.70	200.10
Available Phosphorus (g/kg)	4.50	4.64	4.50	4.61	4.50	4.19	4.50	4.16
Free Calcium (g/kg)		8.99		8.72		8.54		8.37
Phytic Acid Content (g/kg)	11.20	11.95	11.20	10.56	8.20	9.96	8.80	8.64
Susceptible Phytate-P(g/kg)	5.70	8.48	7.10	7.81	3.90	7.07	4.60	6.83
Phytase Activity (FTU/kg^{-1})		407		396		470		472
Fat (g/kg)	85.10	76.22	67.60	62.79	55.80	55.44	52.50	48.97
Fibre (g/kg)	41.70		32.50		24.90		25.40	

On arrival birds were individually weighed and allocated to a pen. Pen allocation was randomised across the room. Total pen weight and mean chick weight was calculated, and di*et al*location was arranged to ensure there was no significant difference in body weight by pen across diets. Each pen was individually fed from a pre-weighed labelled bag containing 2.5kg of appropriate diet per week. Total pen weight was determined on d4, 6, 8, 10, 12 and 14. Feed intake was also measured on these days; feed remaining in the trough was poured into the individual bags for each pen and the bag was weighed. Feed conversion ratio of birds aged 0-4, 4-6, 6-8, 8-10, 10-12, 12-14 was calculated based on feed intake divided by body weight gain, taking into account any mortalities.

Feed troughs were topped up an hour before sampling each pen to encourage feeding and resulting gut fill at sampling. Sampling was carried out at the same time each sampling day. On d4, 10 birds per plot (5 birds from each pen per plot) were euthanised by cervical dislocation. On d6, 5 birds per plot were euthanised (3 birds from one pen and 2 from the other pen per plot), and on d8, 10, 12 and 14, 3 birds per plot were euthanised (2 birds from one pen and 1 from the other pen per plot). The weights and feed intakes were divided by the number of birds in the pen to determine mean individual bird weight or intake. Mortalities were accounted for by adjusting the feed intake and body weight gain calculations to take in consideration the amount of birds in each pen each day.

5.3. Determined Parameters

5.3.1. Digesta sample collection and pH determination

Immediately post-euthanasia, two of the euthanised birds per plot, one per pen, were individually weighed and marked with a coloured pen for identification purposes. Gizzard, duodenum, jejunum and ileum pH of these two birds was determined by inserting a spear tip piercing pH electrode (Sensorex, California, USA) directly into the gut lumen as soon as they had been excised, as described in Chapter 2, Section 2.5.1. Readings were repeated three times per section of gut per bird (ensuring the probe did not touch the gut wall) and average pH was calculated. Gizzard, jejunum and ileum digesta contents from all the birds per plot were then collected by gentle digital pressure into one pot

per section of tract per plot, and stored at -20°C prior to freeze drying. Once freeze dried the samples were ground through a 1mm sieve. Titanium dioxide content of digesta and diet were determined by using an ICP-OES assay, as described in Chapter 2, Section 2.5.8. and Chapter 3, Section 3.2.

5.3.2. Total and Soluble Calcium and Phosphorus Determination

For each plot, total and soluble Ca and P content was determined in the freeze-dried gizzard, jejunum and ileum digesta based on the average of 3 readings per sampling site per plot. Diets were also analysed for total and soluble Ca and P; 6 repeats per diet were carried out. Total Ca and P of the feed and freeze-dried digesta was determined by acid digestion with aqua regia, followed by filtration through Whatman #541 filter papers and analysis by Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) set at phosphorus wavelength 213.167 and calcium wavelength 317.933, as described in Chapter 2, Section 2.5.8. Soluble Ca and P content of the freeze-dried digesta, and soluble P content of the feed, was determined by adding ultra-pure water to the samples, shaking and centrifuging them, and then measuring Ca and P content of the supernatant on an ICP-OES set at phosphorus wavelength 213.167 and calcium wavelength 317.933, as described in Chapter 2, Section 2.5.8.1.

5.3.3. Total and Susceptible Phytate

Total phytic acid content of the gizzard and jejunal digesta samples and diet was analysed by the Megazyme K-Phyt™ assay, as described in Chapter 2, Section 2.5.10. Susceptible phytate content of the diet was analysed by adding warmed acetate buffer to the sample, incubating and centrifuging it, neutralising the supernatant with 0.25M NaOH, and subsequently using the Megazyme K-Phyt™ assay to analyse phytic acid content, as described in Chapter 2, Section 2.5.11.

5.3.4. Phytase Activity

Total phytase activity of the diet and ileal digesta was determined by addition of acetate buffer to the samples followed by incubation at 37°C. A phytate substrate solution and molybdate/vanadate/nitric

acid solution was then added to the samples, they were centrifuged, and then optical density was measured at 415nm, as described in Chapter 2, Section 2.5.12.

5.3.5. Data Analysis

All data was analysed using the SPSS software version 21 for Windows (IBM Statistics, 2013). After KS testing to confirm normality, statistical analysis was carried out using Univariate analysis to determine interactions between the analysed factors, and one-way ANOVA to test the equality of the means. Treatment means were separated using Duncan post hoc test. Paired T-Test was used to compare the values of means. In cases where the effect of phytase inclusion level read to be significant, a linear and quadratic contrast was conducted. Correlations were analysed by bivariate correlation using Pearson correlation. Statistical significance was declared at $p<0.05$.

5.4. Results

5.4.1. Bird Performance

Table 5.3. shows there was no significant interaction between bird age and diet on feed intake (FI), body weight gain (BWG) or feed conversion ratio (FCR). Birds fed diet B had significantly higher BWG and feed conversion than birds fed any other diet. Feed conversion was the significantly lower in birds fed diet C compared to the other diets. Feed conversion was significantly higher at bird age d0-4 and d6-8, and lower at d4-6 and d10-12, compared to the other bird ages. BWG and FI were significantly higher at bird age d12-14 compared to the other bird ages.

Table 5.3. Effect of bird age and diet on bird performance

Age (days)	Individual FI(g)	Individual BWG (g)	FCR
0-4	39.53^{g}	31.88^{h}	1.24^{c}
4-6	54.18^{f}	38.43^{gh}	1.41^{ab}
6-8	53.14^{f}	44.66^{g}	1.19^{c}
8-10	80.61^{e}	59.72^{f}	1.35^{b}
10-12	90.69^{e}	62.54^{f}	1.45^{a}
12-14	111.11^{d}	82.31^{e}	1.35^{b}
SEM	9.27	6.35	0.04
Diet			
A	422.12^{ab}	301.10^{b}	1.36^{b}
B	447.86^{a}	353.84^{a}	1.27^{c}
C	438.80^{a}	311.21^{b}	1.41^{a}
D	408.22^{b}	309.81^{b}	1.36^{b}
SEM	5.33	10.21	0.03
P-values			
Diet	0.013	<0.001	0.003
Age	<0.001	<0.001	0.017
Diet x Age	0.642	0.662	0.394

$^{a-h}$Means within the same column, within the same section, with no common superscript differ significantly ($P \leq 0.05$).

5.4.2. Gastrointestinal pH

Table 5.4. shows there was a significant interaction between diet and age on gizzard digesta pH; at d6, d12 and d14 pH was highest in birds fed diet C, whereas at d4, d8 and d10, pH was lowest in birds fed diet C compared to the other diets. For every diet, there was a significant decrease in gizzard pH between d4 and d6. Also in diets A, B and D there was a significant increase in gizzard pH between d6 and d8 to a reading similar to that found at d4. In all diets, gizzard pH was significantly lower at d14 than at d4.

Table 5.5. shows there was no significant interaction between diet and age or effect of diet on duodenal, jejunal or ileal pH. Duodenal, jejunal and ileal pH was significantly lower at d6 than at any other bird age. Ileal pH was also significantly lower at d4 than at d8, 10, 12 or 14.

Table 5.4. Interaction between bird age and diet on gizzard pH

Age (day)	Diet A	Diet B	Diet C	Diet D
Phytic Acid Concentration (g/kg)	11.95	10.56	9.96	8.64
4	2.91[a]	2.90[a]	2.74[a]	2.75[a]
6	2.38[b]	2.50[b]	2.69[b]	2.42[b]
8	2.74[a]	2.94[a]	2.61[b]	3.01[a]
10	2.59[b]	2.93[a]	2.58[b]	2.81[a]
12	2.62[ab]	2.78[a]	2.85[a]	2.33[b]
14	1.99[c]	1.67[c]	2.48[b]	2.30[b]
SEM	0.12	0.18	0.05	0.11
Age		0.025		
Diet		0.046		
Diet x Age		0.037		

[a,b]Means within the same column with no common superscript differ significantly (P ≤ 0.05).

Table 5.5. Effect of bird age and diet on duodenum, jejunum and ileum pH

	Duodenum	Jejunum	Ileum
Age (day)			
4	6.10[a]	6.03[b]	6.74[b]
6	5.99[b]	5.90[c]	6.19[c]
8	6.10[a]	6.13[ab]	7.24[a]
10	6.11[a]	6.08[ab]	7.33[a]
12	6.08[a]	6.08[ab]	7.08[a]
14	6.13[a]	6.15[a]	7.24[a]
SEM	0.02	0.03	0.16
P-values			
Age	0.020	<0.001	<0.001
Diet	0.613	0.660	0.840
Diet x Age	0.670	0.233	0.166

[a,b]Means within the same column with no common superscript differ significantly (P ≤ 0.05).

5.4.3. Total and Soluble Ca and P

Table 5.6., 5.7. and 5.8. show there were no significant interactions between age and diet or effect of diet on total Ca and P or Ca and P solubility in the gizzard, jejunum or ileum. Ca and P solubility in the gizzard and jejunum was significantly higher at bird age d6 than at d4. Total Ca in the gizzard and total P in the ileum was also significantly higher at bird age d6 than at d4. There was a significant increase

in total Ca in the gizzard, total P in the jejunum and soluble Ca in the jejunum between bird age d10 and d12.

Table 5.6. Effect of bird age on Ca and P solubility in the gizzard

	Total (g/kg)		Soluble (g/kg)		Solubility Coefficient	
Age	Ca	P	Ca	P	Ca	P
4	9.04^{c}	6.95	4.65^{c}	2.83^{b}	0.41^{b}	0.38^{b}
6	9.77^{b}	6.98	6.93^{a}	3.79^{a}	0.61^{a}	0.51^{a}
8	10.10^{b}	7.04	6.25^{b}	3.80^{a}	0.55^{a}	0.51^{a}
10	10.46^{b}	7.09	6.48^{a}	3.80^{a}	0.60^{a}	0.51^{a}
12	11.03^{a}	7.29	6.82^{a}	3.87^{a}	0.57^{a}	0.52^{a}
14	11.30^{a}	7.37	6.93^{a}	4.09^{a}	0.61^{a}	0.55^{a}
SEM	0.31	0.06	0.33	0.16	0.03	0.02
P-Values						
Age	<0.001	0.055	<0.001	<0.001	<0.001	<0.001
Diet	0.315	0.070	0.073	0.333	0.945	0.368
Age x Diet	0.593	0.073	0.148	0.792	0.962	0.981

^{a,b}Means within the same column with no common superscript differ significantly (P ≤ 0.05).

Table 5.7. Effect of bird age on Ca and P solubility in the jejunum

	Total (g/kg)		Soluble (g/kg)		Solubility Coefficient	
Age	Ca	P	Ca	P	Ca	P
4	6.50	4.70^{c}	2.52^{c}	1.98^{d}	0.22^{b}	0.27^{c}
6	6.56	5.09^{c}	3.27^{b}	2.48^{c}	0.29^{a}	0.33^{b}
8	6.69	5.31^{b}	3.36^{b}	2.53^{bc}	0.30^{a}	0.34^{ab}
10	6.72	5.36^{b}	3.36^{b}	2.70^{b}	0.30^{a}	0.36^{ab}
12	6.82	5.57^{a}	3.44^{b}	2.81^{a}	0.30^{a}	0.38^{ab}
14	6.94	5.79^{a}	3.61^{a}	2.92^{a}	0.32^{a}	0.39^{a}
SEM	0.06	0.14	0.14	0.12	0.01	0.02
P-Values						
Age	0.260	<0.001	<0.001	<0.001	<0.001	<0.001
Diet	0.296	0.407	0.352	0.716	0.410	0.146
Age x Diet	0.162	0.618	0.202	0.721	0.843	0.720

^{a,b}Means within the same column with no common superscript differ significantly (P ≤ 0.05).

Table 5.8. Effect of bird age on Ca and P solubility in the ileum

	Total (g/kg)		Soluble (g/kg)		Solubility Coefficient	
Age	Ca	P	Ca	P	Ca	P
4	4.24	3.07[d]	1.59	1.41	0.14	0.19
6	4.32	3.13[c]	1.70	1.56	0.15	0.21
8	4.48	3.24[bc]	1.82	1.71	0.16	0.23
10	4.53	3.39[ab]	1.93	1.71	0.17	0.23
12	4.84	3.45[ab]	1.93	1.71	0.17	0.23
14	5.16	3.60[a]	2.04	1.86	0.18	0.25
SEM	0.15	0.08	0.06	0.06	0.01	0.01
P-Values						
Age	0.101	0.002	0.671	0.576	0.576	0.574
Diet	0.121	0.091	0.200	0.258	0.258	0.281
Age x Diet	0.211	0.168	0.975	0.258	0.951	0.224

[a,b]Means within the same column with no common superscript differ significantly (P ≤ 0.05).

5.4.4. Ileal Ca and P digestibility

Table 5.9. shows there was no significant interaction or effect of diet on ileal digestibility of Ca. Ileal Ca digestibility was significantly higher at bird age d14 than at any other bird age. There was also a significant increase in ileal digestibility of Ca between d4 and d6.

Table 5.10. shows there was a significant interaction between diet and bird age on apparent ileal digestibility of P; at d4, 6, 10 and 14 ileal digestibility of P was the highest in birds fed diet A and at d6, 10, 12 and 14 it was lowest in birds fed diet D.

Table 5.9. Effect of bird age on apparent ileal Ca digestibility

Age (day)	Apparent ileal digestibility coefficient
4	0.56[c]
6	0.63[b]
8	0.61[bc]
10	0.62[b]
12	0.67[b]
14	0.72[a]
SEM	0.02
P-values	
Age	0.001
Diet	0.209
Age x Diet	0.617

[a,b]Means within the same column with no common superscript differ significantly (P ≤ 0.05).

Table 5.10. Effect of bird age and diet on apparent ileal P digestibility

	Apparent ileal digestibility coefficient			
Age (day)	A	B	C	D
4	0.67[c]	0.63[c]	0.64[b]	0.66[b]
6	0.76[b]	0.73[b]	0.73[a]	0.72[ab]
8	0.76[b]	0.76[b]	0.76[a]	0.77[a]
10	0.82[a]	0.75[b]	0.77[a]	0.70[b]
12	0.79[ab]	0.82[a]	0.71[a]	0.69[b]
14	0.79[ab]	0.77[ab]	0.74[a]	0.73[ab]
SEM	0.02	0.02	0.05	0.01
P-values				
Age		<0.001		
Diet		0.017		
Age x Diet		0.001		

[a,b]Means within the same column with no common superscript differ significantly (P ≤ 0.05).

5.4.5. Ca and P digested

Table 5.11. shows there was a significant interaction between age and diet on the amount of Ca digested in the jejunum; at d14 significantly more Ca was digested by birds fed diet C and significantly less Ca was digested by birds fed diet A than birds fed any of the other diets.

Tables 5.12. and 5.13. show there were no significant interactions between age and diet or effect of diet on Ca and P digested in the ileum or P digested in the jejunum. At bird age d6, d8 and d10 significantly more Ca was digested in the jejunum by birds fed diet A than those fed any other diet. The amount of Ca and P digested in the ileum and P digested in the jejunum was significantly lower at bird age d4 than at any other bird age. The amount of P digested in the jejunum was significantly higher at d12 and d14 than at any other bird age.

Table 5.11. Effect of bird age and diet on amount of dietary Ca digested in the jejunum (g/kg diet)

	Ca			
Age (day)	A	B	C	D
4	1.80^{d}	1.85^{c}	1.99^{d}	1.94^{c}
6	2.18^{c}	1.94^{c}	2.13^{d}	2.10^{c}
8	2.29^{bc}	2.26^{b}	2.14^{d}	2.10^{c}
10	2.42^{b}	2.27^{b}	2.27^{c}	2.26^{bc}
12	2.61^{ab}	2.74^{a}	2.69^{b}	2.47^{b}
14	2.90^{a}	2.95^{a}	3.27^{a}	2.93^{a}
SEM	0.14	0.16	0.18	0.13
P-values				
Age		0.001		
Diet		0.163		
Age x Diet		0.002		

^{a,b}Means within the same column with no common superscript differ significantly (P ≤ 0.05).

Table 5.12. Effect of bird age on amount of dietary P digested in the jejunum (g/kg diet)

Age (day)	P
4	1.29^{d}
6	1.67^{c}
8	1.80^{bc}
10	1.90^{b}
12	2.37^{a}
14	2.45^{a}
SEM	0.15
P-values	
Age	<0.001
Diet	0.721
Age x Diet	0.919

^{a,b}Means within the same column with no common superscript differ significantly (P ≤ 0.05).

Table 5.13. Effect of bird age on amount of dietary Ca and P digested in the ileum (g/kg)

Age (day)	Ca	P
4	3.59^{d}	2.29^{c}
6	4.36^{c}	2.61^{b}
8	4.74^{bc}	2.80^{ab}
10	4.93^{bc}	2.81^{ab}
12	5.31^{ab}	2.94^{a}
14	5.41^{a}	2.95^{a}
SEM	0.25	0.09
P-values		
Age	<0.001	0.002
Diet	0.223	0.185
Age x Diet	0.910	0.444

^{a,b}Means within the same column with no common superscript differ significantly (P ≤ 0.05).

5.4.6. Phytic Acid Hydrolysed

Table 5.14. shows there were no significant interactions between age and diet or effect of diet on the amount of dietary phytic acid that was hydrolysed in the gizzard, jejunum or ileum. There was a significant increase in phytic acid hydrolysed between d4 and d6 and between d10 and d12 in the gizzard, jejunum and ileum.

Table 5.14: Effect of bird age on total dietary phytate hydrolysed measured in the gizzard, jejunum and ileum (g/kg)

Age (day)	Gizzard	Jejunum	Ileum
4	1.20^{c}	2.00^{c}	3.12^{d}
6	1.33^{b}	3.21^{b}	3.39^{c}
8	1.44^{b}	3.20^{b}	3.46^{c}
10	1.46^{b}	3.32^{b}	3.61^{bc}
12	1.51^{a}	3.41^{a}	3.79^{ab}
14	1.52^{a}	3.47^{a}	3.96^{a}
SEM	0.04	0.21	0.11
Diet			
A	1.39	3.23	3.59
B	1.36	3.15	3.57
C	1.36	3.11	3.56
D	1.26	3.07	3.52
SEM	0.03	0.03	0.01
P-values			
Age	0.003	<0.001	<0.001
Diet	0.846	0.544	0.786
Age x Diet	0.059	0.070	0.690

^{a,b}Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

5.4.7. Ileal Phytase Activity

Table 5.15. shows there were no significant interactions between age and diet or effect of diet on ileal phytase activity. There was a significant increase in ileal phytase activity between d4 and d6 and ileal phytase activity was higher at d12 and d14 than at any other bird age.

Table 5.15. Effect of bird age on phytase activity in the ileum (U/kg)

Age (day)	Ileal Phytase Activity (U/kg)
4	22.03[e]
6	38.49[d]
8	40.21[cd]
10	41.67[bc]
12	43.30[ab]
14	44.99[a]
SEM	1.68
Diet	
A	38.78
B	38.10
C	38.23
D	38.68
P-values	
Age	<0.001
Diet	0.770
Age x Diet	0.759

[a,b]Means within the same column with no common superscript differ significantly (P ≤ 0.05).

5.5. Discussion

Findings from this study suggest that endogenous phytase, from intestinal bacteria, intestinal mucosa and from dietary cereals, contribute towards degradation of phytate at age d4, resulting in the hydrolysis of over 1g of phytate /kg diet. The amount of total dietary phytate hydrolysed ranged from 21% to 36% in diet with sufficient P availability, but this value may be greater in diets limited in P. Phytase activity increases gradually with bird age, but there appears to be a greater influx, with associated increase in phytic acid hydrolysis and mineral utilisation, between d10 and d12. There were strong correlations between phytic acid hydrolysed in both the gizzard and jejunum and measured

ileal phytase activity (Figure 5.1), suggesting that endogenous phytase contributes towards phytate degradation.

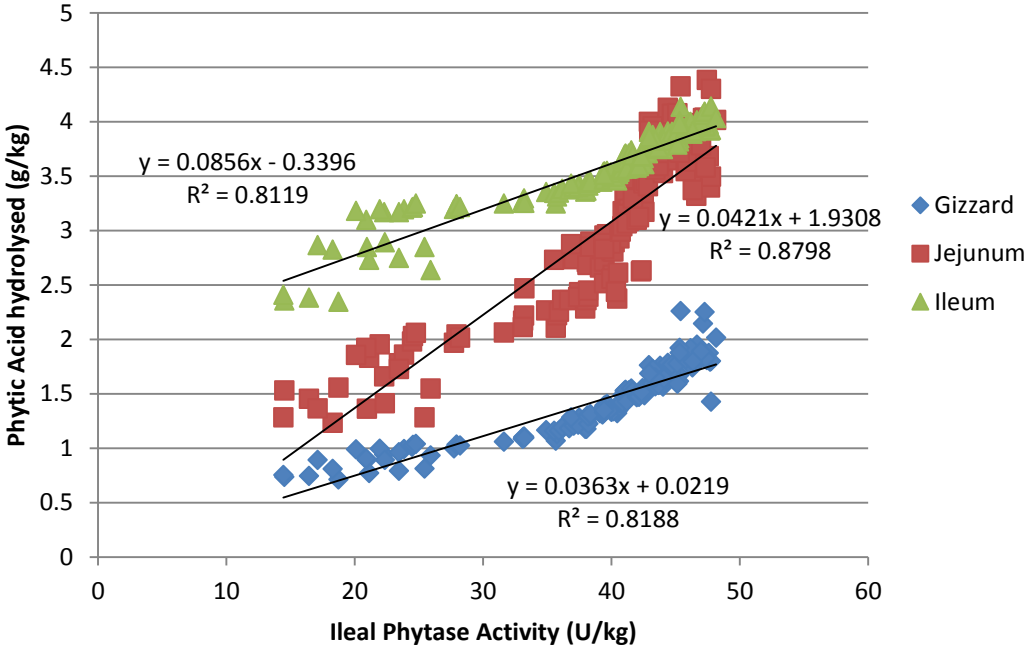

Figure 5.1. Correlation between phytic acid hydrolysed and phytase activity of individual birds

5.5.1. Performance

There was a strong correlation between ileal phytase activity and feed intake ($r=0.770$, $p<0.001$). Thus individual feed intake and body weight gain were probably highest between d12 and d14 post-hatch because the birds were bigger at this age. The transition from dependence on the yolk for nutrients to reliance on feed contributes to the low feed intake at d0-4. Supplies from the yolk can last only 4-5 days (Sell *et al.,* 1991) which suggests that the observed significant increase in Ca and P utilisation between d4 and d6 may be representative of a combination of the shift to dependence on dietary intake of minerals, increased gut maturity and heightened endogenous phytase activity. Birds younger than d4 post-hatch were not analysed in this study due to the large number required to obtain sufficient digesta, as analysed in Chapter 3, Section 3.1. The accuracy of the presented feed intake data may however be questionable as the quantities of feed measured were very small, hence any spillages would have had a huge impact on the readings. An increase in intestinal weight also occurs

as a result of the transition to nutrient supplies from feed; the increase in intestinal weight occurs more rapidly than the body mass of the whole bird (Sklan, 2001). P digestibility was highest but BWG was lowest in birds fed diet A possibly because intestinal phytase activity and intestinal surface area, and hence P solubility and absorption, was greater in birds fed this diet, but more energy was being put into gut development than bird growth so did not transpire into improved BWG.

As expected, feed intake was lowest in birds fed diet D because this diet contained the highest levels of rye. Rye contains approximately 3.5% arabinoxylans (Sárossy *et al.,* 2012), so birds fed diet D were more likely to have had increased digesta viscosity and resulting reduced feed intake. Feed conversion and body weight gain were highest in birds fed diet B possibly because this diet had the highest protein content. It also had the highest P content which may have increased skeletal weight. Birds fed diet C had the lowest feed conversion, possibly due to reduced accuracy associated with collecting feed intake data from very young birds in such a short time period. Feed intake and BWG increased significantly at d8 to d10 possibly because at d10 villus volume in the jejunum and ileum reaches is peak (Noy and Sklan, 1997) so nutrient absorption in relation to bird size is greatest at this age.

5.5.2. Gastrointestinal pH

There were no significant correlations between ileal phytase activity or phytate hydrolysis and pH in any section of the tract measured. However, duodenum, jejunum and ileum pH was lowest at d6; optimum phytase activity in the small intestine occurs at pH 5.5-6 (Maenz and Classen, 1998) so it is possible that the reduction in pH may have instigated the increase in ileal phytase activity and hence hydrolysis of phytate at this age. The higher gizzard pH observed in the d4 birds compared to the d6 birds is potentially due to the immaturity of the proventriculus and gizzard. In the older birds the increased feed intake may have led to increased pH due to the increase in dietary limestone ingested; analysis of a digestibility marker in the digesta would be required to confirm limestone digestibility.

The observed significant decrease in gizzard pH of birds fed diet A and B at d14 compared to birds fed diets C and D may be because diets A and B had lower limestone content, so the buffering capacity was lower compared to the other study diets. Also, between d12 and d14 the amount of Ca in the

gizzards of birds fed diets C and D reduced and hence the buffering capacity of these diets was higher. At d4 it may be that the diet had very little impact on pH in the gizzard due to the very small quantities consumed. The significant decrease in gizzard pH observed between d4 and 6 in all diets coincides with an increase in phytic acid hydrolysis and Ca solubility, meaning less Ca was present in the gut lumen to influence pH. The onset of endogenous phytase activity between d4 and 6 may account for the observed decrease in duodenum and jejunum pH at this time period, again due to increased Ca absorption, as shown by correlations between gizzard Ca solubility and jejunum and ileum pH ($r=0.251$, $p=0.025$ and $r=0.281$, $p<0.001$ respectively). The effect at d8 and 10 may suggest selective Ca consumption of the mash diets or modified diet consumption based on Ca requirements (Wilkinson *et al.* 2011).

5.5.3. Mineral Solubility and Absorption

Observed strong correlations between ileal phytase activity and total Ca and P on the gizzard ($r=0.698$, $p<0.001$ and $r=0.888$, $p=0.002$ respectively) and total jejunum Ca and P ($r=0.288$, $p<0.001$ and $r=0.281$, $p<0.001$ respectively) illustrates the impact that endogenous phytase, coupled with increased feed intake, has on mineral utilisation. Increased ileal Ca digestibility at d14 compared to younger birds is possibly due to increased ileal phytase presence. Endogenous phytase activity and phytate hydrolysis increased between d10 and d12 post-hatch due to increased gut maturity (Torok *et al.,* 2011). For example, Nitsan *et al.* (1991) found that chymotrypsin activity reached a maximum level at approximately d11 post-hatch, and chymotrypsin is inhibited by phytate and activated and stimulated by calcium. Mucosal alkaline phosphatase is not secreted into the gut lumen until approximately d5 (Sabatakou *et al.,* 2007); the pH optimum for alkaline phosphatase lowers as the concentration of phytate lowers, due to presence of phytase. This partly explains why apparent ileal digestibility of Ca and amount of Ca digested in the ileum increased between d4 and d6.

Ileal P digestibility was highest in birds fed diet A at bird age d4, 6, 10 and 14 possibly because diet A had the highest susceptible phytate value meaning that, although this diet had the highest total

phytate content due to its high level of rapeseed meal, the phytate present was most susceptible to the effects of phytase. This coincides with the findings of Akinmusire and Adeola (2009) who found that microbial phytase has more effect on improving true P digestibility in birds fed RSM rather than SBM. Diet A also had the lowest Ca:P ratio at 1.49:1. P utilisation is reduced as the Ca:P ratio increases, and both Mitchell and Edwards (1996) and Dieckmann (2004) state that the optimum Ca: P ratio to be 1.5:1 in relation to P digestibility. Additionally, release of P from phytate is more difficult when the phytate is also bound to protein as phytate-protein-phosphorus complexes, so the lower protein content of diet A may have resulted in comparatively increased P release from phytate in birds fed this diet (Jacob *et al.,* 2000). Furthermore, GIT pH was lower in all sections of the tract in birds fed diet A which again suggests that P release from phytate was higher in birds fed this diet because the complexes were more soluble, as the further away the pH is from neutral the less likely ternary phytate-protein-mineral complexes are to form. Also, diet A had higher fat digestibility because it had the highest fat content and highest phytate content; phytate raises solubility of bile acids (Yuangklang *et al.,* 2005) and inositols which are generated from phytate hydrolysis are involved in fat metabolism and transport. It is therefore likely that serum cholesterol was higher in birds fed diet A; high serum cholesterol has been shown to increase total P digestibility (Huff *et al.,* 1998; Liu *et al.,* 2009) by forming chylomicrons with fatty acids. Diet A also contained more fibre than the other diets which may have improved gizzard development and enhanced bacterial fermentation which regulates P digestibility and absorption. Bacterial P availability is reduced in the large intestine by phytase-mediated heightened P absorption in the small intestine, hence phytase improves fibre digestibility as shown in this study by a strong correlation between ileal phytase activity and apparent ileal P digestibility ($r=0.976, p=0.031$).

Intestinal brush border phytase activity may be subject to regulation in response to dietary P status of the bird, as illustrated by strong correlations between ileal phytase activity and P solubility in the gizzard and ileum ($r=0.989$, $p<0.001$ and $r=0.921$, $p<0.001$ respectively). The lower ileal P digestibility observed in birds fed diet D at age d6, 10, 12 and 14 is likely because diet D had the lowest total and

soluble Ca and P, and the phytate in this diet was least susceptible to the effects of phytase so P release from phytate-complexes was lowest. Diet D also had the had the lowest fat content which, as previously mentioned, results in reduced serum cholesterol levels and hence P digestibility.

5.6. Conclusion

This study proves that intestinal and cereal derived phytase activity is quantifiable in regulating phytate-P digestion. Endogenous phytase, from the diet and intestinal bacteria and mucosa, contributes significantly towards degradation of phytate at bird age d4. It then continues to increase with age, reaching activity levels of approximately 45 U/kg by d14. Further investigation is required to evaluate the observed significant increase in phytase activity at d12 compared d10 post-hatch, and to determine the extent of the impact of endogenous phytase in older birds.

5.7. Implications

This study shows that endogenous phytase activity in the tract has a significant effect on phytate degradation. Although this effect of endogenous phytase is small compared to that provided by supplemented exogenous phytases, knowledge of activity levels produced is useful for formulating pre-starter, starter and layer diets based on phytase activity and phytate concentration.

CHAPTER 6: Effect of feeding broilers diets differing in susceptible phytate content

6.1. Introduction

Insoluble phytate–mineral precipitates and soluble mineral–phytate complexes may be resistant to hydrolysis by phytase (Maenz *et al.,* 1999). In calculating the P available from a diet following phytase addition, the total phytate-P concentration of a diet may therefore be misleading because the total amount of phytate in the diet does not represent the volume of phosphorus available for release. This suggests relative solubility and susceptibility of phytate to phytase should therefore be accounted for when formulating with phytase as this may account for anecdotal reports of apparent 'phytase failure' in diets. This is of particular importance if reliance on phytase for dietary P availability is high. As illustrated in Chapter 3 Section 3.5.1., phytate susceptibility varies considerably between diets and is dependent upon the ingredients used, ingredient variability and solubility of the phytate. Gastrointestinal pH also potentially has a significant impact of phytate susceptibility because it is addition of H+ ions to the weak acid phosphate groups of phytate that convert it from being resistant to susceptible to the effects of phytase. In this study, the total and susceptible phytate contents of the individual feed ingredients being fed were analysed and diets were formulated based on these values. Diets were diagnosed to examine the following hypothesis: degree of phytate susceptibility rather than total phytate will dictate level of response to phytase enzyme supplementation.

Aim

- To determine if 'susceptible' phytate as opposed to 'total' phytate should be measured when investigating phytase efficacy and formulating diets that provide maximum mineral utilisation
- To examine the relationships between factors that influence and respond to phytate degradation

6.2. Trial Procedure

240 Ross 308 broiler chicks, from a breeder flock aged 42 weeks, were obtained on day of hatch and housed in 80 x 80cm floor pens in an environmentally controlled room with wood shavings as litter until d28. Birds were distributed into 48 pens with 5 birds per pen. One pen was classified as one replicate. 4 birds per pen were sampled in the study and there was one spare bird per pen. Feed was provided ad libitum via troughs attached to the front of the pen and water by nipple drinkers. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Nottingham Trent University College of Science ethical review committee.

Birds were fed one of 4 dietary treatments in a 2 x 2 factorial; 2 diets with high or low susceptible phytate content, containing 0 or 500FTU/kg phytase. Each treatment was fed to 12 pens. Diets were mixed in house using a ribbon mixer. Dry ingredients were mixed for 5 minutes prior to addition of oil, and then mixed for a further 5 minutes. The experimental diets were fed to the birds from Day 0 (1 day posthatch) to day 28.

Table 6.1. Composition of diets (g/kg)

Ingredient	High Susceptible Phytate	Low Susceptible Phytate
Wheat	531.30	485.60
Soybean meal 46	312.80	318.80
Wheat Bran		100.00
Rice Bran	80.00	
Soy oil	38.70	59.70
Salt	4.70	4.60
DL Methionine	2.90	2.90
Lysine HCl	2.30	2.10
Threonine	0.80	0.70
Limestone	5.40	4.90
Dicalcium Phos	17.80	18.40
Coccidiostat (Coban - monensin)	0.20	0.20
Vitamin premix*	4.00	4.00
Titanium Dioxide	5.00	5.00

* Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.2 mg; retinol, 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 µg; folic acid, 1.5 mg; and biotin 125 mg.

Table 6.2. Calculated and analysed content of diets

	High 0FTU/kg		High 500FTU/kg		Low 0FTU/kg		Low 500FTU/kg	
	Calculated	Analysed	Calculated	Analysed	Calculated	Analysed	Calculated	Analysed
GE Content (MJ/kg DM)		20.43		20.57		21.11		21.13
Total P Content (g/kg DM)	9.90	9.70	9.90	9.74	8.90	8.66	8.90	8.74
Total Ca Content (g/kg DM)	8.40	8.31	8.40	8.22	8.40	8.20	8.40	8.18
Ash Content (g/kg)		6.75		6.80		6.84		6.77
DM Content (g/kg)		863.30		866.18		887.71		869.18
Protein Content (g/kg DM)	225.90	223.74	225.90	224.08	227.70	229.29	227.70	229.97
Free Phosphorus (g/kg)	3.50	3.80	3.50	3.87	3.50	3.77	3.50	3.72
Supplemented Phytase Activity (FTU/kg)	0	15.91	500	872.62	0	36.03	500	835.39
Total Phytase Activity (FTU/kg)[1]		58.23		1428.62		21.57		1380.38
Phytic Acid Content (g/kg)	13.10	14.90	13.10	14.60	11.10	12.19	11.10	12.70
Susceptible Phytate-P (g/kg)	8.30	8.61	8.30	8.47	5.30	5.65	5.30	5.88
Fat (g/kg)	64.80	70.67	64.80	70.74	74.00	70.75	74.00	70.81
Fibre (g/kg)	28.30		28.30		29.50		29.50	

[1] Dietary cereal phytase activity and supplemented phytase activity combined, analysed by ISO 30024

On arrival birds were individually weighed and randomly allocated to a pen. Total pen weight was calculated and pen/di*et al*locations were altered to ensure there was no significant difference in starting body weights across diet.

Total pen weight and pen feed intake was determined on d7, 14, 21 and 28. The weights and feed intakes were divided by the number of birds in the pen to determine mean individual bird weight or intake. Mortalities were accounted for by adjusting the feed intake and body weight gain calculations to take into consideration the amount of birds in each pen each day. Sampling was carried out at the same time each sampling day. On d14 and d28 two birds per pen were euthanised by cervical dislocation and individually weighed and marked with a coloured pen for identification purposes. Feed troughs were topped up an hour before sampling each pen to encourage feed and hence gut fill at sampling.

6.3. Determined Parameters

6.3.1. Digesta sample collection and pH determination

Gizzard, duodenum, jejunum and ileum pH of the two euthanised birds were determined by inserting a spear tip piercing pH electrode (Sensorex, California, USA) directly into the gut lumen immediately after excision, as described in Chapter 2, Section 2.5.1. Readings were repeated three times (ensuring the probe did not touch the gut wall) and average pH was determined for each gut section. The pH readings were recorded for each individual bird to allow individual weight to be considered in the analysis. Gizzard, jejunum and ileum digesta contents from each individual bird were then collected into one pot per section of tract per bird. The pots were then weighed, stored at -20°C and then freeze-dried to determine digesta sample dry matter content, as described in Chapter 2, Section 2.5.2. Once freeze dried the samples were ground with a pestle and mortar. Titanium dioxide content of digesta and diet were determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES) as described in Chapter 2 Section 3.2 (Morgan *et al*., 2014).

6.3.2. Total and Susceptible Phytate

Total phytic acid content of the gizzard, jejunum and ileum digesta and diets was analysed by the Megazyme K-Phyt™ assay, as described in Chapter 2, Section 2.5.10. Susceptible phytate content of the diets was analysed by adding warmed acetate buffer to the sample, incubating and centrifuging it, neutralising the supernatant with 0.25M NaOH, and subsequently using the Megazyme K-Phyt™ assay to analyse phytic acid content, as described in Chapter 2, Section 2.5.11.

6.3.3. Total and Soluble Calcium and Phosphorus Determination

For each individual bird sampled, total Ca and P content was determined in the freeze dried gizzard, jejunum and ileum digesta samples in triplicate. The diets were also analysed for Ca and P content in triplicate. Total Ca and P was determined by ICP-OES after acid digestion with aqua regia, as described in Chapter 2, Section 2.5.8. Soluble Ca and P content of the freeze-dried digesta and feed was determined by adding ultra-pure water to the samples, shaking and centrifuging them, and then measuring Ca and P content of the supernatant on an ICP-OES set at phosphorus wavelength 213.167 and calcium wavelength 317.933, as described in Chapter 2, Section 2.5.8.1.

6.3.4. Tibia and Femur Bone Strength

The right tibia and femur was collected from each individual bird sampled. The tibia bones were separated at the tibiotarsal junction, where the feet were removed, and the tibiofemoral junction. Femur bones were separated at the tibiofemoral junction and the hip. The bones were defleshed of muscle and tissue by hand using a scalpel. The length and width of each bone was measured using digital callipers and recorded. Bone strength was analysed using a TA.XT plus texture analyser (Stable Microsystems, Guildford, UK) set up with a 50kg load cell and 3 point-bend fixture (Park *et al*., 2003, Taylor *et al*., 2003; Shaw *et al*., 2010) , as described in Chapter 2, Section 2.5.7. The texture analyser was set to measure force in compression, test speed was set at 1mm/sec, and trigger force was set at 7g (0.069N). The texture analyser was calibrated using a 5kg weight and the peak force in Newtons was recorded for each bone.

6.3.5. Tibia and Femur Mineralisation and and Ca and P content

The tibia and femur bones were wrapped individually in foil and autoclaved for 15 minutes at 121°C. Any remaining flesh and connective tissue was removed by hand and the bones oven dried at 110°C for approximately 4 days until constant weight. The dried bones were then weighed into pre-weighed ceramic crucibles and ashed for approximately 14 hours at 650°C (Hall *et al*., 2003). The crucibles were then left to cool in a desiccator and reweighed. Bone ash was calculated as ash weight as a percentage of dry bone weight, as described in Chapter 2, Section 2.5.3.

The ashed bone samples were acid-digested with aqua regia, filtered, diluted and analysed for Ca and P content by ICP-OES set at phosphorus wavelength 213.167 and calcium wavelength 317.933, as described in Chapter 2, Section 2.5.8.

6.3.6. Phytase Activity

Supplemented phytase activity of the diet and ileal digesta was determined using a Quantiplate^{TM} Kit for Quantum phytase, as described in Chapter 2, Section 2.5.13. Total phytase activity of the diet and ileal digesta was determined by adding acetate buffer to the samples, incubating it at 37°C, adding a phytate substrate solution and molybdate/vanadate/nitric acid solution to the samples before centrifuging them and measuring the optical density at 415nm, as described in Chapter 2, Section 2.5.12.

6.3.7. Pepsin Activity

Pepsin activity in the gizzard digesta was determined using 2% bovine haemoglobin as the substrate, based on the method presented by Liu and Cowieson (2011), as described in Chapter 2, Section 2.5.15. Briefly, 5ml of 2% haemoglobin solution was added to approximately 1g digesta sample and the solution was incubated at 37°C for 10 minutes. 1ml of pepsin solution was then added to the solution and it was incubated for a further 10 minutes at 37°C. The reaction was stopped by addition of 10ml of 5% trichloroacetic acid followed by incubation for 5 minutes at 37°C and filtration through a 0.45

µm syringe filter. The optical density of the solution was then read on a UV spectrophotometer (Unicam Helios, USA) set at 280nm.

6.3.8. Data Analysis

All data were analysed using SPSS (v.21). Pen served as the experimental unit for all parameters measured. The statistical model included diet and bird age. Multiple ANOVA test were used to test the equality of the means and Univariate analysis was used to determine interactions between the analysed factors. When differences were significant, means were separated using Duncan post hoc test. The relationship between individual factors was investigated using Pearson product-moment correlation coefficient and interpretations of the strength of the relationship between the two methods was based on guidelines by Cohen (1988); weak relationship r = 0.10 to 0.29, medium relationship r = 0.30 to 0.49 and strong relationship r = 0.50 to 1.0. Statistical significance was declared at $p<0.05$. In the event that interactions were not significant, main effects were discussed.

6.4. Results

6.4.1 Bird Performance

Table 6.3. shows there were no significant interactions between bird age and diet on weekly feed intake or feed conversion, and no effect of diet on weekly feed intake or feed conversion. As bird age increased feed intake significantly increased linearly, but feed conversion was significantly better at d7-14 and significantly worse at d14-21 compared to the other bird ages. There was an interaction between bird age and diet on weekly BWG; at d0-7 and d21-28 BWG was lowest in birds fed Low 0 FTU/kg but at d7-14 and d14-21 it was lowest in birds fed High 0 FTU/kg and Low 0FTU/kg. At d7-14 and d21-28 BWG was highest in birds fed High 500 FTU/kg.

Table 6.3. Effect of phytate susceptibility and phytase supplementation on bird feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR) at d7, 14, 21 and 28

Age (days)[a]	Diet	Individual FI (g)	Individual BWG (g)	FCR
0-7	High 0 FTU	158.34	106.27[a]	1.49
	High 500 FTU	148.72	106.32[a]	1.41
	Low 0 FTU	144.53	93.29[c]	1.57
	Low 500 FTU	158.22	96.14[b]	1.68
	SEM	3.01	2.94	0.05
7-14	High 0 FTU	309.71	263.33[c]	1.19
	High 500 FTU	326.00	277.40[a]	1.18
	Low 0 FTU	306.79	265.39[bc]	1.15
	Low 500 FTU	291.95	268.13[b]	1.12
	SEM	6.04	2.69	0.02
14-21	High 0 FTU	666.86	403.62[b]	1.67
	High 500 FTU	701.83	454.39[a]	1.58
	Low 0 FTU	696.13	400.03[b]	1.75
	Low 500 FTU	718.92	460.13[a]	1.57
	SEM	9.38	13.91	0.04
21-28	High 0 FTU	884.94	640.89[b]	1.39
	High 500 FTU	920.75	710.39[a]	1.30
	Low 0 FTU	920.51	610.35[c]	1.51
	Low 500 FTU	887.61	641.25[b]	1.39
	SEM	8.60	18.33	0.04
Age				
0-7		152.45[d]	100.50	1.54[b]
7-14		308.61[c]	268.56	1.16[d]
14-21		695.93[b]	429.54	1.64[a]
21-28		903.45[a]	650.72	1.40[c]
SEM		149.51	101.56	0.09
P-values				
Age		<0.001	<0.001	<0.001
Diet		0.597	<0.001	0.063
Diet x Age		0.490	0.002	0.092

[a,b] Means within the same column, within the same age group, with no common subscript differ significantly (P<0.05)

Table 6.4. shows there were no interactions between phytate susceptibility and phytase inclusion on cumulative feed intake, BWG or feed conversion at bird age d0-28. BWG and feed conversion was significantly better in birds fed the diets with high phytate susceptibility, and the diets supplemented with phytase at age d0-28.

Table 6.4. Effect of phytate susceptibility and phytase supplementation on feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR) at d0-28

Diet	Individual FI (g)	Individual BWG (g)	FCR
High 0 FTU	2019.86	1414.10	1.43
High 500 FTU	2097.30	1548.50	1.36
Low 0 FTU	2067.95	1369.06	1.51
Low 500 FTU	2056.69	1465.65	1.40
SEM	13.87	33.34	0.03
Susceptibility			
High	2058.58	1481.30[a]	1.40[b]
Low	2062.32	1417.36[b]	1.46[a]
SEM	1.32	22.61	0.02
Phytase			
0 FTU	2043.90	1391.58[b]	1.47[a]
500 FTU	2077.00	1507.08[a]	1.38[b]
SEM	11.70	40.83	0.030
P-values			
Susceptibility	0.256	0.015	0.003
Phytase	0.294	<0.001	0.001
Susceptibility x Phytase	0.487	0.457	0.802

[a,b] Means within the same column with no common subscript differ significantly (P<0.05)

6.4.2. Gastrointestinal pH

Table 6.5. shows there was an interaction between phytate susceptibility and phytase inclusion on duodenum pH at bird age d28; duodenum pH was significantly higher in birds fed High 500 FTU/kg than those fed High 0 FTU/kg and Low 500 FTU/kg. There were no interactions between phytate susceptibility and phytase inclusion on gizzard, jejunum or ileum pH at bird age d28, or on gizzard, duodenum, jejunum or ileum pH at bird age d14. Duodenum pH was higher in birds fed the high susceptible phytate diets than those fed the low susceptible phytate diets at age d14.

Table 6.5. Effect of phytate susceptibility and phytase supplementation on gizzard, duodenum, jejunum and ileum pH

	Gizzard		Duodenum		Jejunum		Ileum	
Diet	d14	d28	d14	d28	d14	d28	d14	d28
High 0 FTU	2.58	2.97	6.23	6.07[b]	5.92	6.17	6.82	7.12
High 500 FTU	2.45	2.65	6.16	6.17[a]	5.97	6.07	6.97	7.22
Low 0 FTU	2.46	2.84	6.10	6.14[ab]	6.00	6.10	7.13	7.31
Low 500 FTU	2.33	2.79	6.05	6.05[b]	5.97	6.06	6.97	7.20
SEM	0.04	0.06	0.03	0.02	0.01	0.02	0.06	0.03
Susceptibility								
High	2.52	2.81	6.19[a]	6.12	5.94	6.12	6.89	7.17
Low	2.40	2.82	6.07[b]	6.10	5.99	6.08	7.05	7.26
SEM	0.04	0.00	0.04	0.01	0.01	0.01	0.06	0.03
P-values								
Susceptibility	0.226	0.963	<0.001	0.445	0.469	0.510	0.133	0.390
Phytase	0.076	0.285	0.076	0.807	0.930	0.229	0.940	0.947
Susceptibility x Phytase	0.827	0.455	0.827	0.005	0.523	0.595	0.147	0.294

[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

6.4.3. Total and Soluble Ca and P

Table 6.6. shows there was an interaction between phytate susceptibility and phytase inclusion on the amount of soluble Ca present and the Ca solubility coefficient measured in the gizzard at bird age d28; Ca solubility was significantly higher in birds fed both the diets supplemented with 500 FTU phytase and significantly lower in birds fed the Low 0 FTU diet than those fed the High 0 FTU diet. Gizzard soluble P content at d28 was significantly higher in birds the diets with high susceptible phytate compared to those fed the diets with low susceptible phytate. Also, total P content at d28, soluble P content at d14 and d28, the solubility Ca coefficient at d14 and the solubility P coefficient at d14 and d28 was significantly higher in birds fed the diets with 500 FTU phytase compared to those fed diets containing 0 FTU phytase.

Table 6.7. shows there was also an interaction between phytate susceptibility and phytase inclusion on the Ca solubility coefficient measured in the jejunum at bird age d28; the solubility coefficient was significantly higher in birds fed the High 500 FTU diet and significantly lower in birds fed the diets containing 0 FTU phytase. Jejunum soluble Ca and P content was significantly higher in birds fed the diets with high susceptible phytate compared to those fed the diets with low susceptible phytate at age d28. Soluble Ca content at d28, soluble P content at d14 and d28, the Ca solubility coefficient at

d14 and P solubility coefficient at d14 and d28 were all significantly higher in the jejunum in birds fed the diets containing 500 FTU phytase compared to those fed the diets with 0 FTU phytase.

Table 6.8. shows there were no interactions between phytate susceptibility and phytase inclusion on ileal total and soluble Ca and P content. Ileum soluble Ca and P content and the Ca solubility coefficient were significantly higher in birds fed the diets with high susceptible phytate compared to those fed the diets with low susceptible phytate at age d28. Soluble Ca and P content at d14 and d28, the Ca solubility coefficient at d14 and d28 and the P solubility coefficient at d28 were all significantly higher in the jejunum in birds fed the diets containing 500 FTU phytase compared to those fed the diets with 0 FTU phytase.

Table 6.6. Effect of phytate susceptibility and phytase supplementation on Ca and P solubility in the gizzard

	Total (g/kg)				Soluble (g/kg)				Solubility Coefficient			
	Ca		P		Ca		P		Ca		P	
Diet	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28
High 0 FTU	8.15	10.75	4.27	7.28	7.17	6.87[b]	4.13	3.67	0.87	0.83[b]	0.45	0.40
High 500 FTU	8.35	10.82	4.50	7.50	7.33	7.43[a]	4.66	4.34	0.90	0.90[a]	0.50	0.46
Low 0 FTU	8.02	10.66	4.23	7.02	7.12	6.54[c]	4.10	3.55	0.86	0.80[c]	0.45	0.39
Low 500 FTU	8.23	10.75	4.49	7.46	7.25	7.36[a]	4.62	4.22	0.88	0.90[a]	0.50	0.47
SEM	0.06	0.03	0.06	0.09	0.04	0.18	0.13	0.17	0.01	0.02	0.01	0.02
Susceptibility												
High	8.25	10.79	4.39	7.39	7.25	7.15	4.40	4.01[b]	0.89	0.87	0.48	0.43
Low	8.12	10.71	4.36	7.24	7.19	6.95	4.36	3.89[c]	0.87	0.85	0.48	0.43
SEM	0.04	0.03	0.01	0.05	0.03	0.07	0.01	0.04	0.01	0.01	0.02	0.01
Phytase												
0 FTU	8.08	10.71	4.25	7.15[b]	7.15	6.71	4.12[b]	3.61[c]	0.87[b]	0.82	0.45[b]	0.40[b]
500 FTU	8.29	10.79	4.50	7.48[a]	7.29	7.40	4.64[a]	4.28[a]	0.89[a]	0.90	0.50[a]	0.47[a]
SEM	0.07	0.03	0.09	0.12	0.05	0.24	0.18	0.24	0.01	0.03	0.01	0.03
P-Values												
Susceptibility	0.407	0.465	0.859	0.092	0.487	<0.001	0.569	0.007	0.279	0.004	0.728	0.961
Phytase	0.606	0.466	0.096	0.001	0.155	<0.001	<0.001	<0.001	0.044	<0.001	<0.001	<0.001
Susceptibility x Phytase	0.990	0.925	0.927	0.222	0.896	0.006	0.964	0.871	0.150	0.028	0.970	0.223

[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

Table 6.7. Effect of phytate susceptibility and phytase supplementation on Ca and P solubility in the jejunum

	Total (g/kg)				Soluble (g/kg)				Solubility Coefficient			
	Ca		P		Ca		P		Ca		P	
Diet	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28
High 0 FTU	6.76	9.47	4.30	5.79	3.57	3.90	2.13	2.89	0.43	0.47[c]	0.23	0.32
High 500 FTU	7.19	9.86	4.57	5.94	3.65	4.26	2.34	3.24	0.44	0.52[a]	0.25	0.35
Low 0 FTU	6.72	9.27	4.24	5.62	3.53	3.88	2.11	2.84	0.43	0.47[c]	0.23	0.31
Low 500 FTU	6.85	9.79	4.50	5.84	3.59	4.09	2.33	3.14	0.44	0.50[b]	0.25	0.34
SEM	0.09	0.12	0.07	0.06	0.02	0.08	0.05	0.08	0.01	0.01	0.01	0.01
Susceptibility												
High	6.98	9.66	4.44	5.87	3.61	4.08[a]	2.23	3.07[a]	0.44	0.49	0.24	0.33
Low	6.79	9.53	4.37	5.73	3.56	3.99[b]	2.22	2.99[b]	0.43	0.49	0.24	0.33
SEM	0.07	0.05	0.02	0.05	0.02	0.03	0.01	0.03	0.01	0.01	0.01	0.01
Phytase												
0 FTU	6.74	9.37	4.27	5.71	3.55	3.89[b]	2.12[b]	2.87[b]	0.43[b]	0.47	0.23[b]	0.31[b]
500 FTU	7.02	9.83	4.54	5.89	3.62	4.18[a]	2.33[a]	3.19[a]	0.44[a]	0.51	0.25[a]	0.35[a]
SEM	0.10	0.16	0.09	0.06	0.02	0.10	0.08	0.11	0.01	0.01	0.01	0.01
P-Values												
Susceptibility	0.314	0.652	0.651	0.447	0.195	0.018	0.536	0.003	0.627	0.128	0.706	0.203
Phytase	0.149	0.126	0.077	0.324	0.114	<0.001	<0.001	<0.001	0.032	<0.001	<0.001	<0.001
Susceptibility x Phytase	0.436	0.841	0.985	0.839	0.786	0.073	0.949	0.247	0.517	0.030	0.974	0.656

[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

Table 6.8. Effect of phytate susceptibility and phytase supplementation on Ca and P solubility in the ileum

	Total (g/kg)				Soluble (g/kg)				Solubility Coefficient			
	Ca		P		Ca		P		Ca		P	
Diet	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28
High 0 FTU	4.76	6.17	1.88	3.53	1.19	2.62	0.80	1.12	0.14	0.32	0.09	0.12
High 500 FTU	5.33	6.24	2.19	3.63	1.24	2.94	0.83	1.25	0.15	0.36	0.09	0.14
Low 0 FTU	4.64	6.14	1.86	3.51	1.14	2.41	0.77	1.07	0.14	0.29	0.08	0.12
Low 500 FTU	5.07	6.16	2.03	3.54	1.21	2.76	0.81	1.22	0.15	0.34	0.09	0.13
SEM	0.14	0.02	0.07	0.02	0.02	0.10	0.01	0.04	0.01	0.01	0.01	0.01
Susceptibility												
High	5.04	6.20	2.04	3.58	1.21	2.78[a]	0.81	1.19[a]	0.15	0.34[a]	0.09	0.13
Low	4.86	6.15	1.94	3.53	1.18	2.58[b]	0.79	1.15[b]	0.14	0.32[b]	0.09	0.13
SEM	0.07	0.02	0.03	0.02	0.01	0.07	0.01	0.01	0.01	0.01	0.00	0.00
Phytase												
0 FTU	4.70	6.15	1.87	3.52	1.17[b]	2.52[b]	0.78[b]	1.10[b]	0.14[b]	0.30[b]	0.09	0.12[b]
500 FTU	5.20	6.20	2.11	3.59	1.22[a]	2.85[a]	0.82[a]	1.24[a]	0.15[a]	0.35[a]	0.09	0.13[a]
SEM	0.18	0.02	0.08	0.02	0.02	0.12	0.01	0.05	0.00	0.02	0.00	0.01
P-Values												
Susceptibility	0.523	0.645	0.453	0.386	0.012	<0.001	0.127	0.001	0.028	<0.001	0.255	0.179
Phytase	0.095	0.679	0.064	0.280	<0.001	<0.001	0.022	<0.001	<0.001	<0.001	0.086	<0.001
Susceptibility x Phytase	0.797	0.822	0.583	0.630	0.441	0.518	0.642	0.299	0.996	0.875	0.544	0.604

[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

6.4.4. Ileal Ca and P digestibility

Table 6.9. shows there were no interactions between, or effects of, phytate susceptibility and phytase inclusion on apparent ileal digestibility of Ca of P. The digestibility of both nutrients was incredibly high in this study compared with most others which may explain the lack of enzyme effect and this is backed up by the relatively high proportion of phytate hydrolysed at the terminal ileum even in the 0 FTU diets. The apparent ileal Ca and P digestibility values presented in Table 6.9. are higher than would be expected based on comparison with similar published studies.

Table 6.9. Effect of phytate susceptibility and phytase supplementation on apparent ileal Ca and P digestibility

	Apparent Ileal Digestibility Coefficient			
	Ca		P	
Diet	d14	d28	d14	d28
High 0 FTU	0.79	0.75	0.92	0.87
High 500 FTU	0.81	0.76	0.93	0.88
Low 0 FTU	0.78	0.75	0.92	0.87
Low 500 FTU	0.80	0.76	0.93	0.87
SEM	0.01	0.01	0.01	0.01
P-Values				
Susceptibility	0.991	0.880	0.386	0.183
Phytase	0.803	0.233	0.125	0.415
Susceptibility x Phytase	0.130	0.336	0.958	0.283

[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

6.4.5. Ca and P digested

Tables 6.10. and 6.11. show there were no interactions between phytate susceptibility and phytase inclusion on the amount of Ca and P digested in the jejunum and ileum. Phytate susceptibility had no significant effect on the amount of Ca and P digested in the jejunum and ileum. The amount of Ca digested was significantly higher in the jejunum at d14 and d28 and ileum at d28 in birds fed the diets containing 500 FTU phytase compared to those fed the diets with 0 FTU phytase.

Table 6.10. Effect of phytate susceptibility and phytase supplementation on amount of dietary Ca and P digested in the jejunum (g/kg diet)

	Amount of mineral digested (g/kg diet)			
	Ca		P	
Diet	d14	d28	d14	d28
High 0 FTU	4.68	6.20	4.67	7.08
High 500 FTU	5.29	6.29	4.84	7.13
Low 0 FTU	4.43	6.05	4.48	7.01
Low 500 FTU	5.12	6.53	4.76	7.06
SEM	0.17	0.09	0.07	0.02
Phytase				
0 FTU	4.56[b]	6.12[b]	4.57	7.04
500 FTU	5.21[a]	6.41[a]	4.80	7.10
SEM	0.23	0.10	0.08	0.02
P-Values				
Susceptibility	0.469	0.219	0.825	0.687
Phytase	0.027	0.045	0.160	0.752
Susceptibility x Phytase	0.894	0.607	0.340	0.981

[a,b] Means within the same column with no common superscript differ significantly (P ≤ 0.05).

Table 6.11. Effect of phytate susceptibility and phytase supplementation on amount of dietary Ca and P digested in the ileum (g/kg diet)

	Amount of mineral digested (g/kg diet)			
	Ca		P	
Diet	d14	d28	d14	d28
High 0 FTU	5.46	6.15	7.53	7.99
High 500 FTU	5.70	6.23	7.63	8.10
Low 0 FTU	5.46	6.10	7.46	7.98
Low 500 FTU	5.63	6.19	7.55	8.04
SEM	0.05	0.02	0.03	0.02
Phytase				
0 FTU	5.46	6.13[b]	7.49	7.98
500 FTU	5.67	6.21[a]	7.59	8.07
SEM	0.07	0.03	0.03	0.03
P-Values				
Susceptibility	0.726	0.163	0.658	0.847
Phytase	0.055	0.023	0.554	0.575
Susceptibility x Phytase	0.767	0.814	0.977	0.878

[a,b] Means within the same column with no common superscript differ significantly (P ≤ 0.05).

6.4.6. Phytic Acid Hydrolysed

Table 6.12. shows there were no interactions between phytate susceptibility and phytase inclusion on the amount of dietary phytic acid that was hydrolysed in the gizzard, jejunum or ileum. The amount of phytic acid hydrolysed was significantly higher in birds fed the diets containing high susceptible phytate in the jejunum and ileum at age d28 and the ileum at age d14.The amount of phytic acid hydrolysed was significantly higher in all sections of the tract at both bird age d14 and d28 in birds fed the diets with 500 FTU phytase compared to those fed the diets with 0 FTU phytase.

Table 6.12. Effect of phytate susceptibility and phytase supplementation on amount of dietary phytic acid hydrolysed by the gizzard, jejunum and ileum (g/kg)

	Gizzard		Jejunum		Ileum	
Diet	d14	d28	d14	d28	d14	d28
High 0 FTU	1.26	3.75	3.34	5.54	4.50	7.72
High 500 FTU	1.59	4.00	3.72	6.01	4.74	8.11
Low 0 FTU	1.19	3.49	3.11	5.30	4.30	7.61
Low 500 FTU	1.55	3.88	3.44	5.71	4.51	7.77
SEM	0.09	0.09	0.11	0.13	0.08	0.09
Susceptibility						
High	1.42	3.88	3.53	5.78[a]	4.62[a]	7.91[a]
Low	1.37	3.69	3.28	5.51[b]	4.40[b]	7.69[b]
SEM	0.02	0.07	0.09	0.10	0.08	0.08
Phytase						
0 FTU	1.22[b]	3.62[b]	3.22[b]	5.42[b]	4.40[b]	7.67[b]
500 FTU	1.57[a]	3.94[a]	3.58[a]	5.86[a]	4.62[a]	7.94[a]
SEM	0.12	0.11	0.13	0.16	0.08	0.10
P-values						
Susceptibility	0.477	0.242	0.125	<0.001	0.032	0.005
Phytase	<0.001	0.042	0.033	<0.001	0.023	<0.001
Susceptibility x Phytase	0.821	0.628	0.848	0.504	0.883	0.136

[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

6.4.7. Ileal Phytase Activity

Table 6.13. shows there was an interaction between phytate susceptibility and phytase inclusion on total phytase activity at both d14 and d28; total phytase activity was significantly different between each of the diets, with birds fed the High 500 FTU diet having the highest phytase activity followed by those fed Low 500 FTU, then High 0FTU and finally Low 0 FTU. There was no interaction between phytase and phytate susceptibility supplemented phytase activity at either bird age d14 or d28. There was also no effect of phytate susceptibility on phytase activity at either bird age d14 or d28. Supplemented phytase activity level in the ileum was significantly higher in birds fed the diets with 500 FTU phytase compared to those fed the diets with 0 FTU phytase at both bird age d14 and d28.

Total phytase activity was always higher in the high susceptible compared with low susceptible diets regardless of whether exogenous phytase was added or not. Given there was no such effect on the added activity, the difference noted above for total phytase activity probably relates for endogenous phytase activity, either of dietary, mucosal, or ileal microbial origin. The fact that the high susceptible diet encouraged higher endogenous phytase activity is possibly a result of the presence of more susceptible substrate. The hydrolysed phytate data tends to support this finding.

Table 6.13. Effect of phytate susceptibility and phytase supplementation on phytase activity in the ileum

	Supplemented Phytase (FTU/kg)		Total Phytase (FTU/kg)	
Diet	d14	d28	d14	d28
High 0 FTU	2.33	3.67	48.86[c]	59.51[c]
High 500 FTU	214.99	357.70	537.09[a]	664.87[a]
Low 0 FTU	2.41	3.89	42.56[d]	51.79[d]
Low 500 FTU	212.12	383.74	472.13[b]	575.05[b]
SEM	52.80	91.85	115.30	141.97
Susceptibility				
High	108.66	180.69	292.97	362.19
Low	107.26	193.81	257.35	313.42
SEM	0.49	4.64	12.60	17.24
Phytase				
0 FTU	2.37[b]	3.78[b]	45.71	55.65
500 FTU	213.55[a]	370.72[a]	504.61	619.96
SEM	74.66	129.73	162.24	199.51
P-values				
Susceptibility	0.143	0.447	<0.001	<0.001
Phytase	<0.001	<0.001	<0.001	<0.001
Susceptibility x Phytase	0.123	0.454	<0.001	<0.001

[a,b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

6.4.8. Pepsin Activity

Table 6.14. shows there were no interactions between phytate susceptibility and phytase inclusion on pepsin activity. Birds fed the high susceptible phytate diets had higher pepsin activity than those fed the low susceptible phytate diets at both bird age d14 and d28. Pepsin activity was also higher at both bird age d14 and d28 in birds fed the diets with 500 FTU phytase compared to those fed the diets with 0 FTU phytase.

Table 6.14. Effect of phytate susceptibility and phytase supplementation on pepsin activity in the gizzard

	Pepsin Activity (U/kg-1)	
Diet	d14	d28
High 0 FTU	930.83	686.84
High 500 FTU	1029.28	838.65
Low 0 FTU	879.45	677.53
Low 500 FTU	948.43	774.13
SEM	26.92	33.12
Susceptibility		
High	980.05[a]	762.75[a]
Low	913.94[b]	725.83[b]
SEM	23.37	13.05
Phytase		
0 FTU	905.14[b]	682.19[b]
500 FTU	988.86[a]	806.39[a]
SEM	29.60	43.91
P-values		
Susceptibility	<0.001	0.031
Phytase	<0.001	<0.001
Susceptibility x Phytase	0.352	0.129

[a, b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

6.4.9. Tibia and Femur Strength, Mineralisation and Ca and P content

Tables 6.15. and 6.16. show there were no interactions between phytate susceptibility and phytase inclusion on tibia or femur strength, length, width, mineralisation or Ca and P content. Tables 6.15. and 6.16. also show that birds fed the high susceptible phytate diets had significantly higher tibia P content at bird age d28 and femur Ca and P content at both bird age d14 and d28. Table 6.15. shows that birds fed the diets supplemented with phytase had significantly higher tibia Ca and P content at both bird age d14 and d28 and tibia strength and ash content at bird age d28 than those fed the diets without phytase. Table 6.16. shows birds fed the diets supplemented with phytase also had significantly higher femur strength at d28 and Ca and P content at both bird age d14 and d28.

Table 6.15. Effect of phytate susceptibility and phytase supplementation on tibia strength, length, width, mineralisation and Ca and P content

	Strength (N)		Length (mm)		Width (mm)		Weight (g)		Ash (%)		Ca (% of ash)		P (% of ash)	
Diet	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28
High 0 FTU	57.29	246.67	59.04	89.30	4.31	7.68	3.47	13.85	31.79	32.81	22.37	31.42	9.94	13.66
High 500 FTU	60.24	287.03	58.97	89.48	4.33	7.61	3.59	13.55	32.10	35.20	25.13	34.09	10.81	16.59
Low 0 FTU	56.70	241.95	58.50	89.70	4.25	7.38	3.44	13.39	31.96	32.75	22.74	27.96	10.15	12.72
Low 500 FTU	60.25	269.19	57.01	89.23	4.28	7.40	3.25	13.21	31.99	35.11	23.69	32.14	10.57	15.76
SEM	0.82	9.06	0.41	0.09	0.01	0.07	0.06	0.12	0.05	0.59	0.53	1.11	0.17	0.78
Susceptibility														
High	58.77	266.85	59.01	89.39	4.32	7.65	3.53	13.70	31.94	34.01	23.75	32.75	10.38	15.12[b]
Low	58.47	255.57	57.76	89.47	4.27	7.39	3.35	13.30	31.98	33.93	23.21	30.05	10.36	14.24[c]
SEM	0.10	3.99	0.44	0.03	0.02	0.09	0.06	0.14	0.01	0.03	0.19	0.95	0.01	0.31
Phytase														
0 FTU	57.00	244.31[b]	58.77	89.50	4.28	7.53	3.46	13.62	31.88	32.78[b]	22.55[b]	29.69[b]	10.04[b]	13.19[d]
500 FTU	60.24	278.11[a]	57.99	89.36	4.31	7.51	3.42	13.38	32.04	35.15[a]	24.41[a]	33.11[a]	10.69[a]	16.17[a]
SEM	1.15	11.95	0.28	0.05	0.01	0.01	0.01	0.08	0.06	0.84	0.66	1.21	0.23	1.05
P-values														
Susceptibility	0.935	0.356	0.122	0.933	0.513	0.178	0.267	0.500	0.978	0.941	0.323	0.002	0.935	0.018
Phytase	0.366	0.008	0.330	0.881	0.733	0.900	0.800	0.686	0.888	0.028	<0.001	<0.001	0.016	<0.001
Susceptibility x Phytase	0.933	0.591	0.377	0.732	0.963	0.789	0.349	0.923	0.908	0.991	0.098	0.372	0.387	0.875

[a,b]Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

Table 6.16. Effect of phytate susceptibility and phytase supplementation on femur strength, length, width, mineralisation and Ca and P content

	Strength (N)		Length (mm)		Width (mm)		Weight (g)		Ash (%)		Ca (% of ash)		P (% of ash)	
Diet	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28	d14	d28
High 0 FTU	60.22	182.86	45.51	68.40	4.89	8.53	2.58	10.05	33.65	33.63	24.20	33.12	12.86	15.23
High 500 FTU	61.69	219.02	45.20	67.20	4.79	8.91	2.45	10.73	33.73	34.87	29.51	37.88	15.52	17.62
Low 0 FTU	59.71	191.56	45.09	66.82	4.83	8.46	2.57	9.64	33.16	33.45	22.23	31.53	11.96	14.85
Low 500 FTU	63.15	211.44	44.60	67.28	4.88	8.56	2.62	9.84	33.22	34.72	27.14	35.77	14.85	16.45
SEM	0.67	7.30	0.16	0.29	0.02	0.09	0.03	0.21	0.13	0.32	1.39	1.22	0.72	0.54
Susceptibility														
High	60.95	200.94	45.35	67.80	4.84	8.72	2.52	10.39	33.69	34.25	26.85^{b}	35.50^{b}	14.19^{b}	16.42^{b}
Low	61.43	201.50	44.85	67.05	4.85	8.51	2.59	9.74	33.19	34.09	24.69^{c}	33.65^{c}	13.40^{c}	15.65^{c}
SEM	0.17	0.20	0.18	0.27	0.01	0.08	0.03	0.23	0.18	0.06	0.77	0.66	0.28	0.27
Phytase														
0 FTU	59.96	187.21^{b}	45.30	67.61	4.86	8.50	2.58	9.84	33.41	33.54	23.21^{d}	32.33^{c}	12.41^{d}	15.04^{c}
500 FTU	62.42	215.23^{a}	44.90	67.24	4.84	8.74	2.53	10.29	33.47	34.80	28.33^{a}	36.82^{a}	15.18^{a}	17.04^{a}
SEM	0.87	9.91	0.14	0.13	0.01	0.08	0.02	0.16	0.02	0.44	1.81	1.59	0.98	0.71
P-values														
Susceptibility	0.881	0.950	0.258	0.212	0.885	0.172	0.532	0.179	0.611	0.992	0.001	0.011	0.039	0.021
Phytase	0.446	0.003	0.365	0.534	0.824	0.127	0.706	0.355	0.947	0.342	<0.001	<0.001	<0.001	<0.001
Susceptibility x Phytase	0.759	0.368	0.836	0.168	0.520	0.350	0.471	0.615	0.993	0.893	0.746	0.615	0.750	0.223

^{a,b}Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

6.4.10. Correlations between factors

Each parameter measured was correlated against each other for each individual bird, to determine how the different factors and mechanisms impacted on each other on a bird to bird basis. Table 6.17. shows the correlations between the measured factors at bird age d14. Table 6.18. shows the correlations between the measured factors at bird age d28 and Table 6.19. shows the correlations between the measured factors and bone parameters, strength and mineralisation at d14 and d28. Only significant ($P<0.05$) correlations are presented and only strong correlations are discussed. Strong correlations are shown in **red**, medium correlations are shown in *blue* and weak correlations are shown in green, based on the guidelines of Cohen (1988); weak $R^{2=}$0.10 to 0.29, medium $R^{2=}$0.30 to 0.49 and strong $R^{2=}$0.50-1.

Table 6.17. Correlations between factors in individual birds at d14

	Weight (g)	Gizzard pH	Duodenum pH	Jejunum pH	Ileum pH	Gizzard Total Ca (g/kg)	Gizzard Total P (g/kg)	Gizzard Soluble Ca (g/kg)	Gizzard Soluble P (g/kg)	Gizzard Solubility Coefficient Ca	Gizzard Solubility Coefficient P	Jejunum Total Ca (g/kg)	Jejunum Total P (g/kg)	Jejunum Soluble Ca (g/kg)	JejunumSoluble P (g/kg)	Jejunum Solubility Coefficient Ca	Jejunum Solubility Coefficient P	Ileum Total Ca (g/kg)	IleumTotal P (g/kg)	Ileum Soluble Ca (g/kg)	Ileum Soluble P (g/kg)	Ileum Solubility Coefficient Ca	Ileum Solubility Coefficient P	AID Ca	AID P	Jejunum Ca Digested (g/kg diet)	Jejunum P Digested (g/kg diet)	Ileum Ca Digested (g/kg diet)	Ileum P Digested (g/kg diet)	Gizzard Phytate hydrolysed (g/kg)	Jejunum Phytate hydrolysed (g/kg)	Ileum Phytate hydrolysed (g/kg)	Supplemented Phytase (FTU/kg)	Total Phytase (FTU/kg)
Weight (g)																																		
Gizzard pH																																		
Duodenum pH		0.237																																
Jejunum pH																																		
Ileum pH	0.264																																	
Gizzard Total Ca (g/kg)			0.201																															
Gizzard Total P (g/kg)			0.226	-0.070		0.792																												
Gizzard Soluble Ca (g/kg)																																		
Gizzard Soluble P (g/kg)								0.921																										
Gizzard Solubility Coefficient Ca								0.998	0.921																									
Gizzard Solubility Coefficient P								0.907	0.980	0.910																								
Jejunum Total Ca (g/kg)																																		
Jejunum Total P (g/kg)											0.397																							
Jejunum Soluble Ca (g/kg)	0.203																																	
JejunumSoluble P (g/kg)				0.209				0.372	0.315	0.380	0.303			0.703																				
Jejunum Solubility Coefficient Ca														0.994	0.737																			
Jejunum Solubility Coefficient P								0.276	0.228	0.294	0.338			0.475	0.789	0.554																		
Ileum Total Ca (g/kg)	-0.271							0.201	0.226	0.202	0.202																							
IleumTotal P (g/kg)		0.013				0.204												0.841																
Ileum Soluble Ca (g/kg)		-0.252		0.304	0.240									0.235	0.390	0.237																		
Ileum Soluble P (g/kg)	0.197	-0.249	-0.239	0.309	0.259									0.331	0.468	0.332				0.818														
Ileum Solubility Coefficient Ca																				0.491	0.416													
Ileum Solubility Coefficient P	0.242		-0.344			-0.253								0.256	0.284	0.257	0.202	-0.263	-0.279	0.268	0.673	0.615												
AID Ca					0.219															0.234	0.207													
AID P								0.242	0.225		0.199						0.225				0.218			0.754										
Jejunum Ca Digested (g/kg diet)								0.652	0.665	0.650	0.669				-0.253		-0.230			0.241		0.281			-0.183									
Jejunum P Digested (g/kg diet)								-0.279	-0.290	-0.277	-0.320									0.232						0.414								
Ileum Ca Digested (g/kg diet)					0.210										0.320					0.229				0.996	0.749								
Ileum P Digested (g/kg diet)				0.225																							0.312						
Gizzard Phytate hydrolysed (g/kg)	0.220														0.339		0.204			0.325	0.398	0.239	0.329	0.262	0.205			0.242					
Jejunum Phytate hydrolysed (g/kg)					-0.256													0.215					-0.208										
Ileum Phytate hydrolysed (g/kg)																				0.255	0.294												
Supplemented Phytase (FTU/kg)					0.239		0.213								0.652	0.221	0.485		0.223	0.549	0.468	0.292		0.439	0.412		0.231	0.435	0.258	0.488	0.283			
Total Phytase (FTU/kg)					0.238		0.201								0.640	0.226	0.432		0.228	0.534	0.460	0.272		0.446	0.416		0.232	0.442	0.258	0.492	0.288		0.991	
Pepsin Activity (U/kg-1)	0.236				0.209										0.380		0.049			0.389	0.448		0.257	0.305	0.343			0.285		0.252	0.269	0.240	0.505	0.527

Table 6.18. Correlations between factors in individual birds at d28

	Weight (g)	Gizzard pH	Duodenum pH	Jejunum pH	Ileum pH	Gizzard Total Ca (g/kg)	Gizzard Total P (g/kg)	Gizzard Soluble Ca (g/kg)	Gizzard Soluble P (g/kg)	Gizzard Solubility Coefficient Ca	Gizzard Solubility Coefficient P	Jejunum Total Ca (g/kg)	Jejunum Total P (g/kg)	Jejunum Soluble Ca (g/kg)	JejunumSoluble P (g/kg)	Jejunum Solubility Coefficient Ca	Jejunum Solubility Coefficient P	Ileum Total Ca (g/kg)	IleumTotal P (g/kg)	Ileum Soluble Ca (g/kg)	Ileum Soluble P (g/kg)	Ileum Solubility Coefficient Ca	Ileum Solubility Coefficient P	AID Ca	AID P	Jejunum Ca Digested (g/kg diet)	Jejunum P Digested (g/kg diet)	Ileum Ca Digested (g/kg diet)	Ileum P Digested (g/kg diet)	Gizzard Phytate hydrolysed (g/kg)	Jejunum Phytate hydrolysed (g/kg)	Ileum Phytate hydrolysed (g/kg)	Supplemented Phytase (FTU/kg)	Total Phytase (FTU/kg)
Weight (g)																																		
Gizzard pH																																		
Duodenum pH			0.293																															
Jejunum pH																																		
Ileum pH																																		
Gizzard Total Ca (g/kg)																																		
Gizzard Total P (g/kg)						0.270																												
Gizzard Soluble Ca (g/kg)							0.503																											
Gizzard Soluble P (g/kg)							0.560	0.980																										
Gizzard Solubility Coefficient Ca							0.501	1.000	0.979																									
Gizzard Solubility Coefficient P							0.581	0.962	0.983	0.964																								
Jejunum Total Ca (g/kg)								0.238	0.246	0.237	0.217																							
Jejunum Total P (g/kg)								0.235	0.255	0.236	0.230	0.696																						
Jejunum Soluble Ca (g/kg)								*0.424*	*0.393*	*0.426*	*0.332*	0.208	0.235																					
JejunumSoluble P (g/kg)	0.285	0.287						*0.391*	*0.344*	*0.397*	*0.301*		0.276	0.646																				
Jejunum Solubility Coefficient Ca								*0.425*	*0.392*	*0.429*	*0.344*		0.234	0.996	0.663																			
Jejunum Solubility Coefficient P		0.219						*0.301*	0.265	*0.317*	*0.358*			*0.351*	0.704	*0.419*																		
Ileum Total Ca (g/kg)								*0.337*	*0.329*	*0.338*	*0.317*																							
IleumTotal P (g/kg)																		0.277																
Ileum Soluble Ca (g/kg)								0.289	0.266	0.290		0.214		0.584	0.627	0.580	0.225																	
Ileum Soluble P (g/kg)													0.255	*0.439*	0.649	*0.454*	*0.372*																	
Ileum Solubility Coefficient Ca								0.286	0.265	0.287	0.172	0.214		0.586	0.626	0.583	0.228			0.998	0.653													
Ileum Solubility Coefficient P														*0.349*	*0.483*	*0.390*	0.525			*0.477*	*0.449*	0.506												
AID Ca							*0.308*	-0.274	-0.294	-0.272	*-0.312*										0.238		0.207											
AID P															0.258									*0.383*										
Jejunum Ca Digested (g/kg diet)																																		
Jejunum P Digested (g/kg diet)					0.235		0.299								0.232					0.223	0.236	0.216			0.235	*0.334*							
Ileum Ca Digested (g/kg diet)	0.296						*0.312*	-0.237	-0.291	-0.233	*-0.305*												0.224	0.277	0.097	0.219							
Ileum P Digested (g/kg diet)																																		
Gizzard Phytate hydrolysed (g/kg)			-0.295	-0.216			0.225					0.200	0.280	0.214	0.276	0.216				*0.312*	*0.359*	*0.334*	*0.440*											
Jejunum Phytate hydrolysed (g/kg)							*0.304*							0.218	*0.400*	0.217				0.503	0.598	0.492	0.276			0.258							
Ileum Phytate hydrolysed (g/kg)														0.235	*0.348*	0.234				*0.441*	*0.363*	*0.460*	*0.317*					0.214		*0.319*	0.220			
Supplemented Phytase (FTU/kg)		*0.316*					*0.303*	0.234		0.245			0.219	0.571	0.742	0.611	0.635			0.669	0.741	0.663	0.526				0.245	0.283		0.277	0.530	*0.332*		
Total Phytase (FTU/kg)		*0.318*					0.272	0.247	0.200	0.257			0.241	0.610	0.804	0.646	0.599			0.743	0.806	0.739	0.621	0.207		0.207	0.287	0.296	0.220	*0.330*	0.576	*0.394*	0.937	
Pepsin Activity (U/kg-1)		*0.312*						*0.309*	0.278	*0.315*				*0.439*	0.554	*0.455*	*0.343*			0.546	0.589	0.547	0.506							0.205	*0.421*	*0.396*	0.573	0.599

Table 6.19. Correlations between factors and bone parameters, strength and mineralisation in individual birds at d14 and d28

		Tibia Length (mm)	Tibia Width (mm)	Tibia Weight (g)	Tibia Strength (N)	Tibia Ash (%)	Tibia Ca (% of ash)	Tibia P (% of ash)	Femur Length (mm)	Femur Width (mm)	Femur Weight (g)	Femur Strength (N)	Femur Ash (%)	Femur Ca (% of ash)	Femur P (% of ash)	Weight (g)	Gizzard pH	Duodenum pH	Jejunum pH	Ileum pH	Gizzard Total Ca (g/kg)	Gizzard Total P (g/kg)	Gizzard Soluble Ca (g/kg)	Gizzard Soluble P (g/kg)	Gizzard Solubility Coefficient Ca	Gizzard Solubility Coefficient P	Jejunum Total Ca (g/kg)	Jejunum Total P (g/kg)	Jejunum Soluble Ca (g/kg)	JejunumSoluble P (g/kg)	Jejunum Solubility Coefficient Ca	Jejunum Solubility Coefficient P	Ileum Total Ca (g/kg)	IleumTotal P (g/kg)	Ileum Soluble Ca (g/kg)	Ileum Soluble P (g/kg)	Ileum Solubility Coefficient P	AID Ca	AID P	Jejunum Ca digested (g/kg diet)	Jejunum P digested (g/kg diet)	Ileum Ca Digested (g/kg diet)	Ileum P Digested (g/kg diet)	Gizzard Phytate hydrolysed (g/kg)	Jejunum Phytate hydrolysed (g/kg)	Ileum Phytate hydrolysed (g/kg)	Supplemented Phytase (FTU/kg)	Total Phytase (FTU/kg)	Pepsin Activity (U/kg-1)
d14	Tibia Length (mm)															0.416															-0.143																	
	Tibia Width (mm)	0.429														0.550										0.242		0.220		0.214												0.285					
	Tibia Weight (g)	0.727	0.532													0.542																								-0.212					
	Tibia Strength (N)	0.377	0.659	0.5												0.642			0.281									0.237	0.237	0.240		-0.227	-0.202			0.302							0.261	0.251				
	Tibia Ash (%)			-0.272	0.45											0.283			0.245										0.213		0.234				0.231		0.200					0.239					
	Tibia Ca (% of ash)							0.827						0.310	0.312		0.236		0.213				0.380	0.346	0.383	0.315				0.354	0.216							0.274	0.249	0.314		0.284				0.295	0.301	
	Tibia P (% of ash)													0.331	0.309		0.321		0.297					0.301	0.325	0.283				0.213											0.319					0.226	0.220	
	Femur Length (mm)	0.526	0.517	0.552	0.544	0.211										0.501							-0.235			-0.232																				
	Femur Width (mm)	0.326	0.572	0.431	0.674	0.348			0.568							0.504			0.203											0.217																	
	Femur Weight (g)	0.346	0.471	0.506	0.506	0.217			0.675	0.614						0.476														0.217																	
	Femur Strength (N)	0.418	0.599	0.563	0.797	0.395			0.582	0.621	0.491					0.691			0.205									0.209	0.199	0.213		-0.205											0.200					
	Femur Ash (%)		0.247		0.323				0.238			0.372				0.346													0.259																	
	Femur Ca (% of ash)														0.651					0.249	0.206				0.244			0.228	0.440					0.391		0.260		0.275	0.271		0.312	0.329		0.616	0.614	0.324
	Femur P (% of ash)																			0.277	0.311	0.290	0.255	0.294	0.213				0.439	0.222		0.356	0.316	0.303			0.369	0.341			0.382		0.301	0.241		0.619	0.625	0.302
d28	Tibia Length (mm)															0.680																															
	Tibia Width (mm)	0.328																																												
	Tibia Weight (g)	0.721	0.427													0.553																	-0.206	0.234												
	Tibia Strength (N)	0.427	0.453	0.326												0.634												0.166	0.343		0.210				0.229	0.245					0.241					0.295	0.234
	Tibia Ash (%)			-0.217	0.170																															0.230									0.271	0.267	
	Tibia Ca (% of ash)				0.292					0.284			0.214			0.272					0.459	0.317	0.372	0.371	0.428				0.214					0.286	0.421		0.269		0.283	0.314	0.244			0.568		0.362	0.391	
	Tibia P (% of ash)													0.571	0.496							0.438	0.427	0.442	0.389	0.255	0.321	0.450	0.578	0.459	0.361			0.533	0.495							0.218	0.377	0.338	0.536	0.589	0.218
	Femur Length (mm)	0.396	0.214	0.256	0.262											0.422			0.294																						0.220							-0.287
	Femur Width (mm)	0.374	0.369	0.341	0.334				0.376							0.507																									0.247							
	Femur Weight (g)	0.562	0.359	0.500	0.333				0.593	0.611						0.574			0.206																													
	Femur Strength (N)	0.298		0.235	0.524				0.217		0.293					0.558													0.355		0.291				0.200	0.224				0.255					0.300	0.325	0.215
	Femur Ash (%)	-0.273		0.248							-0.51											0.371	0.350	0.372	0.351			0.297		0.296																	
	Femur Ca (% of ash)	0.266											0.274		0.880		0.212					0.591	0.570	0.593	0.512			0.582		0.584	0.302			0.597	0.477	0.268			0.232				0.360	0.217	0.549	0.627	0.477
	Femur P (% of ash)																0.232					0.525	0.499	0.526	0.454			0.454	0.478	0.458	0.260	0.221		0.489	0.368									0.295	0.447	0.490	0.474

6.5. Discussion

6.5.1. Effect of phytate susceptibility and phytase supplementation on bird performance, GIT pH, mineral utilisation, pepsin activity and bone strength and mineralisation

6.5.1.1. Performance

Interestingly, overall (d0 to 28) BWG was significantly higher and FCR significantly improved in birds fed the diets with high susceptible phytate compared to those fed diets with low susceptible phytate. Among other factors instigated by the differences between the compositions of the diets, this may be because mineral solubility, total ileal phytase activity and phytate degradation was higher in birds fed the high susceptible phytate diets. BWG and FCR was also significantly higher in birds fed diets with supplemented phytase and there were strong correlations between phytase activity and BWG ($r=0.555$, $p=0.031$) and FCR ($r=0.559$, $p<0.001$) at bird age d28. This experiment presented slightly confounding results in that pepsin activity increased with phytase presence. A possible explanation for this is that phytate reduces pepsin activity which results in endogenous flow of amino acids and minerals, reduced reabsorption of sodium and damage to membrane transporters (Liu *et al.,* 2008). Pepsin releases protein from protein-phytate complexes and phytase increases the rate of this release and prevents complex formations occurring, thus improving protein digestibility (Kies *et al.,* 2006). It is possible therefore that performance was greater in birds fed the high susceptible diets because there was comparatively less precipitation of protein with phytate in birds fed these diets.

Approximately only 30% (d14) to 50% (d28) of the total phytate hydrolysed was hydrolysed in the gizzard, suggesting that susceptibility of phytate in the small intestine is as essential as its susceptibility in the gizzard. This suggests the assay for measuring phytate susceptibility may require amendment as involves just exposing the feed samples to conditions that mimic the gastric phase. The solubility of phytate complexes is dependent on a number of factors, including duration of incubation, pH, molar ratio of the mineral to phytate and presence of multiple cations (Maenz *et al.,* 1999). The high susceptible phytate diets contained rice bran which has been shown to increase digesta viscosity

above that of wheat bran (Farrell and Martin, 1998), suggesting that gastric residence time may have been greater in birds fed the high susceptible phytate diets, so there was greater time for degradation of phytate mineral complexes to occur. Gizzard pH was sufficiently low in all diets in this study to allow phytic acid hydrolysis to occur. Increases in phytate hydrolysis in the jejunum and ileum with bird age were small compared to those in the gizzard, suggesting it was increased digesta retention time in the gizzard that caused the observed increased phytate hydrolysis with age. The positive effect of phytate susceptibility on bird performance may not be just due to a direct effect on mineral and protein availability because binding of phytate to metallic cations not only makes them unavailable as nutritional components but also has an impact on cell vesicular trafficking, DNA signalling and repair and endocytosis (Bohn *et al.,* 2008).

Birds fed the diets with high susceptible phytate hydrolysed significantly more phytate in the jejunum at d28 and the ileum at both d14 and d28 than birds fed the diets with low susceptible phytate. This is partly due to the difference in compositions between the two diets; the high susceptible phytate diets contained rice bran and the diet with low susceptible phytate diets contained wheat bran. Rice bran and wheat bran, despite having high levels of Ca (0.64% and 0.91% respectively) and P (1.69% and 0.70% respectively), are viewed as undesirable cereals for poultry diets, predominantly because they have high phytate values (approximately 5.3% and 2.5% respectively). This is because milling to produce bran fractions concentrates the phytate. Approximately 47% of the phytate in wheat bran is susceptible to the effects of phytase, whereas approximately 94% of the phytate in rice bran is susceptible to hydrolysis by phytase, which suggests that that supplementing poultry diets with phytase could enable rice bran to be used more successfully as an ingredient in poultry diets. This is demonstrated in this study, in that performance results for birds fed the rice bran based diets were similar to or better than found in previous studies where birds were fed standard commercial wheat-soyabean based diets when supplemented with phytase.

6.5.1.2. Gastrointestinal pH

At d14 duodenum pH was significantly higher in birds fed the diet with high susceptible phytate diets compared to the low susceptible phytate diets possibly due to increased Ca release from phytate-Ca complexes. Duodenum pH was highest at d28 in birds fed the high susceptible phytate diets with 500 FTU/kg phytase which suggests that bicarbonate ion secretion into the duodenum was increased to overcome heightened acidity of the digesta from the gizzard. This decreased acidity may have been caused in part by the higher levels of phytate in the gizzard of birds fed this diet which caused increased secretion of HCl in response to a higher presence of intact protein. The decreased acidity in birds fed diets with phytase may be because gastric residence time was lower as less intervention was needed by the bird to provide optimum pH for pepsin activity. Pepsin activity was higher at this pH possibly because the phosphate groups from phytate were able to interact with α-NH_2 more readily in this environment, so more pepsin was secreted to compensate for lost proteolytic capacity in response to increased phytate-protein complexes (Selle *et al.,* 2000). It appears that susceptibility of phytate to phytase has an impact only when the pH range is outside the range for phytate solubility. This again illustrates that phytate susceptibility influences phytate hydrolysis in the small intestine as well as the gizzard, and hence possibly dictates the efficacy of endogenous intestinal bacteria and mucosa phytase efficacy. Pepsin activity was also greatest in birds fed the diets with high susceptible phytate as there was more pepsin present to compensate for the higher total phytate concentration and because gizzard pH was closer to the optimum of 2.8 for pepsin activity (Walk *et al.,* 2012). The higher water holding capacity of rice bran compared to wheat bran may have increased pH in birds fed the high susceptible phytate diets (Silveira and Badiale-Furlong, 2009).

6.5.1.3. Mineral Solubility and Absorption

The greater amount of soluble P in the gizzard and more soluble Ca and P in the jejunum and ileum in birds fed the high susceptible diets at d28 illustrates the greater response of this diet to phytase effects. When phytate is in excess, formation of soluble complexes between phytic acid and a metal ion produces predominantly 1:1 relative quantities of reactants and products, whereas when the relative

quantities are between 1:2 and 1:5, pure phytic acid metal salts precipitate. When metal ions are in excess, phytic acid becomes phytate (Vasca *et al*., 2002). Only very low or very high metal: ion ratios are able to then increase phytate solubility (Bohn *et al.,* 2008). In this study phytate was in greater excess in the high susceptible diet than the low susceptible diet, meaning the phytate was more soluble, hence phytate hydrolysis was greater.

At d14 Ca and P presence in the gizzard directly influenced mineral presence in the jejunum ($r=0.782$, $p<0.001$ and $r=0.771$, $p<0.001$ respectively), but mineral presence in the jejunum had no effect on the ileum due to absorption of minerals in the jejunum ($r=0.051$, $p=0.088$). At d28 however the opposite was true, Ca and P presence in the ileum was effected by that in the jejunum, whereas mineral presence in the gizzard had no impact on the jejunum. This suggests that bird response to phytase and mineral utilisation may vary with age depending on stage of growth and requirement. The high susceptible diet contained more wheat than the low susceptible diet, and the endogenous phytases in wheat are not inhibited by Ca^{2+} unlike endogenous phytases in many other cereals (Tang *et al.,* 2006). This potentially explains why the higher Ca levels in the high susceptible diet did not have a negative impact on phytate-P hydrolysis.

The lower mineral solubility in birds fed the low susceptible phytate diets compared to the high susceptible phytate diets may be because there was incomplete dephosphorylation of the inositol ring of phytate in birds fed this diet. This may have resulted in a higher presence of IP_4 and IP_3, and therefore less IP_6 for phytase to act on. It may also have increased the presence of IP_5 which is able to complex to cations. This suggests there may have been only partial release of Ca which may have promoted Ca-phosphate and Ca-phytate precipitation. Unlike IP_5, IP_4 and lower inositols do not reduce mineral absorption directly, but instead interact with phytate and contribute towards negative mineral absorption (Sandberg *et al*., 1999).This requires further investigation as the lower inositol esters were not measured in this study. Absorption of Ca was greater in birds fed the diets supplemented with phytase which potentially had a direct impact on performance due to reduced formation of insoluble calcium soap with fatty acids in the gut lumen (Diarra *et al.,* 2010). Also, phytate

forms complexes with Ca ions which are required for endogenous α-amylase activation, thus increased Ca availability increases carbohydrate metabolism (Liu *et al.*, 2008).

6.5.1.4. Pepsin Activity

In this study pepsin activity increased with phytase presence which is in agreement with the work of Liu *et al.* (2009) who found that phytase inclusion of 500 FTU/kg increased the activity of pepsin by 12%. This finding is however in contrast with some of the literature in which it is stated that phytase may spare excess pepsin secretion by hydrolysing phytate and alleviating the negative effect of phytate (Cowieson *et al.,* 2007); for example found Walk *et al.* (2012) found phytase level of 5000 FTU/kg reduced pepsin production by increasing its efficiency and Esmaeilipour *et al.* (2011) found that phytase increased stability of pepsin by 6.5% and hence reduced its production. Also, birds fed the high susceptible phytate diets had higher pepsin activity at both d14 and d28 than those fed the diets with low susceptible phytate. This may be because more phytate-complexes could be readily dissolved in birds fed the high susceptible phytate diets so there was a reduced inhibitory effect of phytate on pepsin. There was also more protein present for pepsin to hydrolyse, resulting in increased pepsin secretion. Pepsin activity was also strongly correlated with soluble P content in the gizzard ($r=0.676$, $p<0.001$), jejunum ($r=0.720$, $p<0.001$) and ileum ($r=0.743$, $p<0.001$) and soluble Ca content in the jejunum ($r=0.641$, $p<0.001$) and ileum ($r=0.722$, $p<0.001$) at bird age d28. This may be because pepsin releases protein from phytate-protein-mineral complexes (formed both in the gastrointestinal tract and in cereals as globoids) which weakens the complex and releases minerals. The observed effects may be partly because phytase neutralises the negative effects of phytate on protease activity and hence increases the presence of peptide end-products from protease digestion which are involved in protein digestion regulation. Phytase may therefore have increased pepsin activity by alleviating the suppressive effect of phytate on proteolytic enzymes, although this would require further investigation. Pepsin activity was higher at d14 than d28 possibly because digesta residence time in the younger birds was shorter and hence proportionately more pepsin per gram of digesta was secreted.

Phytase prevents precipitation of protein with phytate by increasing the rate of release of protein from a phytate-protein precipitate and inhibiting complex formation (Kies *et al.,* 2006) and phytate-protein aggregates leave unsuitable protein substrates for pepsin (Yu *et al.,* 2012). There were strong correlations between pepsin activity and both total and supplemented phytase activity at bird age d14 ($r=0.590$, $p<0.001$ and $r=0.566$, $p<0.001$ respectively) and d28 ($r=0.730$, $p<0.001$ and $r=0.701$, $p=0.001$) and between pepsin activity and the amount of phytate hydrolysed in the jejunum and ileum at d28 ($r=0.666$, $p<0.001$ and $r=0.546$, $p<0.001$ respectively). Pepsin is able to release protein from precipitates alone, but the process is speeded up by phytase.

At gastric pH, Ca has the same solubilising effects on protein and phytate as pepsin (Pontoppida *et al.,* 2007) and Ca ions dissociate insoluble phytate-protein complexes which form soluble Ca-phytate. This occurs through competition for binding to the phosphate groups of phytate between divalent cations and the positively charged sites of the protein. Low pH promotes pepsin activity and hence increases protein digestion, particularly digestion of amino acids that are associated with pepsin activity such as serine, glutamine, leucine, and tyrosine (Walk *et al*., 2012). The observed increased performance in birds fed the diets containing phytase may also be because hydrolysis of phytate leads to reduced mucin loss, increased mucosal protection from microbial growth, improved pancreatic enzyme activity and heightened amino acid digestibility. This is because phytate removes Ca from the mucin, due to its requirement for divalent cations, which alters its fluidity because Ca makes mucin smaller and more dense through to loss of intramolecular water (Moran, 2006).

6.5.1.5. Bone Strength and Mineralisation

The direct effect of phytase on both increasing hydrolysis of phytate-bound Ca and P and elimination of the antinutritional effects of phytate on divalent cations and other mineral chelators explains why birds fed the diets with phytase had increased tibia and femur Ca and P at d14 and d28 and improved bone strength at d28. Bone strength was more sensitive than ash content to dietary effects in this study potentially due to the methodology used (Rath *et al*., 2000). In this study susceptible phytate content had no impact on bone strength or ash content at either bird age studied. Birds fed the diets

supplemented with phytase had better tibia and femur strength at d28 possibly because phytase promoted bone development and helped maintain serum Ca values at their optimum in the blood, resulting in optimum bone development.

Biochemical adaptations mean that the proximal epiphysis of both the tibia and femur is affected most by diet, due to the impact and pressure put on the bones by ligament action and muscle tension to support the muscles during growth (Wideman and Pevzner, 2012). This explains why at bird age d28 there were strong correlations between BWG and tibia and femur length ($r=0.503$ $p<0.038$ and $r=0.549$, $p<0.001$ respectively), strength ($r=0.571$, $p<0.001$ and $r=0.418$, $p=0.003$) $p<0.001$ respectively) and width ($r=0.533$, $p=0.011$ and $r=530$, $p<0.001$ respectively). Femur length, strength, weight and width also correlated strongly with pepsin activity ($r=0.728$, $r=0.993$, $r=603$ and $r=702$ respectively, $p<0.001$ for all). This is possibly because, as stated previously, more Ca and P can be released from protein-phytate-mineral complexes because pepsin releases the protein from these complexes, subsequently weakening the bond between the phytate and minerals, and phytase helps speed up this process. Phytase presence may also have increased glycine and serine availability and digestion, resulting in increased collagen strength (Selle *et al.,* 2007). This, coupled with increased mineral solubility, resulted in more Ca and P present for bone formation, hence why there were also strong correlations between femur length, strength, weight and width and Ca solubility in the jejunum ($r=0.713$, $r=0.628$, $r=0.553$ and $r=0.687$ respectively, $p<0.001$ for all) and ileum ($r=0.816$, $r=0.710$, $r=0.681$ and $r=0.764$ respectively, $p<0.001$ for all) at bird age d28. In the growth phase the majority of Ca is used for bone formation in order to support body weight, with P deposition following Ca deposition (Barreiro *et al.,* 2009). The lower femur Ca and P content at bird age d14 and d28 and tibia P content at d28 in birds fed the low susceptible diet is largely due to reduced release of minerals from phytate-mineral complexes, but may also be partly because this diet had slightly higher fat content, which may have reduced intestinal absorption of minerals.

There are conflicting views as to whether the tibia or femur is most sensitive to the Ca:P ratio and the impacts of phytate on phosphorus. In this study the tibia was more sensitive than the femur to phytase

effects but the femur was more sensitive to phytate susceptibility than the tibia. This may be because the femur reacts most to changes in bodyweight and skeletal mineralisation partly because external torsion on femurs is greater than that of tibias, suggesting the effects observed in the femur may have been primarily a response to the indirect effects of phytate susceptibility on increasing bodyweight, as opposed to a direct effect of phytate hydrolysis on mineral availability (Applegate and Lilburn, 2002). Cellular sensitivity is however greater in the tibia than in the femur, especially at the epiphyseal end (Kocamis *et al.,* 2000) so the tibia response is a result of increased release of minerals from phytate by phytase. This difference emphasises why it is important to measure both tibia and femur mineralisation and strength in this type of study.

6.5.1.6. Conclusion on effect of feeding broilers diets differing in susceptible phytate content

The organic P component of feed ingredients fed to poultry exists in both phytase-susceptible and phytase-resistant forms, and binding of divalent cations to phytate can cause a portion of dietary phytate to be resistant to hydrolysis by phytase. This study showed that birds fed diets with high susceptible phytate content had significantly higher cumulative BWG and FCR, P solubility and phytate degradation in the jejunum and ileum, pepsin activity and femur Ca and P content at bird age d28 than birds fed diets with low susceptible phytate content. Consequently, accuracy of diet formulations and phytase matrix values could potentially be improved by measuring reactive phytate content of the individual ingredient being fed as opposed to an accepted total phytate content value for the ingredient, although further investigation is required to confirm this. This study also suggests that the effect phytase supplementation will have on bird health and performance could potentially be anticipated by measuring how much of the total phytate in a diet is susceptible to the effects of phytase *in vitro* prior to feeding.

6.5.2. Interactions between factors

The correlations seen between total and supplemented phytase activity and pepsin at both d14 and d28 are possibly because phytate binds to the peptide that activates pepsin, and hence obstructs

conversion of the zymogen to pepsin (Yu *et al.,* 2012) and because phytase alleviates the suppressive effect of phytate on pepsin. Phytase efficiency is dependent on phytate solubility which is affected by intestinal pH and Ca levels as illustrated by correlations between total and supplemented phytase activity and the Ca solubility in the jejunum and ileum at d14 and d28, ileum pH at d14 and gizzard pH and soluble Ca content at d28. As shown in this study, gastrointestinal pH varies considerably between individual birds resulting in large variation in Ca and P solubility and hence availability for absorption between individuals.

The ability of phytase to degrade phytate is negatively affected by high dietary Ca levels and high Ca: AvP ratios. The observed correlation between supplemented and total phytase activity and Ca and P solubility possibly relates to the ability of phytase to alleviate binding of minerals to phytate and hydrolyse phytate, which in turn balances the soluble Ca and P in the small intestine, reduces digestible P to Ca ratios and reduces dietary buffering capacity. The consequence of this is decreased precipitation of calcium phytate and calcium phosphate and hence improved digestibility of Ca and P and therefore improved performance and bone mineralisation, observed in this study as correlations between jejunum and ileum Ca and P solubility and both bodyweight and measured bone parameters. The correlation between phytate hydrolysed in the jejunum and ileal mineral solubility may be partly because phytate binds to metal ions that are required as cofactors for digestion. For example, α-amylase requires Ca as a cofactor (Cowieson *et al*., 2006) so increased secretion of pancreatic α-amylase, in response to presence of undigested food in the small intestine, would result in increased secretion of Ca, although these effects are likely to be very small and requires further investigation. Consequently, the secretion of cofactors is increased through negative feedback mechanisms due to phytate binding to the cofactors as well as to dietary proteins and digestive enzymes directly. Reduced apparent mineral digestibility by phytate may be due to reduced availability of dietary minerals for absorption, increased endogenous secretion of minerals in the gut lumen or a combination of the two. The correlation between pepsin activity and P solubility in the jejunum and ileum and Ca solubility in the ileum at d28 may be because phytase combats the negative effect of phytate on enzyme activity

and mucin production in the small intestine. Additionally there may have been reduced gizzard pH because HCl production was high in response to the presence of phytate-pepsin complexes and also the diets contained wheat and rice bran, so digesta retention time may have been high. It should however be noted that mineral solubility in water does not necessarily correlate with bioavailability, partly because a highly soluble minerals are more susceptible to dissociation and resulting binding to phytate.

The correlations between individual bird tibia and femur strength and body weight at d14 and d28 illustrates that birds with higher body weights may have stronger bones and thus body weight potentially dictates bone strength. Bone strength is also determined by degree of mineralisation of the bone matrix and volume and structure of the bone tissue (Boivin and Meunier, 2002), as seen by correlations between tibia and femur strength, width and length at d14. This is dictated by physiological maturity of the bird as well as nutrition, genes for expression of collagen and proteins, quantity and quality of the organic and inorganic material and structure of the bone (Rath *et al*., 1999; Boskey *et al*., 1999). It should however be noted that in this study bone length and width were measured with callipers which does not indicate bone curvature so this may not have been the most appropriate method; measuring bone length and width using a pachymeter may be a more accurate alternative (Soares Da Silva Araujo *et al.,* 2003).

In this study there were no correlations between bone strength and ash, Ca or P content in either the tibias or femurs at either bird age. This may be because in this study bone strength was measured by three-point bend test, which measures the ultimate load put on the bone until it breaks. This alone may not completely quantify bone strength, as it reflects just the rigidity of the bone and elastic deformation until the point where the bone is no longer resistant. It is therefore imperative to measure both bone strength and bone mineralisation to obtain a complete assessment of bone strength at different ages, as bone mineralisation indicates compressional strength. For example, Rath *et al.* (2000) found bone strength and ash peaked between d21 and d35, followed by reduced strength but not reduced bone ash.

The amount of phytate hydrolysed in the ileum and ileal soluble P content correlated with tibia and femur ash and Ca and P content at d28 but not at d14, possibly because bone density does not reach its peak until between d21 and d28 post-hatch (Talaty *et al*., 2010) and the amount eaten by the birds is lower and more varied between individuals at d14. Hypocalcaemia, due to reduced dietary Ca availability, leads to weaker bones and lower mineralisation through a reduction in collagen crosslinking and instigation of PTH secretion and vitamin D synthesis which releases bone minerals (Talaty *et al*., 2009). The interaction between Ca, P, vitamin D and other calcitropic hormones is complex and requires that Ca and P in poultry diets is balanced as Ca combines with P to form calcium-phosphate crystals. This is demonstrated by strong correlations between Ca and P in the tibia at d28 and femur at d14 and d28 and between tibia P and Ca and femur P and Ca at both d14 and d28. Dietary protein level also has a direct effect on bone strength as excess protein intake can cause negative Ca balance and hence inhibit bone growth, so it is necessary to supplement Ca with protein to maintain optimal Ca balance and bone health. The protein levels in the diets in this study were at a level that facilitated bone formation.

6.5.2.1. Conclusion on the relationships between factors that influence and respond to phytate degradation

This study showed that total and supplemented phytase activity directly increased pepsin activity at both d14 and d28, possibly due to hydrolysis of phytate which inhibits pepsin. This study also showed that total and supplemented phytase and pepsin activity increase jejunum and ileum soluble Ca and P content due to release of minerals from phytate-mineral and phytate-protein-mineral complexes. Interestingly, in this study it was phytate hydrolysis in the jejunum as opposed to the gizzard that significantly correlated with total and supplemented phytase, pepsin and tibia Ca content. Phytate susceptibility appears to impact on phytate hydrolysis in the small intestine as well as in the gizzard. This suggests that phytate susceptibility potentially influences endogenous bacteria and mucosa phytase efficacy, and that the assay used to measure susceptible phytate may need to be modified to

encompass exposing the samples to small intestine phase environmental conditions as well as gastric phase.

6.6. Implications

It is widely accepted that response to phytase is largely dictated by the concentration of phytate, level of supplemented phytase, the intrinsic properties and source of the phytase and feed particle size (Amerah and Ravindran, 2009). This study however suggests that the susceptibility of phytate to the effects of phytase should also potentially be considered when developing phytase matrix values and formulating diets as could act as a tool to indicate the response to phytase. Measurements of susceptible phytate content could therefore enable the effect phytase supplementation will have on bird performance to be anticipated prior to feeding, although this requires further investigation. This study also suggests a deeper understanding of the impact of supplemented phytase on pepsin activity is required, as it has been demonstrated that they work synergistically to enhance mineral availability.

CHAPTER 7: Conclusions and Recommendations

Phytate reactivity, and hence phytase efficacy, is dictated by conformation and configuration of the phytate molecule, the pH of the tract environment, the presence of substrates that may compete with phytate for mineral binding, the source of phytate and the volume of phytate ingested (Cowieson *et al.,* 2006). In this section the potential importance of determining phytate susceptibility to phytase and the effect of supplementing high levels of phytase in broiler diets will be discussed. The impact of pH on phytate susceptibility and protein-phytate interactions in the broiler gastrointestinal environment, and the impact of endogenous intestinal phytase on phytate degradation will also be discussed.

7.1. Availability and reactivity of phytate in ingredients and diets fed to broilers, and in the different environments of the broiler digestive tract

7.1.1. Phytate susceptibility to the effects of phytase

Measurements of total phytate concentration do not necessarily indicate substrate availability for phytase (Dayyani *et al*., 2013); this study suggests it may instead be measurements of phytate that is susceptible to the effects of phytase that could enable accurate determination of phosphorus availability for the bird. Measuring phosphorus release from phytate-P complexes after the diets have been exposed to conditions that mimic the GIT may therefore be the most accurate way to predict phytate degradation and enhance accuracy of phytase matrix values. This is illustrated through data presented in Chapter 6 in that BWG, FCR, P solubility and phytate degradation in both the jejunum and ileum, pepsin activity and tibia and femur Ca and P content was significantly higher in birds fed diets containing high susceptible phytate compared to those fed diets containing low susceptible phytate, despite a greater total phytate content in the high susceptible phytate diets. As shown in the study observing susceptible phytic acid content of common feed ingredients fed to poultry in the UK (Chapter 3 Section 3.5.), phytate susceptibility varies considerably between ingredients and between batches of ingredients, and total phytate content appears to bear no relationship to the susceptible

phytate content of an ingredient. Further investigation is required to determine feed ingredient combinations which have the greatest susceptibility to the effects of phytase. Both total and susceptible phytate content can be measured using simple colorimetric assays and measuring susceptible phytate takes little more time than that required to measure total phytate.

7.1.2. Superdosing phytase

The current phytase dosage in most commercial broilers is approximately 500 FTU/kg of *E. coli* phytase equivalents, but doses higher than this result in further improvements in bird performance, as illustrated in Chapter 4 when doses of 5000 FTU/kg were fed. Rather than just increasing release of minerals and amino acids from phytate-complexes, higher phytase doses increase phytate destruction so the extra-phosphoric anti-nutritional effects of phytate are alleviated. Phytate destruction results in subsequent production of lower inositol-phosphate esters (IP_1-IP_4) which are more soluble in the small intestine than IP_6 and IP_5 and have a lower capacity to chelate divalent cations. This suggests that feed ingredients currently viewed as undesirable due to their high phytate content could potentially be usable in poultry diets when combined with high phytase doses.

7.1.3. Gastrointestinal pH

This project illustrates that gastrointestinal pH has possibly the greatest impact on the interaction between phytate and feed components (illustrated in Chapter 4 Section 4.4.3., Chapter 5 Section 5.4.2. and Chapter 6 Section 6.4.2), alongside the presence of components that may compete with phytate for mineral binding and the volume and type of ingested phytate. Findings from this project suggest that phytase appears to reduce the requirements for HCl secretions which makes gastric pH closer to the optimum for pepsin and for phytate-complex degradation (pH <3), and hence improves ileal amino acid digestibility. Additionally, less intervention is required to alter pH which reduces gastric residence time (Maenz, 2001). The study presented in Chapter 4 supports the proposal that high phytase levels assist Ca release, potentially releasing sufficient Ca from Ca-phytate complexes to

maintain performance and bone ash, suggesting it may be advantageous to feed reduced Ca and high phytase diets in order to obtain optimum gastric pH.

7.1.4. Protein-phytate interactions

The isoelectric point of a protein source determines the strength of the interactions between phytate and protein, and hence the ability of phytases to release protein from phytate-protein complexes. At gastric pH, phytate has a strong positive charge and protein has a strong negative charge resulting in binary protein-phytate complexes that are relatively soluble (Selle *et al.,* 2012), hence why phytate is most susceptible to the effects of phytase here. At intestinal pH, both proteins and phytate are negatively charged and have strong electrostatic attractions with a net positive charge, resulting in formation of tertiary protein-phytate complexes which are not easily hydrolysed. In this project intestinal pH was lower, and hence further away from the isoelectric point, in birds fed diets with high phytate and low susceptible phytate content, which may have resulted in heightened presence of pepsin-resistant protein-phytate complexes being formed and hence reduced performance in birds fed these diets. This highlights the significant impact that GIT pH has on protein digestibility and hence bird performance.

7.1.5. Endogenous phytase

There is a common misconception that poultry lack any endogenous phytase, however, an investigation within this project (Chapter 5) illustrated phytase activity from intestinal mucosa, bacteria and the diet reaches levels of 45 U/kg in digesta by bird age d14 and the amount of total dietary phytate hydrolysed ranged from 21% to 36%. However, the impact of endogenous phytase remains small compared to the effect of supplemented phytase due to their site of action within the gastrointestinal tract: supplemented phytases work in the gastric phase, where phytate-complexes are relatively soluble, whereas endogenous phytases are produced mainly in the duodenum where phytate solubility is greatly reduced (Maenz and Classen, 1998), thereby reducing their potential efficacy.

7.2. Future Research and Development

The use of phytase in poultry feed, and hence interest in phytase research, is anticipated to increase globally in the future. This is driven largely by depletion of global phosphate supplies and increasing focus on sustainability. Inorganic phosphate supplies are non-renewable, the quantity and quality are declining and production costs are increasing. Increased efficiency of phytate-P utilisation could aid the preservation of global P reserves (Selle *et al.,* 2007). Research into phytase is not only driven by its economic and environmental impact on generating bioavailable P but also by recognition of its extra-phosphoric effects and the development of matrix values for amino acids and energy, as demonstrated in this project and other recent publications. This section explores the future of phytase and makes recommendations on how key findings from this project may be applied.

7.2.1. Zinc deficiency as a tool for measuring phytate effects

Zinc is the cation most vulnerable to formation of complexes with phytate and zinc deficiency in broilers occurs only when phytate is present (Kornegay, 2001). This suggests the degree of zinc deficiency could be used as an indicator of the chelating capacity and hence anti-nutritional impact of phytate, and phytate susceptibility to phytase. It may be advantageous therefore to measure zinc concentration in the duodenum when analysing the impact of phytate in nutrition studies. This is because the shift of pH in the duodenum causes phytate to readily seek cations (chiefly zinc) with which to complex, and subsequently the rate of zinc secretion into the duodenum via the pancreas exceeds the rate at which dietary zinc is consumed. This also suggests that zinc deficiency could be completely eliminated by supplementing diets with high levels of phytase. A valuable area of future research is the use of zinc responses as a tool to investigate phytate susceptibility.

7.2.2. Mineral chelators

Mineral chelators, such as EDTA and citrate, can assist the conversion of phytate from phytase-resistant to phytase-susceptible form in the gastrointestinal environment (Maenz *et al.,* 1999). Such chelators can remove the divalent cations that bind to phytate making that portion of it resistant to

phytase and hence increase susceptibility to phytase. It would be advantageous to determine the most effective competitive chelator, or combination of chelators, and the optimum supplementation level in a range of diets, with regards to enhancing phytase activity and improving phytate susceptibility. Chelators can also improve mineral solubility via competitive chelation; the chelator binds to minerals, therefore decreasing the amount of minerals available to form insoluble phytate-mineral complexes. Mineral-chelate complexes are soluble (Vieira, 2008) and can therefore be absorbed intact, or the minerals can be released to binding sites on the brush border membrane of intestinal epithelium. It is probable that chelators have a greater beneficial effect on diets that have high phytate, low endogenous phytase and high multivalent mineral levels, but this requires further analysis.

7.2.3. Improved analysis of tibia and femur health

This project has emphasised the need for clarity in measuring bone parameters. The 3-point bend method used in this project to analyse bone breaking strength was successful at detecting some dietary effects on bone formation. As this method measures only the ultimate load on the bone prior to breaking, only deformity and elasticity rather than compressional strength experienced by the bird are measured, suggesting its ability to truly reflect bone health may be questionable. This method also assumes that the bone is a compact cylinder and considers only external diameter of the bone, and hence does not take into account the considerable variability in internal composition of bones (Winter, 2008). Shear or torsion testing may be more advantageous methods for measuring bone breaking strength as these approaches account more for bone geometry and generate data that are more representative of the types of injuries seen at processing, although this would require extensive validation before the method could be universally adopted for measuring bone strength in poultry.

Bone mineralisation is considered to quantify compressional strength (Rath *et al.,* 2000), so bone ash content is often used as an indicator of strength. Bone mineral density, which is measured by methods including X-ray and ultrasound, is considered to more accurately reflect bone mineral content and quality and is valued because it is non-invasive (Barreiro *et al.,* 2011). Bone density is however subject to change based on the organic content the bone whereas ash content does not, and analysis of bone

density is more costly compared to analysis of ash content. This suggests that further investigation is needed into the most accurate method for measuring both bone breaking strength and mineral content. It is, however, clear that both need to be measured as alone these factors are not able to ascertain bone health; for example, a deformed bone will have different breaking strength compared to a normal bone even if they have similar mineral and organic matrices.

It would also be advantageous to measure other minerals in bones besides Ca and P as an indicator of bone health. With regards to measuring mineralisation, many authors have found that hydroxyapatite and aluminium have similar density, so it is potentially possible to reduce time spent measuring bone Ca and P content by measuring aluminium content using radiology and comparing it to the amount of Ca and P using a pre-determined scale (Almeida Paz and Bruno, 2006).

It may be beneficial to measure zinc in broiler bones because, as stated earlier, phytate is a potent zinc chelator and zinc is important for stimulating osteoblast activity and for suppressing osteoclast activity (Park *et al.,* 2013). Measurements of magnesium content in bones could indicate increased precipitation of Ca and P caused by phytate. This is because high levels of Ca and P cause manganese deficiency because precipitation of calcium phosphate in the intestinal tract leads to decreased absorption of magnesium (Bao *et al.,* 2010). It may also be valuable to measure sodium in bones because phytate has a negative effect on acid-base balance, due to its impact on Ca, which affects the impact that sodium has on acid-base balance and lowers osmotic pressure. The result of this is soft bones and retarded bone growth. Also, copper causes bones to become more fragile, epiphyseal cartilage to become thicker and vascular diffusion of thickened cartilage to be reduced (Mikulski *et al.,* 2009). Zinc, magnesium, copper and sodium deficiency can result in shortening and thickening of leg bones suggesting measurements of the length, width and strength could directly indicate the extent of the anti-nutritional effects of phytate. Additionally, there is evidence to suggest that silicon is localised in active calcification sites in bones and hence dietary silicon supplementation may improve bone development in broilers, suggesting it may also be advantageous to measure silicon in broiler leg bones (Elliot and Edwards, 1991). These minerals can all be measured at once using the ICP-OES

assay used to measure bone Ca and P content, therefore providing a powerful profile for no additional cost or time.

7.2.4. Improved phytase efficacy

Research effort is required to develop microbial phytases with greater capabilities for degrading phytate, resulting in a further decrease in P excretion and production of better extra-phosphoric responses. These phytases need to have higher specific catalytic activity (per unit of protein) and higher activity in wider pH ranges (Marquardt and Bedford, 2001).

There is evidence to suggest that the feeding of other enzymes alongside phytase could improve nutrient utilisation. For example, Ravindran *et al.* (1999) and Zyla *et al.* (1999) found that combinations of phytase and xylanase in wheat-based diets was advantageous, possibly because the xylanase facilitates substrate access for phytase and increases the absorption of nutrients liberated by phytase. Also, supplementing acid phosphatase with phytase, and combinations of 3-phytases, 6-phytases and acid phosphatases could accelerate dephosphorylation of phytate (Zyla *et al.,* 2004).

It is well established that higher phytase doses increase phytate degradation, but further investigation is needed into the maximum level that can be supplemented with beneficial results. Shirley and Edwards (2003) found that supplementing maize-soyabean meal diets with a maximum of 12,000 FTU/kg phytase resulted in phytate degradation of 94.8% which had a significant impact on bird performance, tibia ash and AME. Investigation is required into whether inclusion levels this high have any negative effects of broiler health, whether it is worth the extra financial cost and whether efficacy can be increased even further to achieve complete dephosphorylation of phytate.

7.2.5. Feeding ingredients with reduced phytate content

Recent identification of genes that suppress phytate synthesis, resulting in low-phytate seeds, suggest it may be possible to diminish phytate accumulation in feed ingredients (Nyannor *et al.,* 2007; Raboy, 2007) and hence reduce the requirement for P supplementation in poultry diets. Low-phytate seeds, including corn, barley and soyabeans, and high phytase crops, particularly wheat, have been shown to

have increased P bioavailability, but the adoption of this approach is dependent on global acceptance of genetically modified crops (Sands *et al.,* 2003; Hatzack *et al.,* 1999) and how readily available these crops are which will dictate the cost efficiency. Another possible method is to pre-treat feed ingredients with phytase to reduce their phytate content. This has been found to be a particularly advantageous approach in oilseed meals, for example Newkirk and Classen (2001) found that feeding broilers diets containing rapeseed meal that had been pre-treated with phytase resulted in higher amino acid digestibility. Additionally, the development of transgenic birds that are able to produce greater levels of endogenous phytase could be beneficial, although again this is dependent on public acceptance (Debnath *et al.,* 2005). The costs and advantages of these approaches however require further research.

7.3. Recommendations

- Dietary phytate that is susceptible to phytase effects as opposed total phytate content should potentially be considered when determining phytase matrix values. Both total and susceptible phytate content can be measured using simple colorimetric assays. For optimum response to phytase it may be beneficial to formulate diets based on the susceptible phytate content of the individual batch of ingredients being fed.
- Some feed ingredients have high total phytate content but also high susceptible phytate content, meaning they have the potential to be used as replacements for more expensive, unsustainable feed ingredients such as soyabean if used in the presence of phytase. This is particularly the case in diets supplemented with high doses of phytase (>500 FTU/kg), because high phytase doses alleviate the extra-phosphoric anti-nutritional effects of phytate as well as increase mineral availability.
- Intestinal and cereal-derived phytase activity is quantifiable in regulating phytate-P digestion, and should hence be considered when developing phytase matrix values.

- Excessive dietary limestone levels in poultry diets, and possibly in other species, potentially has negative implications on gastrointestinal pH due to the high buffering capacity of limestone (Morgan *et al.,* 2014).

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Appendix A

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animal

A comparison of two methods for determining titanium dioxide marker content in broiler digestibility studies

N. K. Morgan†, D. V. Scholey and E. J. Burton

School of Animal, Rural and Environmental Science, Nottingham Trent University, Southwell, Nottinghamshire, England, NG25 0QF, UK

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The use of inert markers in broiler diets eliminates the need to quantitatively evaluate feed intake and excreta output to determine diet digestibility, and enables nutrient uptake at specific points along the gastrointestinal tract to be examined. Titanium dioxide (TiO_2) is commonly used for this purpose and measured using a UV-spectrophotometric assay. Two experiments were conducted to observe whether an inductively coupled plasma optical emission spectrophotometer (ICP-OES) assay is able to replace the UV-spectroscopy assay for rapid analysis of TiO_2 in broiler feed and ileal digesta samples. In the first experiment, TiO_2 was added at 5 g/kg to 19 broiler diets. Ross 308 male broilers ($n = 452$) fed these diets were involved in a series of digestion studies to determine ileal digesta recovery of TiO_2. In the second experiment, defined amounts of TiO_2 were added to ileal digesta samples from Ross 308 male broilers ($n = 176$) and TiO_2 recoveries were determined. The feed and ileal samples from both experiments were analysed by both UV-spectroscopy and ICP-OES, and relatedness of the findings from the two assays was determined. Overall relatedness of the two assays was strong for determination of TiO_2 concentration in both the broiler diets and ileal digesta samples ($r = 0.908$ and $r = 0.884$, respectively). Overall recovery of supplemented TiO_2 was 97.62% by the UV-spectroscopy assay and 98.77% by the ICP-OES assay. The ICP-OES assay in this study was as accurate as spectrophotometric determination for the quantification of TiO_2 content. The ICP-OES method can also be used to analyse several elements within one assay, with a single preparation step, and thus the measurement of TiO_2 may be incorporated into the analysis of other minerals. Time and resources dedicated to determining diet digestibility in broilers could be minimised by using the ICP-OES assay to replace the UV-spectroscopy assay when measuring TiO_2 concentration.

Keywords: broiler, titanium dioxide, digestibility, methodology

Implications

Titanium dioxide (TiO_2) is commonly added as an inert marker to broiler diets to enable diet digestibility to be determined. This study demonstrates that an inductively coupled plasma optical emission spectrophotometer (ICP-OES) assay could replace the commonly used UV-spectroscopy assay for the determination of TiO_2 concentration in poultry diets and ileal digesta. This is advantageous because the ICP-OES assay used in this study has comparatively greater detection limits and sensitivity than the UV-spectroscopy assay. In addition, the ICP-OES assay enables TiO_2 determination to be incorporated into other mineral concentration analyses.

Introduction

Inert digestibility markers added to broiler diets eliminate the need to evaluate quantitative feed intake and excreta output, and enable nutrient utilisation to be examined along the gastrointestinal tract (Short *et al.*, 1996). Inert markers must maintain digestive transit at the same speed as other dietary nutrients in the tract and be physiologically inactive, as well as being non-toxic, easily analysed, able to be homogenously mixed into a diet, indigestible and non-absorbed (Jagger *et al.*, 1992; Titgemeyer *et al.*, 2001). Titanium dioxide (TiO_2) has some advantages over the commonly used chromic oxide (Cr_2O_3), with studies showing improvements in reproducibility and homogeneity (Jagger *et al.*, 1992). TiO_2 is also approved for use as a feed additive by the Food and Drug Administration, unlike Cr_2O_3 (Titgemeyer *et al.*, 2001). Another commonly used marker is acid-insoluble ash, but it has been suggested that its digestive transit does not accurately reflect that of feed passage (Cheng and Coon, 1990).

The method most widely used to determine TiO_2 concentration is UV-spectroscopy, primarily based around the method of Short *et al.* (1996). This method involves the initial hydrolysis of the sample with sulphuric acid (H_2SO_4) followed by a colour reaction. An intense orange/yellow colour results

† E-mail: nat.morgan@ntu.ac.uk

from the addition of hydrogen peroxide (H_2O_2) to an acidic titanium (Ti) solution, and the colour intensity can be quantified by UV-spectrometry. This method has been used successfully in several species including poultry (Short *et al.*, 1996), cattle (Titgemeyer *et al.*, 2001) and pigs (Jagger *et al.*, 1992), but some authors reported being unable to achieve reliable results using this process (Myers *et al.*, 2004).

In poultry research, TiO_2 as a dietary marker has been used successfully to determine calcium and phosphorus utilisation (Walk *et al.*, 2012). Mineral digestibility and utilisation in poultry is frequently analysed by inductively coupled plasma optical emission spectrophotometer (ICP-OES) in preference to UV methods as the ICP-OES assay can be used to analyse many elements in one preparation. Ti concentration can be detected by ICP-OES, which suggests that there is potential for TiO_2 measurement to be made concurrently with mineral content, thus reducing analysis time and resource use.

A comparison between a UV-spectroscopy assay and ICP-OES assay for determination of TiO_2 has previously been investigated by Boguhn *et al.* (2009) in turkey diets and digesta. In this paper, it was suggested that there was incomplete recovery of TiO_2 for both assays used, and hence values read to be lower than expected. However, detailed inspection of the results of the turkey data presented by Boguhn *et al.* (2009) confirms that for some of the samples the readings were higher than expected when the UV-spectroscopy assay was used, and lower than expected when the ICP-OES assay was used. This suggests that potentially neither, or just one, of the assays is producing values that are representative of the TiO_2 concentration in the sample. It is possible that the UV-spectroscopy assay is amplifying the value, and the ICP-OES assay is not detecting all the TiO_2 in the sample. The conclusion made by Boguhn *et al.* (2009) that both assays can be used to determine TiO_2 may therefore be questionable. Rodehutscord *et al.* (2012) have subsequently used ICP-OES to analyse TiO_2 concentration in broiler ileal digesta, indicating that the new ICP methodology is an attractive prospect to workers in the field, but highlighting that this is an area that requires further validation. Therefore, the aim of this study was to investigate consistency of TiO_2 recovery from an ICP-OES and a UV-spectroscopy assay, and evaluate whether the ICP-OES assay can be used as an alternative to the UV-spectroscopy assay for the determination of TiO_2 as a marker in poultry digestibility studies.

Material and methods

Birds and husbandry

For experiment 1, Ross 308 male broilers (n = 452) were involved in a series of digestion studies to determine ileal digesta recovery of TiO_2 either by UV-spectroscopy by the method of Short *et al.* (1996), or by an ICP-OES assay. Birds were fed 1 of 19 experimental diets in mash form, each with TiO_2 added at 5 g/kg: 6 semi-synthetic starch dextrose-based diets and 13 more commercial style diets based on cereals including wheat, rapeseed, maize and rye, and soya bean meal. All the 19 diets were analysed for TiO_2 concentration. Each diet was fed to a minimum of 20 birds. All birds were from breeder flocks aged 42 to 45 weeks and were obtained from a commercial hatchery on the day of hatch. Chicks were randomised by weight and placed in 0.64 m^2 floor pens in groups of four, bedded on clean wood shavings. Birds were allowed *ad libitum* access to the treatment diets and water for the duration of the trials, which spanned between 2 and 4 weeks. The room was thermostatically controlled to produce an initial temperature of 32°C and reduced to 21°C by day 21. The lighting regimen used was 24 h light on day 1, with darkness increasing by 1 h/day until 6 h of darkness was reached and this was maintained throughout the remainder of the study. Birds were euthanised by cervical dislocation. Digesta sample collection was carried out on a total of 144 14-day-old birds, 144 21-day-old birds and 164 28-day-old birds. At each bird age, digesta was pooled per pen of four birds, and averaged across diet. Digesta content was removed from the intestinal section distal to the Meckel's diverticulum and proximal to the ileo-ceco-colonic junction of each bird. The digesta samples were then freeze-dried and ground through a 1 mm screen.

For experiment 2, Ross 308 male broilers (n = 176) were fed a diet that contained no TiO_2 from day 0 to 42. The birds were from a breeder flock aged 43 weeks, and were obtained from a commercial hatchery on the day of hatch. Chick placing, room temperature and lighting regime were as previously described. Birds were allowed *ad libitum* access to the treatment diets and water for the duration of the trial. Digesta content was removed from the intestinal section distal to the Meckel's diverticulum and proximal to the ileo-ceco-colonic junction of each bird. The samples were freeze-dried and ground through a 1 mm screen. TiO_2 was subsequently added to the digesta samples at 0, 5, 10, 15 and 20 g/kg to encompass the range found in poultry digestibility studies.

All feed and digesta samples from both experiments 1 and 2 were analysed for TiO_2 concentration by both the UV-spectroscopy and ICP-OES assays described below.

Calibration standards

About 250 mg TiO_2 was dissolved in 100 ml of 7.4 M H_2SO_4 and diluted to 500 ml with distilled water to produce a standard Ti solution of 0.5 mg/ml. This standard solution was used to prepare the calibration curve for both the UV-spectroscopy and ICP-OES assays. For the ICP-OES assay, the TiO_2 standard solution was diluted with ultrapure water in varying increments to produce standards between 0 and 10 ppm. These standards were measured on an ICP-OES (Optima 2100 DV ICP-OES, model PQ Excell VG Elemental; Perkin-Elmer, Shelton, CT, USA) set to detect Ti at wavelength 334.936 nm, and a calibration curve was derived from the readings. For the UV-spectroscopy assay, graded volumes of TiO_2 standard solution was pipetted into individual 100 ml volumetric flasks and made up to 10 ml with 7.4 M H_2SO_4. About 10 ml 30% H_2O_2 was then added to the solutions

and the contents were made up to 100 ml with distilled water before measurement on a spectrophotometer (Unicam Helios, Berkshire, UK) set at 410 nm.

UV-spectroscopy assay

The UV-spectroscopy assay was based on the study by Short *et al.* (1996). Briefly, triplicate aliquots (~0.3 g) of each digesta sample and five replicates of each of the 19 feed samples were ashed in porcelain crucibles for 16 h at 650°C. Once cooled, 10 ml H_2SO_4 (7.4 M) was added to each crucible and the samples were heated for ~1 h until completely dissolved. The contents were then transferred quantitatively into 100 ml volumetric flasks via filter papers (Whatman 541) using distilled water. About 10 ml of 30% H_2O_2 was then added to each flask and the flasks made to volume with distilled water. Solutions were thoroughly mixed before reading on a spectrophotometer set at 410 nm. Sample analysis was repeated if the *Z*-value between the same samples exceeded 5%.

ICP-OES assay

For the ICP-OES assay, an aqua regia digestion step was carried out according to Association of Official Analytical Chemists 985.01. Briefly, 10 ml of aqua regia (35.5 to 37.5% hydrochloric acid and 68 to 72% nitric acid at a ratio of 3 : 1) was added to 50 ml glass conical flasks containing triplicate aliquots (~0.5 g) of each digesta sample and five replicates of each feed sample, and left at room temperature (14.4°C ± 0.15 s.e.m.) for a minimum of 12 h. The samples were then boiled until completely dissolved, for ~1 h. The contents were then filtered through Whatman 541 filter papers into 50 ml volumetric flasks and made to volume with ultrapure water, before transfering into 15 ml tubes. The samples were assayed on an ICP-OES set to detect Ti at wavelength 334.936. Sample analysis was repeated if the *Z*-value between the same samples exceeded 5%. Four digesta samples were repeated using a reduced sample size (~0.2 g) with eight replicates to assess whether smaller quantities of material were viable for the assay.

Statistical analysis

All data were analysed using IBM SPSS statistics version 21. *T*-tests were conducted to differentiate between means. The relatedness of the readings from each assay was investigated using Pearson product–moment correlation coefficient, and interpretations of the strength of the relationship between the two methods were based on guidelines by Cohen (1988); weak relationship $r = 0.10$ to 0.29, medium relationship $r = 0.30$ to 0.49 and strong relationship $r = 0.50$ to 1.0. Linear regressions were calculated using the true and measured Ti concentrations. Significance was accepted at $P < 0.05$.

Results and discussion

There were no significant differences between any TiO_2 concentrations measured by the UV-spectroscopy assay and the ICP-OES assay. There were consistently strong relationships between the two methods for analysis of TiO_2 concentration in the diets (Table 1) and ileal digesta (Table 2). This suggests that the ICP-OES assay used in this study is successful at identifying diet and ileal digesta TiO_2 concentration, and hence has the potential to replace the widely used UV-spectroscopy assay.

Table 1 *Relatedness of an ICP-OES assay and UV-spectroscopy assay for determination of TiO_2 concentration in broiler diets[a] (experiment 1)*

	Method of TiO_2 determination (g/kg)		
Diet	ICP-OES	UV spectroscopy	Relatedness[b]
Semi-synthetic starch dextrose[c]	6.03	6.29	0.684
Wheat soya bean[d]	5.93	5.09	0.794
Wheat soya bean (0 FTU/kg phytase)	5.85	5.97	0.778
Wheat soya bean (500 FTU/kg phytase)	5.71	6.08	0.759
Wheat soya bean (5000 FTU/kg phytase)	6.64	6.97	0.708
Wheat rapeseed (0 FTU/kg phytase)	6.11	6.53	0.886
Wheat rapeseed (500 FTU/kg phytase)	4.90	5.08	0.866
Wheat rapeseed (5000 FTU/kg phytase)	6.49	6.53	0.963
Maize rapeseed	6.87	6.98	0.995
Maize soya bean	4.99	4.88	0.956
Maize, rye, wheat, soya bean	4.87	5.16	0.758
Maize, rye, soya bean	5.75	5.47	0.689
s.e.m.	0.14	0.23	

ICP-OES = inductively coupled plasma optical emission spectrophotometer; TiO_2 = titanium dioxide; Ti = titanium.
[a]The average of a minimum of five replicates per diet, measured as per kg feed.
[b]Strength of the relationship between the ICP-OES and UV-spectroscopy method for Ti measured in each diet where confidence is the result is $P < 0.05$.
[c]The average measured TiO_2 content of six semi-synthetic starch dextrose-based diets.
[d]The average measured TiO_2 content of three wheat soya bean meal-based diets.

Table 2 *Relatedness of an ICP-OES assay and UV-spectroscopy assay for the determination of TiO_2 concentration in broiler ileal digesta[a] (experiment 1)*

	Method of TiO_2 determination (g/kg)		
Diet	ICP-OES	UV spectroscopy	Relatedness[b]
Semi-synthetic starch dextrose[c]	13.58	13.40	0.776
Wheat soya bean[d]	13.99	13.53	0.550
Wheat soya bean (0 FTU/kg phytase)	13.43	13.65	0.512
Wheat soya bean (500 FTU/kg phytase)	15.63	15.87	0.822
Wheat soya bean (5000 FTU/kg phytase)	13.32	12.42	0.887
Wheat rapeseed (0 FTU/kg phytase)	13.16	12.48	0.529
Wheat rapeseed (500 FTU/kg phytase)	14.19	14.95	0.613
Wheat rapeseed (5000 FTU/kg phytase)	12.92	12.71	0.858
Maize rapeseed	12.23	12.01	0.584
Maize soya bean	12.49	12.99	0.726
Maize, rye, wheat, soya bean	12.33	12.04	0.563
Maize, rye, soya bean	12.19	12.06	0.646
s.e.m.	0.20	0.26	

ICP-OES = inductively coupled plasma optical emission spectrophotometer; TiO_2 = titanium dioxide; Ti = titanium.
[a]The average response of a minimum of 28 birds per diet, 452 birds in total, with digesta samples collected at age 14, 21 or 28 days post-hatch. Analysis was replicated a minimum of three times per digesta sample.
[b]Strength of the relationship between the ICP-OES and UV-spectroscopy method for Ti measured in each digesta sample where confidence in the result is $P<0.05$.
[c]The average measured TiO_2 content of ileal digesta from birds fed one of 6 semi-synthetic starch dextrose-based diets, from 32 birds per diet, 192 birds in total, fed as 8 pens of 4 birds per diet.
[d]The average measured TiO_2 content of ileal digesta from birds fed 1 of 3 wheat soya bean meal-based diets, from 64 birds per diet, 192 birds in total, fed as 16 pens of 4 birds per diet.

The ICP-OES assay had to be modified to analyse ileal digesta samples in experiment 1 as some of the samples contained TiO_2 levels that saturated the ICP-OES detector, which compromised the sensitivity of the measurement. When a smaller sample size (0.2 g) was analysed, the samples all read in the optimum necessary range for detection by the ICP-OES, and therefore smaller quantities can be universally used to avoid any need to dilute the samples with ultrapure water. Coefficients of variation for the smaller sample size were <5%.

Relatedness between the two methods in determination of ileal digesta TiO_2 was numerically greater when phytase was included in the diets (Table 2). Phytase improves digestibility and therefore increases TiO_2 digesta content (Rutherfurd *et al*., 2004). The sensitivity of the UV-spectroscopy assay decreases as TiO_2 concentration decreases (Boguhn *et al*., 2009), whereas the sensitivity of the ICP-OES assay is consistent and not dictated by concentration in the sample. This suggests that in the presence of high TiO_2 concentration, such as in the digesta samples from birds fed phytase, the two assays were similar in sensitivity; however, in the samples with lower TiO_2 concentration, the similarity in sensitivity between the two assays reduced, and the UV-spectroscopy assay was comparatively less reliable. This also potentially explains why observed deviances in TiO_2 level in the diet away from the supplemented 5 g/kg were greater when analysed by UV-spectroscopy than by ICP-OES. The observed deviances are likely because dietary TiO_2 levels were measured per kg feed.

In this study, there were no significant differences between the measured values, or between the calculated slopes determined by the two assays for the analytical recoveries of TiO_2, whereas previous research has shown marked differences between the two assays (Boguhn *et al*., 2009). In addition, Boguhn *et al*. (2009) found that the values from the ICP-OES assay were lower than the expected values, which was not the case in this study (Tables 1 and 2). This may be because of the shorter digestion time used (25 min in contrast to 60 min), and therefore there may have been incomplete dissolution of the samples. Further verification of full Ti recovery was made in the second study where known amounts of Ti were added to digesta before quantification analysis via both methods. This found consistently strong relationships between the two methods at the different TiO_2 supplementation levels in the digesta samples (Table 3) and that the slopes produced by both methods were almost identical. The observed recovery of supplemented TiO_2 was 97.62% by the UV-spectroscopy assay and 98.77% by the ICP-OES assay in this study.

The main advantage of the ICP-OES assay when compared with the UV-spectroscopy is that the former has been shown to be more sensitive at quantitative analysis with improved detection limits. The ICP-OES assay is also less time-consuming, and the ICP-OES enables several elements to be detected in parallel, which reduces preparation time and the amount of sample, and hence potentially the number of birds required.

There are, however, some advantages to the UV-spectroscopy assay compared with the ICP-OES assay. The

Table 3 *Calculated slopes of linear regressions and relatedness of an ICP-OES assay and UV-spectroscopy assay for the determination of TiO_2 recovery at different levels in broiler ileal digesta[1] (± s.e.m.) (experiment 2)*

TiO_2 added to sample (g/kg)	Method of TiO_2 determination (g/kg)		
	ICP-OES	UV spectroscopy	Relatedness[3]
0	0.13 (±0.01)	0.15 (±0.03)	0.952
5	4.94 (±0.24)	4.79 (±0.32)	0.745
10	10.06 (±0.29)	9.84 (±0.21)	0.868
15	14.80 (±0.23)	14.63 (±0.27)	0.918
20	20.04 (±0.20)	19.74 (±0.44)	0.734
Slope[4]	0.999	0.998	

ICP-OES = inductively coupled plasma optical emission spectrophotometer; TiO_2 = titanium dioxide; Ti = titanium.
[1]The average response of spiked digesta pooled from 176 birds aged 42 days post-hatch. Analysis was replicated 10 times per sample.
[3]Strength of the relationship between the ICP-OES and UV-spectroscopy method for Ti measured in each digesta sample where confidence in the result is $P < 0.05$.
[4]Linear regressions where *y* was the measured Ti concentration and *x* was the true Ti concentration.

ICP-OES assay is more expensive owing to the cost to run the ICP-OES and to maintain the argon gas supplies, although this is mitigated by the potential for concurrent mineral analysis. The ICP-OES assay is also more hazardous as it involves the use of aqua regia, which is moderately more corrosive than H_2SO_4. Furthermore, the detection range is greater in the UV-spectroscopy method which reduces any potential need for dilution of samples; however, in this study, a reduced sample weight (0.2 g) was shown to overcome any requirement for dilution with the ICP-OES method.

In conclusion, the ICP-OES assay used in this study was successful in determining TiO_2 added as an inert marker in broiler digestibility studies and could replace the widely used UV-spectroscopy assay. The ICP-OES assay is more sensitive at quantitatively analysing TiO_2 concentration, consumes less time than the UV-spectroscopy assay and allows the TiO_2 determination to be carried out concurrently with other mineral analysis by ICP-OES. However, it is essential that the current sample weight (0.2 g digesta) is used for detection.

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Appendix B

The effect of dietary calcium inclusion on broiler gastrointestinal pH: Quantification and method optimization

N. K. Morgan,*[1] C. L. Walk,† M. R. Bedford,† and E. J. Burton*

*School of Animal, Rural and Environmental Science, Nottingham Trent University, Southwell, Nottinghamshire, England, NG25 0QF, United Kingdom; and †AB Vista Feed Ingredients, Woodstock Court, Blenheim Road, Marlborough Business Park, Marlborough, Wiltshire, SN8 4AN, United Kingdom

ABSTRACT There is little consensus as to the most appropriate methodology for the measurement of gastrointestinal pH in chickens. An experiment was conducted to establish the optimum sampling method for the determination of broiler digesta pH in birds fed differing levels of dietary calcium. Ross 308 broilers (n = 60) were fed 1 of 2 experimental diets, one containing 0.8% monocalcium phosphate and 2% limestone and one containing 0.4% monocalcium phosphate and 1% limestone. Four factors were investigated to determine the most appropriate method of measuring broiler gastrointestinal digesta pH: removal from the tract, prolonged air exposure, altering the temperature of the assay, and controlling the water content of the digesta. The conditions were assessed at bird ages from 7 to 42 d posthatch. Dietary Ca content had no significant effect on in situ pH, but it contributed toward variance in ex situ pH of both gizzard and duodenum digesta. Digesta pH read higher when the digesta was removed from the tract, but the amount of time the digesta was exposed to air did not affect the reading. Digesta pH read higher when measured at room temperature than when measured at 41°C; temperature made the strongest unique contribution to explaining variance in duodenum pH, and the second strongest contribution to explaining variance in gizzard pH, after diet. When water was added to the digesta, before pH determination, the pH of the digesta read higher ($P < 0.001$) than when measured in situ. The method that resulted in pH readings that were most representative of bird gastrointestinal environment was insertion of a pH probe directly into the gut lumen posteuthanasia, because measurement ex situ likely encourages dissociation of carbonic acid, the major buffer in the gastrointestinal tract, which causes pH to read to be higher than when measured in situ. This study shows that the method of pH measurement needs careful consideration to ensure the validity of the result.

Key words: broiler, dietary calcium, methodology, pH

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INTRODUCTION

Digesta pH is one of the major gastrointestinal (GI) factors that influence nutrient bioavailability (Pang and Applegate, 2007) and the intestinal microbiota (Hajati and Rezaei, 2010). It is imperative that broiler GI pH is kept at a constant optimal level as small changes outside the normal pH ranges (gizzard 1.2–4 and duodenum 5.7–6.5; Pang and Applegate, 2007; Jiménez-Moreno et al., 2009; Walk et al., 2012) can have significant negative implications on digestion and mineral absorption (Bristol, 2003). Accurate determination of digesta pH in broiler chickens could therefore act as a tool to indicate the potential for optimum gut health and maximum nutrient absorption.

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[1]Corresponding author: neil.morgan@ntu.ac.uk

The current methodologies used for digesta pH determination in broilers are based predominantly on historic techniques, with the most frequently cited being almost 30 yr old (Hurwitz, 1980; Clunies and Leeson, 1984). The majority of methods involve the use of a pH meter with a handheld probe, but sample handling before pH testing varies among studies; in particular whether the measurement is determined in situ or ex situ. To investigate limestone and phytase effects on intestinal pH, measurements were taken directly from the digesta contents in the lumen by Walk et al. (2012). In this study, a pH probe (Sensorex S175CD, Garden Grove, CA) was inserted directly into the gut lumen, through openings made by separating the sections of GI tract, immediately posteuthanasia. In this study, gizzard pH ranged from 1.76 to 2.63 and duodenum pH ranged from 5.86 to 6.24. A similar in situ method was carried out by Zou et al. (2009), based on the method of Manzanilla et al. (2006), to explore the effects of sodium butyrate in the GI tract in which a unipolar

electrode (no further details specified) was inserted through small incisions made in the gut wall. The gizzard pH in that study ranged from 3.02 to 3.21 and duodenum pH ranged from 6.16 to 6.20. Winget et al. (1962), however, measured GI pH in vivo to investigate the effect of fasting on GI pH in laying hens. To acquire small intestine pH, a pH electrode (GK2021, Radiometer, Copenhagen, Denmark) was inserted into an incision made in the small intestine under anesthetic, and to obtain gizzard pH the bird swallowed a pH electrode (Radiometer, G282A), and it was forced through the esophagus into the gizzard. Radiographs were taken to ensure the probes were in the correct position. In this study, gastric pH ranged from 3.17 to 3.48 duodenal pH ranged from 5.77 to 7.10. Although this method minimizes alteration of the GI environment through air exposure, its invasiveness precludes general use.

In contrast to the in situ methods discussed above, Engberg et al. (2004), González-Alvarado et al. (2008), and Jiménez-Moreno et al. (2009) removed the digesta before measuring pH to explore the effects of fiber source and heat processing, and the effects of whole wheat and xylanase, on GI pH. Gizzard pH ranged from 3.14 to 3.56 and duodenum pH ranged from 5.72 to 5.93 in these studies. These findings indicate that gizzard pH tends to be higher when measured ex situ than in situ. This suggests that the impact of removing the digesta from the tract is potentially a key factor affecting GI pH determination. This may be because exposure to air causes carbonate from dietary limestone, blood buffering capacity, and pancreatic secretions to dissociate to CO_2 and water (Guinotte et al., 1995), thus resulting in removal of hydrogen ions from the milieu (Zhang and Coon, 1997).

Some of the methods presently used to determine poultry GI pH involve the addition of water to the digesta before pH determination. For example, to investigate the effect of copper on the GI environment, digesta was removed and 9-fold dilution of deionized water was added, based on the digesta weight before pH determination (Pang and Applegate, 2007). In this study, pH in the gizzard ranged from 3.07 to 3.28 and in the duodenum, from 6.22 to 6.31. The same method was carried out by Hosslumand et al. (2011) and Esmaeilipour et al. (2011) to investigate the effect of nonantibiotic feed additives and the effect of xylanase and citric acid, respectively, on the GI environment; gizzard and duodenum pH ranged from 2.85 to 4.22 and 5.92 to 6.26, respectively, in these studies. To examine the effects of dietary Ca and fat on intestinal pH, Shafey et al. (1991) flushed the GI tract from the base of the gizzard with 2 mL of distilled water, and then added an additional 5 mL to the digesta before measuring pH; duodenal pH in this study ranged from 5.86 to 6.24. Also, to investigate the effect of citric acid and phytase on GI pH, Nourmohammadi et al. (2011) added 90 mL of sterilized physiological saline (1:10 dilution) to 10 g of digesta content; gizzard pH ranged from 3.09 to 3.23 and duodenum from 5.71 to 5.80. Methods involving diluting digesta samples before pH determination have been observed as far back as 1969, when Bowen and Waldroup (1969) examined the influence of propylene glycol on GI pH. In that study, the gizzard pH ranged from 2.47 to 3.06, and the duodenum pH ranged from 5.46 to 6.65. It can be noted from these results that pH generally reads higher in diluted digesta samples than those determined in situ. This indicates that a further potential issue to consider is variation between samples based on hydrogen ion concentration, that is, how diluted the digesta is by recent water consumption or by addition of water to digesta before pH determination.

In laying hens, the impact of varying volume and source of limestone in a diet has been extensively researched, but in broilers there are limited published data. There is a perception that there are no issues surrounding overinclusion of limestone in broiler diets. However, a combination of both the high buffering capacity of carbonate and an elevated pH caused by presence of Ca leads to raised digesta pH levels (Ekmay and Coon, 2010). An increase in GI pH in broilers fed high Ca from limestone reduced apparent ileal CP digestibility (Walk et al., 2012). Although mineral research tends to prioritize P, as it is nonrenewable and hence increasingly expensive, the potential negative effects of incorrect limestone supplementation, especially with regard to GI pH, should not be discounted.

The aims of this study were to establish the optimum sampling method for the determination of broiler digesta pH that is most representative of the GI environment and, subsequently, to determine the effect of dietary limestone inclusion level on digesta pH. The sampling methods assessed were the effect on pH of removing the digesta from the gut, subjecting the digesta to prolonged air exposure, altering temperature of the digesta pH assay, and controlling the amount of water present in the digesta, in birds fed 1 of 2 dietary limestone levels.

MATERIALS AND METHODS

Birds and Husbandry

Ross 308, male broilers (n = 60) from a 42-wk-old breeder flock were obtained from a commercial hatchery at day of hatch. Chicks were randomized by weight and placed in 0.64 m^2 floor pens in groups of 6, bedded on clean wood shavings. Birds were allowed ad libitum access to the treatment diets and water for the duration of the trial. The room was thermostatically controlled to produce an initial temperature of 32°C, reduced to 21°C by d 21. The lighting regimen used was 24 h of light on d 1, with darkness increasing by 1 h a day until 6 h of darkness was reached, and this was maintained throughout the remainder of the study. All birds sampled were euthanized by cervical dislocation. This occurred at the same time each sampling day, after at least 6 h of light, to ensure maximal gut fill. Institutional and national guidelines for the care and use of

Table 1. Composition and nutrient content of experimental diets

Item	High diet	Low diet
Ingredient, %		
Wheat	66.4	67.8
Soybean meal, 48% CP	25.0	25.0
Lysine	0.30	0.30
Methionine	0.25	0.25
Soy oil	4.00	4.00
Limestone	2.00	1.00
Monocalcium phosphate	0.80	0.40
Sodium chloride	0.25	0.25
Sodium bicarbonate	0.15	0.15
Broiler trial supplement[1]	0.40	0.40
TiO_2	0.50	0.50
Calculated composition		
CP, %	20.1	20.3
Gross energy, kcal/kg	4,660	4,660
Total P, %	0.55	0.46
Total Ca, %	1.10	0.65
Lys, %	1.23	1.24
Met, %	0.52	0.53
TSAA, %	0.84	0.84
Sodium, %	0.18	0.18
Potassium, %	0.80	0.80
Chloride, %	0.27	0.27
Analyzed composition		
CP, %	20.8	20.4
Gross energy, kcal/kg	4,610	4,750
Total P, %	0.78	0.44
Total Ca, %	1.27	1.31

[1]Supplied per kilogram of diet: manganese (manganese sulfide and manganous oxide), 100 mg; zinc (zinc oxide), 80 mg; iron (ferrous sulfate), 20 mg; copper (copper sulfate), 10 mg; iodine (calcium iodate), 1 mg; molybdenum (sodium molybdate), 0.48 mg; selenium (sodium selenite), 0.25 mg; folic (folic acid), 1.5 mg; vitamin A (retinyl acetate), 13.5 mg; vitamin E (DL-α tocopherol acetate), 100 mg; vitamin D_3 (cholecalciferol), 5 mg; vitamin B_2 (thiamine mononitrate), 3 mg; vitamin B_2 (riboflavin), 10 mg; vitamin B_3 (niacinamide), 60 mg; vitamin B_5 (calcium pantothenate), 15 mg; vitamin B_6 (pyridoxine HCl), 3 mg; vitamin B_{12} (cyanocobalamin), 30 mg; vitamin K (menadione sodium bisulphate complex), 5.0 mg; biotin, 125 mg.

animals were followed, and all experimental procedures involving animals were approved by the University College of Science ethical review committee.

Dietary Treatments

Experimental diets were formulated to be as nutritionally similar as possible, with the exceptions of P and Ca, and to meet the requirements of the age and strain of bird. The low diet was formulated at a low level of Ca and P (0.4% monocalcium phosphate and 1% limestone), and the high diet was formulated to contain double the inclusion levels of Ca and P (0.8% monocalcium phosphate and 2% limestone). These levels were chosen to produce a measurable difference in digesta buffering. This resulted in 2 dietary treatment groups with each treatment replicated by 5 pens of 6 chicks each (30 chicks/dietary treatment). After dietary treatment allocation, individual birds within pens were subsequently assigned to a designated sampling method as detailed in the methodology below for each experiment.

Table 2. Acid-binding capacity (ABC) and buffering capacity (BUF) of the experimental diets

Item	High limestone diet[1]	Low limestone diet[2]
pH[3]	6.31	6.29
SEM	0.04	0.09
ABC-2[4]	5,300	4,000
ABC-3[5]	2,900	2,400
ABC-4[6]	1,800	1,350
SEM	70.7	75.0
BUF-2[7]	1,208	860
BUF-3[8]	887	747
BUF-4[9]	675	707
SEM	26.3	40.0

[1]High limestone diet contained 0.80% monocalcium phosphate and 2% limestone.

[2]Low limestone diet contained 0.40% monocalcium phosphate and 1% limestone.

[3]Initial pH of samples.

[4]Acid-binding capacity to pH 2.

[5]Acid-binding capacity to pH 3.

[6]Acid-binding capacity to pH 4.

[7]Buffering capacity to pH 2.

[8]Buffering capacity to pH 3.

[9]Buffering capacity to pH 4.

The limestone in the diets had a particle size of 1 to 2 mm (average US standard screen number 14). Sodium bicarbonate was added to the diets to reduce total chloride content. Diets were fed in mash form, mixed in house, and were analyzed for gross energy by bomb calorimetry (Robbins and Firman, 2006), DM, and protein content (calculated as nitrogen multiplied by 6.25) by the AOAC International (1995, 2001) standard methods (930.15 and 990.03, respectively). Phosphorus and Ca content of the diets were analyzed by inductively coupled plasma-optical emission spectroscopy following an aqua regia digestion step (AOAC 985.01). Calculated and analyzed values for each diet are shown in Table 1.

Acid binding capacity and buffering capacity of the diets were determined based on the assay of Lawlor et al. (2005). A 0.5-g sample of diet was suspended in 50 mL of ultra-pure water with continuous stirring. The suspension was then titrated with 0.1 mol/L of HCl so that approximately 10 additions of titrant were required to reach pH 2.0. The pH readings after each addition were recorded following equilibration for 3 min. Acid-binding capacity was calculated as the amount of acid in milliequivalents required to lower the pH of 1 kg of food to pH 2, 3, and 4. This was repeated 5 times per diet. The analyzed values are presented in Table 2.

Experiment 1: Effect of Diet and Age on Gizzard and Duodenum Digesta pH In Situ

Forty-eight birds were used to assess the effect of varying dietary limestone content and the effect of bird

age on digesta pH. Sampling was carried out on 8 birds per day (4 birds on each diet per day), on d 7, 14, 21, 28, 35, and 42 posthatch. Immediately posteuthanasia, the gizzard was removed intact and a digital pH meter (Mettler-Toledo, UK) with a spear tip piercing pH electrode (Sensorex S175CD) was directly inserted into the digesta in the lumen of the proximal gizzard (proventricular opening), while ensuring the pH electrode did not touch the gizzard wall, and the pH was recorded. This was repeated 6 times, putting the probe in different areas of the gizzard each time (mean variability ± 0.07 SEM). The probe was rinsed with ultra-pure water once all 6 readings had been taken. The process was then repeated in the duodenal loop of the same bird. Readings were taken at the distal end of the duodenum; based on average length of the duodenum across the bird ages, the duodenum was cut at a point 30 cm from the gizzard (Yadav et al., 2010), and the pH electrode was inserted directly into this opening. Again, measurements were repeated 6 times (mean variability ± 0.04 SEM). The tip of the pH probe was stored in pH 4 solution when not in use.

Experiment 2: Effect of Removing Digesta from the GI Tract on Determining Digesta pH

Twenty-four birds were used to assess the effect of removing the digesta from the GI tract on measuring digesta pH. Sampling was carried out on 8 birds per day (4 birds on each diet per day), on d 7, 14, and 28 posthatch. Immediately posteuthanasia, in situ gizzard and duodenal pH were determined, as previously described, for every bird on each sampling day (mean variability ± 0.06 SEM and ± 0.03 SEM, respectively). For one-half of the birds (n = 4; 2 on each diet, per sampling day), the digesta was removed immediately after in situ pH had been determined and was put into centrifuge tubes that had been maintained at room temperature (14.4°C ± 0.15 SEM). A stop watch was started the instant the digesta was put into the centrifuge tubes, and pH was recorded every 15 s for 3 min using a spear-tip electrode and digital pH meter. This entire process was carried out on the other half of the birds (n = 4; 2 on each diet, per sampling day), except the digesta was put into centrifuge tubes that had been previously warmed to 41°C in a water bath.

Experiment 3: Effect of Digesta Water Content on Digesta pH

Thirty-six birds were used to assess the effect of digesta water content on digesta pH. Sampling was carried out on 12 birds per day (6 on each diet per day), on d 21, 35, and 42 posthatch. Immediately posteuthanasia, in situ gizzard pH was determined as previously described (mean variability ± 0.04 SEM). The digesta contents were then transferred into 7-mL containers and weighed, and then immediately snap frozen using a dry ice/industrial methylated spirit mix. The frozen samples were freeze-dried, reweighed, and the average water content across all the samples was calculated. This process was repeated in the duodenum of the same bird (mean variation in situ ± 0.07 SEM). For each section of the tract, after freeze drying, the samples were reconstituted with a corresponding volume of deionized water (pH 6.95 ± 0.02 SEM) to ensure uniform water content equal to the average of all samples collected. The pH of the reconstituted digesta samples was then measured directly with 6 replicate readings per sample for the gizzard and duodenum (mean variability ± 0.06 and ± 0.03 SEM, respectively).

Statistical Analysis

All data were analyzed using IBM SPSS statistics version 21. In experiment 1, an ANOVA was conducted to determine 2-way interactions between bird age and dietary limestone content on in situ gizzard and duodenum pH. When means were significantly different, *t*-tests were conducted to differentiate between means. Statistical power calculations were used to predict sample size that would be required to predict differences in dietary limestone content effect at different pH measures. In experiment 2, multiple linear regressions, with individual bird number as a covariate, were used to determine the unique contribution and relatedness of time exposed to air (log time in seconds), digesta temperature, and diet on variance in gizzard and duodenal pH at d 7, 14, and 28. Interpretations of the strength between the relationships were based on those of Cohen (1988): small r = 0.1–0.29, medium r = 0.30–0.39, and large r = 0.50 to 1.0. *t*-Tests were conducted to make statistical comparisons between in situ pH and pH at the exponential time point where digesta pH ceased to fluctuate postremoval from the tract. Two-, 3-, and 4-way interactions between diet, time exposed to air, digesta temperature, and bird age were determined by multiple ANOVA. In experiment 3, *t*-tests were conducted to make statistical comparisons between in situ pH and the pH readings of the samples that had been reconstituted with water. Two- and 3-way interactions among diet, bird age, and sampling method (in situ or reconstituted with known water content) were determined by multiple ANOVA. Multiple linear regressions, with individual bird as a covariate, were used to determine the unique contribution and relatedness of digesta water content and diet on variance in gizzard and duodenal pH at d 21, 35, and 42. Pearson product-moment correlation coefficient was carried out to investigate the relationship between in situ pH and digesta DM at d 21, 35, and 42. Significance was always accepted at $P < 0.05$.

RESULTS AND DISCUSSION

This series of experiments investigated both the effect of dietary Ca level on digesta pH in broilers, and also whether pH is altered by sample retrieval methods. Only significant interactions are presented in the tables and discussed. If the interactions were not significant, the main effects were discussed.

Effect of Diet and Age on In Situ Gizzard and Duodenum pH

The in situ gizzard and duodenal pH values in this study were not significantly different from those found throughout the literature, such as those presented by both Zou et al. (2009) and Walk et al. (2012). In experiment 1, an interaction ($P < 0.05$) was observed between bird age and dietary limestone content on in situ gizzard pH (Table 3). In general, gizzard pH fluctuated substantially (1.8 to 3.6) among the days measured. This may be partly due to the time that the birds were euthanized before sample collection; the anterior tract is emptied during dark periods, suggesting that feed intake (May et al., 1990), and thus retention time in the tract, may vary between birds. Another possible explanation for this variation is that the birds were fed a mash diet and hence may have selected Ca from the diets (Wilkinson et al., 2011) and modified diet consumption based on Ca requirements.

There was no relationship between gizzard pH and bird age, which is in agreement with the work of Angel et al. (2010). Gizzard pH was, however, significantly higher in birds fed the high limestone diet compared with birds fed the low limestone diet on d 7, 14, and 35 (Table 3). This may be largely due to the greater buffering capacity of the high limestone diets compared with the low limestone diets (Table 2). Similar findings have been observed throughout the literature; for example, gizzard pH was 2.37 compared with 2.52 in birds (age 0–16 d) fed either a diet containing 0.64 or 1.03% Ca, respectively, in a study conducted by Walk et al. (2012), and in a study by Guinotte et al. (1995) gizzard pH in immature birds was 2.70 compared with 3.82 in diets containing either 10 or 36 g/kg of Ca, respectively. This observed increase in pH with higher dietary limestone content in the gizzards of generally younger birds may be because they are more vulnerable to alterations in the GI environment, and they are unable to react to the increased bicarbonate load by increasing proventricular HCl secretion because of the immaturity of the gizzard (Coutu and Craig, 1988; Winkler et al., 1996). This, however, does not explain the re-emergence of this observed finding in the d 35 birds.

Conversely, on d 28 and d 42, gizzard pH was higher in birds fed the low limestone diet, and diet had no influence on gizzard pH at d 21. This finding is difficult to reconcile alongside findings from other ages. A possible explanation is that feed intake was increased and gizzard retention time reduced to meet the high demand for Ca (Zhang and Coon, 1997), thereby exceeding capacity to secrete sufficient HCl to maintain acidity of digesta in the gizzard. Unfortunately, feed intake, relative gizzard size, and digesta transit rate were not measured in this study, so this theory cannot be verified at this point. The low sampling sizes, and high variability in gizzard pH, suggest that further investigation with more birds is needed to fully evaluate these findings.

In the duodenum no interactions or significant effects of dietary Ca level or bird age were observed on digesta pH (data not shown). Previous studies have suggested that alteration in gizzard pH subsequently affected duodenal pH via manipulation of bacterial colonization of the lower digestive tract (Duke, 1992; Fernandez et al., 2002). However, the current study does not reflect

Table 3. Influence of dietary calcium level and bird age on in situ gizzard pH of broilers (experiment 1)[1]

Item	High limestone diet[2]	Low limestone diet[3]	No. of birds required to predict dietary differences[4]
Age, d			
7	2.42[c]	3.33[a]	10
14	2.71[b]	2.42[c]	17
21	1.88[e]	1.86[e]	28
28	2.18[d]	2.30[d]	22
35	3.84[a]	2.22[c]	9
42	2.47[c]	3.39[b]	6
SEM		0.20	
Diet × age		<0.001	

[a–e]Means within a row with no common superscript are different ($P < 0.05$).

[1]Means represent the average of 8 birds per day, 48 birds total, with 4 birds per diet each day.

[2]High limestone diet contained 0.86% monocalcium phosphate and 2% limestone.

[3]Low limestone diet contained 0.46% monocalcium phosphate and 1% limestone.

[4]Number of birds necessary to predict differences between the high limestone and low limestone effect on gizzard pH, based on statistical power calculation.

Table 4. Influence of dietary calcium level, bird age, method, and digesta temperature on gizzard and digesta pH of broilers measured ex situ over a 3-min time period (experiment 2)[1]

	High limestone diet[2]				Low limestone diet[3]			
		Ex situ				Ex situ		
Item	In situ	Ambient temperature, 14°C	Water bath, 41°C		In situ	Ambient temperature, 14°C	Water bath, 41°C	
Gizzard, d of age								
7	2.42[f]	2.65[e]	2.40[f]		2.33[f]	2.56[e]	2.37[f]	
14	2.71[d]	2.84[c]	2.78[d]		2.42[e]	2.69[d]	2.60[e]	
28	2.18[g]	2.27[f]	2.19[g]		2.36[e]	2.31[f]	2.21[g]	
SEM				0.079				
Diet × age				0.017				
Temperature × age				0.001				
Temperature × method[4] × diet				0.003				
Duodenum, d of age								
7	5.85[f]	5.98[e]	5.86[f]		5.80[f]	6.12[b,c]	5.98[e]	
14	6.14[b]	6.24[a]	6.16[b]		6.10[b]	6.26[a]	6.15[b]	
28	5.93[g]	5.81[h]	5.89[g]		5.78[h]	5.82[h]	5.87[h]	
SEM				0.100				
Temperature × age				0.048				
Method[4]				0.033				

[a–h]Means within a row, and within a column, with no common superscript are different ($P < 0.05$).

[1]Means represent the average of 8 birds per day, 24 birds total, with 4 birds per diet each day.

[2]High limestone diet contains 0.80% monocalcium phosphate and 2% limestone.

[3]Low limestone diet contains 0.45% monocalcium phosphate and 1% limestone.

[4]pH measured in situ or at 75 s (the highest exponential point) after the digesta had been removed from the tract.

this finding. This may be due to methodical differences in the sample handling before pH measurement. The number of birds necessary to predict differences in duodenum pH between the 2 diets was highest at d 21 and lowest at d 35, with 25 birds and 9 birds required, respectively. Similar figures were also observed in the gizzard (Table 3) highlighting that variation between individual birds, regarding the effect of diet on GI pH, is detectable in both the gizzard and duodenum. This, however, requires further investigation, because there was slight variation between numbers of birds required at the other bird ages.

Effect of Removing Digesta from the GI Tract on Determining Digesta pH

In experiment 2, there was no effect ($P > 0.05$) of temperature × diet × age on gizzard pH. However, gizzard pH was significantly higher in birds fed the high limestone diet compared with those fed the low limestone diet, but only on d 14. There was no effect of diet on gizzard pH on d 7 or d 28, but there was a numerical increase in gizzard pH in birds fed the high limestone diet at d 7, which resulted in a diet × age interaction ($P < 0.05$; Table 4). This increase in pH caused by high dietary limestone presence has possible negative implications for Ca and P utilization, because at high pH hydrolysis of phytate-Ca complexes is reduced, as most microbial phytases are active only at low pH. Additionally, at low pH Ca and P are relatively soluble, and are hence unlikely to precipitate, but at higher pH phytate-mineral complexes are more insoluble (Selle et al., 2000), so precipitation of Ca, P, and phytate is likely. Gizzard pH decreased from d 7 to 28, which may be due to an increase in DM content of the digesta due to heightened feed intake. The findings from this study suggest that high dietary inclusion levels of limestone potentially has a detrimental effect on gut pH, but further investigation using a larger population of broilers would be needed to fully identify the extent of this effect on phytate.

Maintaining samples at room temperature after removal from the tract led to gizzard pH readings being consistently higher ex situ than in situ, but when the digesta pH was measured ex situ in samples maintained at 41°C, this was not always the case (Table 4). An interaction ($P < 0.05$) was observed between temperature and bird age on digesta pH in the gizzard (Table 4). On d 7 and 28, gizzard pH was significantly higher when measured at room temperature than when measured at 41°C, but temperature had no effect on gizzard digesta pH on d 14. Similar to the gizzard, duodenum pH was numerically higher when measured at room temperature than when measured at 41°C, with the exception of d 28 in birds fed the high diet, where duodenum pH was the lowest and not affected by temperature × age ($P < 0.05$; Table 4). This may be due to the small sampling size, gut maturity or high variability in duodenum pH. Digesta temperature made the strongest unique contribution to duodenum pH, and second strongest contribution to gizzard pH, when the effects of diet and time exposed to air were controlled for, and digesta temperature and pH were correlated (Table 5).

Table 5. Correlations and relative contributions of the effect of time exposed to air, temperature, and dietary limestone on digesta pH and dietary limestone effect on gizzard and duodenal digesta pH of broilers (experiment 2)[1]

	Gizzard pH			Duodenum pH		
Item	d 7 of age	d 14 of age	d 28 of age	d 7 of age	d 14 of age	d 28 of age
Relative contribution						
R-square	0.65	0.95	0.93	0.80	0.24	0.49
Beta[2]						
Time[3]	0.02	0.07	0.05	0.11	0.33	0.10
Temperature[4]	0.81	1.56	0.63	2.39	0.65	4.57
Diet[5]	1.40	3.14	1.79	1.26	0.18	0.52
Correlation[6]						
Time	0.02	0.07	0.00	0.07	0.33	−0.05
Temperature	0.59	0.08	−0.21	0.65	0.33	−0.36
Diet	−0.57	−0.40	0.13	0.39	0.10	−0.52

[1]Represent the average response of 8 birds per age, 24 birds in total, 4 birds on each diet at each sampling point.

[2]Coefficient to indicate statistically significant unique contribution of the factor.

[3]Log time of seconds digesta was exposed to air postremoval from the tract (15 to 180 s).

[4]Digesta measured at either room temperature (14.4°C) or at 41°C.

[5]Digesta of birds fed either the high limestone diet (0.8% monocalcium phosphate and 2% limestone) or low limestone diet (0.4% monocalcium phosphate and 1% limestone).

[6]Correlations between factor and pH readings.

The observed findings may have been confounded by individual bird variation; thus, further investigation is needed to fully consider the interaction between digesta temperature and bird age. This again highlights that measuring digesta pH in situ is likely to provide pH readings that are most representative of the GI tract environment of the bird.

The time of digesta exposure to air had no significant effect on gizzard or duodenum digesta pH, but initial removal of digesta from the tract lead to a numerical rise in pH before the readings plateaued (data not shown). This plateau may indicate the point at which no further CO_2 remains to be released from the carbonate in the digesta. Although time exposed to air had no significant effect on digesta pH, it did make the biggest unique contribution toward the variance observed in duodenal pH in 14-d-old birds (Table 5). This may be because at this bird age there were more Ca ions present in the digesta to influence pH. The effect of time exposure did not, however, significantly affect duodenal pH at this bird age because the factors of diet, time exposed to air, and digesta temperature accounted for only 24% of the variance in duodenal pH (Table 5). The generally observed increase in pH when measured ex situ compared with in situ in both the gizzard and duodenum (Table 4) is potentially attributable to CO_2 release from carbonate buffering pH on exposure to air by altering the equilibrium of carbonic acid dissociation toward water and CO_2. Further investigation is required to confirm this. It can therefore be speculated that a combination of both heightened pH buffering effect and reduced digesta temperature on exposure to air contributed to the observed increase in pH on removal of digesta from the tract. This suggests that measuring pH of digesta that has been removed from the tract may not be providing a true representation of any dietary effects on the GI tract environment.

Effect of Digesta Water Content on Digesta pH

In experiment 3, digesta from both the gizzard and duodenum were standardized with a known volume of water to identify the effect of dilution on the acidity of the sample. This was investigated to identify the influence of variation in water consumption by the bird on digesta pH. A secondary aim of this study was to identify if water addition to the sample before pH determination, as observed in published studies such as Pang and Applegate (2007), Smulikowska et al. (2009), and Mirzaie et al. (2012), was affecting the accuracy of the pH reading.

Diet had no effect on gizzard or duodenal pH in experiment 3 (data not shown). Digesta pH read higher ($P < 0.05$) in the samples that had been reconstituted with water compared with the in situ measurements in the gizzard and the duodenum (Table 6). The addition of water dilutes hydrogen ions, thereby reducing the acidity of the digesta. Despite both studies using the same range in dietary Ca concentration between treatments, Shafey (1999) found a significant effect of dietary Ca on digesta pH, which was not found in this study. This may be due to the substantial amount of distilled water (approximately 7 mL) added to the digesta before pH measurement in the study conducted by Shafey (1999). In the current study, the observed higher pH in the reconstituted samples suggests that adding water to digesta, coupled with removing the digesta from the tract before reading the pH, potentially reduces the accuracy of the reading and does not necessarily reflect the GI tract environment within the bird. Further investigation is needed into the influence that variation in water consumption may have on digesta pH because the method used in this study observes only the impact of a singular level of reconstitution on digesta pH.

Table 6. Influence of method and bird age on gizzard and duodenum pH of broilers (experiment 3)[1]

	Gizzard		Duodenum	
Item	In situ	Reconstituted[2]	In situ	Reconstituted[2]
Age, d				
21	2.14	2.65	5.87	6.39
35	2.92	3.51	6.17	6.48
42	3.04	3.73	6.05	6.29
SEM	0.18		0.06	
Method[3]	<0.001		<0.001	
Bird age	<0.001		0.039	

[1]The mean represents the average of 12 birds per age, 36 birds in total, 6 birds on each diet at each sampling point.

[2]Digesta samples that had been removed from the tract, snap frozen, freeze-dried, and reconstituted with a volume of water equal to the average of all samples collected for that section of tract.

[3]pH measured in situ or in samples that had been standardized with a known volume of water.

It is likely that freeze-drying had little direct effect on the pH of the digesta, or influence on the higher pH observed in the reconstituted samples (Table 6). This is based on the general acceptance that chemical reactivity in solid form corresponds to the pH of the aqueous solution before freeze-drying, referred to as pH memory (Govindarajan et al., 2006). Numerous studies observing the impact of freeze-drying on sample pH, for example Costantino et al. (1997) and Vakos et al. (2000), found that pH and behavior of proteins in an aqueous states were similar to those presented in the same solution after freeze-drying.

Digesta DM content of both the gizzard and jejunum was numerically higher in birds fed the low limestone diet compared with those fed the high limestone diet over bird age d 21, 35, and 42 (gizzard 502.50 g/kg ± 12.98 SEM and 467.74 g/kg ± 19.23 SEM, respectively, and duodenum 396.37 g/kg ± 12.49 SEM and 393.71 g/kg ± 14.77 SEM, respectively). This may be because feed intake of the low limestone diet was higher, to meet the demands for Ca. There were strong correlations between digesta DM and in situ pH in the gizzard at d 21 ($r = -0.765$), d 35 ($r = -0.649$), and d 42 ($r = -0.682$), and in the duodenum at d 21 ($r = 0.550$), d 35 ($r = 0.720$), and d 42 ($r = -0.741$), where confidence in the result was always $P < 0.05$. This supports the supposition that digesta water content influences GI tract pH. This is also illustrated in Table 7, whereby reconstitution with water was shown to make the biggest unique contribution toward the variance observed in duodenal pH, when the contribution of diet was accounted for, at all the bird ages in this experiment. Reconstitution with water also made the biggest unique contribution to gizzard at d 42, and made relatively high contributions in the other bird ages in this experiment. As bird age increased, effect of water content and diet on the variance in pH in both the gizzard and duodenum decreased (Table 7), likely due to increased gut maturity and hence ability to respond to alterations to the GI environment.

Sample handling profoundly affects pH determination in digesta. A key factor seems to be removal of the digesta sample from the tract because this appears to cause pH to alter from the in situ value. Removal of digesta from the bird also affects pH via an associated temperature reduction, which can be partially

Table 7. Correlations and relative contribution of reconstitution and dietary limestone on gizzard and duodenal digesta pH of broilers (experiment 3)[1]

	Gizzard pH			Duodenum pH		
Item	d 21 of age	d 35 of age	d 42 of age	d 21 of age	d 35 of age	d 42 of age
Relative contributions						
R-square	0.63	0.36	0.10	0.53	0.40	0.17
Beta[2]						
Reconstitution[3]	0.50	0.45	0.33	0.60	0.54	0.41
Diet[4]	0.82	0.70	0.04	0.47	0.48	0.35
Correlation[5]						
Reconstitution	0.51	0.39	0.33	0.66	0.57	0.36
Diet	−0.30	−0.35	0.04	−0.17	−0.30	0.22

[1]Data represent the average response of 12 birds per age, 36 birds in total, 6 birds on each diet at each sampling point.

[2]Coefficient to indicate statistically significant unique contribution of the factor.

[3]Digesta samples that had been removed from the tract, snap frozen, freeze-dried, and reconstituted with a volume of water equal to the average of all samples collected for that section of tract.

[4]Digesta of birds fed either the high limestone diet (0.8% monocalcium phosphate and 2% limestone) or low limestone diet (0.4% monocalcium phosphate and 1% limestone).

[5]Correlations between factor and pH readings.

mitigated through use of a water bath to maintain bird body temperature. However, this approach is not recommended because the buffering effect on removal cannot be overcome. Water content of the digesta was also shown to have a substantial effect on pH, but this could not be standardized without confounding results by removing the digesta from the tract. It can be concluded that the method that gives the most accurate representation of broiler GI tract environment when determining digesta pH is to insert a pH probe directly in situ into the gut lumen immediately postexsanguination. Generally, pH was higher in birds fed the high limestone diet compared with birds fed the low limestone diet, suggesting that excessive dietary limestone levels in broiler diets potentially has negative implications on GI tract pH. However, this conclusion requires verification in a larger study using the optimum sampling techniques described above and a wider range of limestone levels.

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Appendix C

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In vitro versus *in situ* evaluation of the effect of phytase supplementation on calcium and phosphorus solubility in soya bean and rapeseed meal broiler diets

N. K. MORGAN, C. L. WALK[1], M. R. BEDFORD[1], AND E. J. BURTON

School of Animal, Rural and Environmental Science, Nottingham Trent University, Southwell, England, UK, and [1]AB Vista Marlborough, UK

Abstract 1. *In vitro* assays provide a rapid and economical tool to evaluate dietary effects, but have limitations. In this study, the effect of phytase supplementation on solubility, and presumed availability, of calcium (Ca) and phosphorus (P) in soya bean meal (SBM) and rapeseed meal (RSM) based diets were evaluated both *in situ* and by a two-step *in vitro* digestion assay that simulated the gastric and small intestine (SI) phases of digestion.

2. Comparison of the *in vitro* findings to *in situ* findings was used to evaluate the *in vitro* assay. Ross 308 broilers (n = 192) were fed on one of 6 SBM or RSM diets supplemented with 0, 500 or 5000 FTU/kg phytase from 0 to 28 d post hatch. The 6 diets and raw SBM and RSM were exposed to a two-step *in vitro* assay. Ca and P solubility and pH in the gizzard and jejunal digesta and in the gastric and SI phase of *in vitro* digestion were measured.

3. Both *in vitro* and *in situ* analyses detected that Ca solubility was lowest when diets were supplemented with 500 FTU/kg phytase, compared to the control diets and diets supplemented with 5000 FTU/kg phytase. Phosphorus solubility increased with increasing phytase level. Both methods also identified that mineral solubility plateaus in the gastric phase.

4. Overall relationship of the two methods was strong for both determination of gastric phase Ca and P solubility (r = 0.96 and 0.92, respectively) and also SI phase Ca and P solubility (r = 0.71 and 0.82, respectively). However, mineral solubility and pH were higher when measured *in vitro* than *in situ*, and the *in situ* assay identified an interaction among the effects of phase, protein source and phytase inclusion level on Ca solubility that the *in vitro* assay did not detect.

5. This two-step *in vitro* assay successfully predicted phytase efficacy, but to determine detailed response effects in the animal, *in situ* data is still required.

INTRODUCTION

Dietary ingredients, phytate concentration and gastrointestinal pH are among the factors that dictate availability of dietary calcium and phosphorus. Mineral solubility is presumed to indicate availability following the studies of Shafey and McDonald (1991), Tamim *et al.* (2004) and Walk *et al.* (2012*a*). Despite having one of the lowest affinities with phytate, dietary calcium has the greatest practical impact on phytate-P availability due to its high inclusion levels in broiler diets (Maenz *et al.*, 1999). High dietary limestone levels instigate an increase in gastric pH, which shifts the pH away from the optimum (pH 1.8) for pepsin activity. Large ratios of Ca:P have also been associated with reduced phytase efficacy (Tamim and Angel, 2003; Tamim *et al.*, 2004) and hence reduced mineral availability (Selle *et al.*, 2000) and bird performance (Huff *et al.*, 1998; Cabahug *et al.*, 1999; Cowieson *et al.*, 2006).

Low intrinsic phytase activity in the small intestine (SI) phase (from the proximal duodenum to the distal ileum) of poultry, and low phytase activity in common poultry feed ingredients, lead to very limited release of P from phytate-P without the use of

Correspondence to: Natalie K. Morgan, School of Animal, Rural and Environmental Sciences, Nottingham Trent University, Southwell, Nottinghamshire, England NG25 0QF, UK. E-mail: nat.morgan@ntu.ac.uk

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exogenous phytases. However, some endogenous phytase activity has been detected in all sections of the broiler SI, but most particularly in the duodenum, indicating that optimum efficiency of endogenous phytase occurs at pH 5.5–6.5. However, as phytate–mineral complexes are most soluble in the crop, proventriculus and gizzard (collectively referred to as the gastric phase), hydrolysing phytate at these earlier regions of the gastrointestinal tract increases potential for mineral utilisation (Tamim *et al.*, 2004). As the pH of this gastric phase of the tract is less than 4 (Selle *et al.*, 2000), commercially available microbial phytases usually demonstrate optimum efficiency at this pH.

Digesta pH is one of the major gastrointestinal factors influencing Ca and P solubility (Walk *et al.*, 2012*b*). As pH increases along the gastrointestinal tract, the affinity of phytate for Ca is increased and more phytate–Ca complexes are formed. Small deviations from the normal gastrointestinal tract pH ranges (gizzard 1.2–4.0 and duodenum 5.7–6.5) (Pang and Applegate, 2007; Walk *et al.*, 2012*c*), such as those initiated by the high acid buffering capacity of limestone, may significantly reduce Ca and P absorption (Jimenez-Moreno *et al.*, 2009). This may subsequently result in an increase in gastrointestinal pH, and thereby reduce the mineral:phytate molar ratio required to precipitate phytate (Maenz *et al.*, 1999).

The concentration of Ca ions in the SI is thought to determine the rate of hydrolysis of phytate-P by endogenous phytase (Maenz and Classen, 1998). For example, intestinal phytase activity was 9% greater when birds were fed on diets containing 4 g/kg compared to 9 g/kg Ca from either $CaCO_3$ or Ca malate (Applegate *et al.*, 2003). Phytate-P hydrolysis by endogenous phytase is consequently reduced in the presence of high Ca; for example, the apparent absorption of P was 24.9% when broilers were fed on diets containing 5 g/kg Ca (as limestone) compared to 65.2% when Ca was not added (Tamim and Angel, 2003). There is a perception that there are no issues surrounding over-inclusion of limestone in broiler diets, but these findings suggest that the potential impact of high levels of Ca on exogenous phytase efficacy requires consideration and further investigation.

In vitro assays have the potential to act as rapid and cost-effective tools for the evaluation of both phytase efficacy and Ca and P availability in dietary ingredients. There are, however, limitations to *in vitro* methodologies: it is impossible to exactly reconstruct the environmental variability and interactions found *in situ*, so *in vitro* assays are able to measure degradability only and not digestibility. The overall aim of this study was to compare *in vitro* and *in situ* quantification of Ca and P solubility in a range of diets. The first objective was to examine the solubility of Ca and P in pure soya bean meal (SBM) and rapeseed meal (RSM) using a two-step *in vitro* assay procedure developed by Walk *et al.* (2012*a*). The second objective was to determine the influence of three levels of phytase on Ca and P solubility in SBM- and RSM-based diets both *in vitro* and *in situ*. The final objective was to evaluate the two-step *in vitro* assay by comparing Ca and P solubility determined *in vitro* to Ca and P solubility measured *in situ* in both the gastric and SI phase.

MATERIALS AND METHODS

Birds and husbandry

Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Nottingham Trent University College of Science ethical review committee.

Ross 308, male broilers (n = 192) from a 42-week old breeder flock, were obtained from a commercial hatchery on the day of hatch. Chicks were randomised by weight and placed in 0.64 m^2 floor pens in groups of 6, bedded on clean wood shavings. Birds were allowed *ad libitum* access to the treatment diets and water for the duration of the trial (d 0–28). The room was thermostatically controlled to produce an initial temperature of 32°C and reduced to 21°C by d 21. The lighting regimen used was 24 h light on d 1, with darkness increasing by 1 h per d until 6 h of darkness was reached and this was maintained throughout the remainder of the study.

Dietary treatments

Experimental diets were arranged as a 2 × 3 factorial design including two dietary protein sources (SBM or RSM) and three levels of phytase (0, 500 or 5000 FTU/kg). This resulted in a total of 6 treatment groups replicated by 8 pens of 4 chicks each (32 chicks/dietary treatment). Diets were given in mash form and formulated to meet or exceed Ross 308 nutrient requirements (Table 1). Diets were mixed in house and analysed for P and Ca content by ICP-OES (ICP-MS model PQ Excell VG Elemental, Connecticut, USA) following an *aqua regia* digestion step (AOAC, 985.01; Leytem *et al.*, 2008). Titanium dioxide was added as an inert marker for nutrient digestibility evaluation and the dietary content quantified by the method of Short *et al.* (1996). Total phytate content was analysed by a K-Phyt assay (Megazyme™, Wicklow, Ireland, UK) and phytase activity was analysed according to the method of Engelen *et al.* (2001). The formulated and analysed values for each diet are shown in Table 2. The phytase used in the experiment was an *Escherichia coli* 6-phytase with an expected activity of 5000 FTU/g (Quantum Blue™, AB Vista Feed Ingredients, Marlborough, UK).

Table 1. *Ingredient and nutrient composition of experimental diets*

Ingredient, g/kg	SBM diet	RSM diet
Wheat	605.9	605.2
Fishmeal, 72%	30.0	30.0
Rapeseed extruded	0.00	250.0
Soya bean meal, 46%	100.0	0.00
Soya hulls	70.0	0.00
Soya oil	5.59	5.44
Sodium chloride	3.6	3.6
Valine	2.1	2.2
DL-methionine	4.1	2.9
Lysine HCl	5.0	6.5
Threonine	2.4	2.4
L-tryptophan	0.1	0.2
Glycine	5.2	5.1
L-arginine HCl	3.6	3.5
Isoleucine	1.9	2.8
Limestone	10.0	8.5
Dicalcium phosphate	13.0	11.4
Choline oxide	0.2	0.2
Vitamin/trace mineral premix[1]	4.0	4.0
Titanium dioxide	5.0	5.0
Formulated composition		
Crude protein (g/kg)	201.1	198.6
ME (MJ/kg)	12.8	12.8
Calcium (g/kg)	10.0	10.0
Total P (g/kg)	6.8	7.7
Available P (g/kg)	4.5	4.5
Phytate P (g/kg)	5.4	6.4
Lys (g/kg)	13.5	13.5
Analysed composition		
Crude protein (g/kg)	238.7	251.4
ME (MJ/kg)	13.9	13.5
Calcium (g/kg)	15.3	12.7
Total P (g/kg)	6.7	7.5
Phytate P (g/kg)	5.4	6.5

[1]Supplied per kg of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.08 mg; selenium, 0.2 mg; retinol, 15.5 mg; cholecalciferol, 4 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 5 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 μg; folic acid, 1.5 mg; and biotin 125 mg.

Table 2. *Recovery of phytase activity in experimental diets*

Diet	Phytase[1] recovery (FTU/kg diet, as-fed)
Soya bean meal (SBM)	138
SBM + 500 FTU/kg phytase	457
SBM + 5000 FTU/kg phytase	4030
Rapeseed meal (RSM)	<80
RSM + 500 FTU/kg phytase	377
RSM + 5000 FTU/kg phytase	5280

[1]One unit of phytase activity (FTU) is defined as the quantity of enzyme that liberates 1 nmole of inorganic P per min from the bean phytate at pH 4.5 and 37°C. Each result is the mean of three replicates.

In situ procedure

Immediately after killing by cervical dislocation, on d 28, the gizzard was removed intact and a digital pH meter (Mettler-Toledo, Leicester, UK) with spear tip piercing pH electrode (Sensorex, California, USA) was inserted directly into the digesta in the lumen of the proximal gizzard (proventricular opening), ensuring that the pH electrode did not touch the gizzard wall, to record pH. This measure was repeated 6 times in different areas of the gizzard (mean variability ± 0.07). SI pH was recorded in the same way from the medial jejunum (the intestinal section distal to the duodenal loop and proximal to the Meckel's diverticulum; mean variability ± 0.06). After pH measurements were obtained, gizzard or jejunal digesta was pooled per pen and frozen at −20°C prior to freeze drying (LTE Scientific, Lancashire, UK) for 5 d to constant weight.

Soluble Ca and P content were determined in the gizzard and jejunal digesta and in the feed by a method based on Self-Davis and Moore (2000). Briefly, 2 g of sample was weighed into a pre-weighed bottle and 200 ml ultra-pure water (RCW 3000 water purifier for ion chromatograph, Millipore, Watford, UK) was added. The sample was placed on a shaker set at 200 rpm for 60 min prior to being centrifuged at 8600 *g* for 10 min. The supernatant was then filtered through Whatman #541 filter papers before measurement by ICP-OES, with wavelengths for Ca and P set at 317.933 and 213.617 nm, respectively. Six replicates were analysed for each sample. ICP standards were made by diluting 1000 mg/kg standard (Fisher Scientific, Loughborough, UK) and ultra-pure water. If it was not possible to carry out the ICP analysis immediately, 8 drops of concentrated HCl per 20 ml extract were added to acidify the samples. The percent Ca or P solubility was calculated according to the following equation:

$$(\text{Soluble Ca or P in digesta supernatant} / \text{Total Ca or P in the diet}) \times 100$$

In vitro procedure

A two-step *in vitro* assay procedure was used to investigate solubility of Ca and P in the gastric and SI phases of digestion in samples of RSM and SBM and in the 6 complete diets given in the previously described *in situ* study. This assay was based on that of Walk *et al.* (2012a) with the following modification: Ca and P concentration of the supernatants were analysed by ICP-OES as opposed to colorimetric analysis. Briefly, each feed ingredient or diet was ground through a 1 mm screen and 2.5 g was weighed into pre-weighed tubes. For each phase, gastric and SI, a minimum of 9 sub-samples were analysed for each diet and feed ingredient. Samples were analysed for Ca and P content in triplicate on the ICP-OES. To mimic the gastric phase, 4.5 ml of 0.13 N HCl, with 2000 U pepsin/ml (Sigma-Aldrich, Dorset, UK) was added to the pre-weighed samples before incubating at 41°C for 20 min. Sample pH was then obtained in triplicate using a spear tip

piercing pH electrode (Sensorex, California, USA) to ensure samples were within the target range of pH 3.5–4.5. Samples were then diluted to 20 ml with 0.1 M HCl and centrifuged at 11 000 × g at 4°C for 1 min before the tubes were weighed. Post-centrifugation, the supernatant was collected into a separate pre-weighed tube. The sample was again diluted to 20 ml with 0.1 M HCl, re-centrifuged and the supernatant collected. The pooled supernatant was filtered through a 0.22 µm filter (Fisher Scientific, Loughborough, UK) and stored at −20°C until further analysis. The filtered supernatant was diluted 1:10 with ultra-pure water and analysed for soluble Ca and P using ICP-OES set at 213.617 nm for P and 317.933 nm for Ca as previously described. The percent gastric Ca or P solubility was then calculated according to the following equation:

$$(\text{Soluble Ca or P in the gastric phase} / \text{Total Ca or P in the diet}) \times 100$$

For the intestinal phase, the samples were weighed and incubated for 20 min at 41°C with HCl and pepsin as for the gastric phase. Immediately after this initial incubation, 1.5 ml of $NaHCO_3$ containing 2 mg of pancreatin/ml (Sigma-Aldrich, Dorset, UK) was added to each sample before mixing and incubating the samples at 41°C for an additional 60 min. The contents of each tube were then made up to 45 ml with 0.32 M $HClO_4$ to stop the enzymatic reaction and weighed before centrifugation at 4°C at 7200 × g for 1 min. The supernatant was immediately filtered through Whatman 541 filter paper and diluted 1:10 with 1 M HNO_3. The diluted samples were then analysed for Ca and P content using ICP-OES as described previously. The percent of soluble Ca or P in the SI was calculated according to the following equation:

$$(\text{Soluble Ca or P in the SI} / \text{Total Ca or P in the diet}) \times 100$$

Statistical analysis

All data were analysed using IBM SPSS statistics version 19. Multiple ANOVA was used to determine the effect of protein source and phytase inclusion on solubility of Ca and P in the gastric and SI phase of both the *in vitro* and *in situ* samples. The statistical model included protein source, phytase inclusion level and digestion phase to investigate all two- and three-way interactions. Independent sample *t*-tests were used to directly compare the findings from the *in vitro* and *in situ* methodologies. The relatedness of the methodologies was investigated using Pearson product–moment correlation coefficient and interpretations of the strength of the relationship between the two methods was based on guidelines provided by Cohen (1988): weak relationship $r = 0.10–0.29$, medium relationship $r = 0.30–0.49$ and strong relationship $r = 0.50–1.0$. Results were only recorded where confidence in the result was $P < 0.05$. Significance was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

Solubility of Ca and P in SBM and RSM determined by *in vitro* methodology

While Table 2 shows the expected levels of phytase activity within manufactured diets, Table 1 showed that the analysed dietary Ca values in this study were higher than the formulated values. As Ca and P solubility levels are influenced by total dietary mineral content (Maenz *et al.*, 1999), this is likely to have reduced mineral solubility; which in turn has been reported to reduce phytase efficacy, increase gastrointestinal pH and reduce mineral absorption (Walk *et al.*, 2012*c*). The *in vitro* solubility of Ca and P in either RSM or SBM were observed to be lower ($P < 0.05$) in the SI phase than in the gastric phase (Table 3), which is in agreement with earlier suggestions that Ca and P solubility reach a plateau in the gastric phase (Walk *et al.*, 2012*a*). Mineral solubility in the gastric phase therefore dictates availability of those minerals in the subsequent regions of the gastrointestinal tract. Phytate, Ca and P are relatively soluble at gastric pH and are hence unlikely to precipitate. However, at higher pH (pH 4–7) phytate–mineral complexes are more insoluble (Selle *et al.*, 2000), so precipitation of Ca, P and phytate is more likely in the SI phase, thereby reducing Ca and P absorption. The *in vitro* pH ranges within this study were similar to those recorded *in situ* (Table 4), and are also similar to those found in published *in situ*

Table 3. *Solubility of P and Ca (g/100 g) liberated from soya bean meal (SBM) or rapeseed meal (RSM) subjected to a two-step in vitro assay procedure*[1]

In vitro phase	Soluble Ca	Soluble P
Gastric		
Soya bean meal	0.89[a]	0.45[a]
Rapeseed meal	0.91[a]	0.48[a]
Small intestine		
Soya bean meal	0.63[b]	0.29[b]
Rapeseed meal	0.67[b]	0.32[b]
SEM	0.02	0.03
P-value		
Protein source	0.042	0.030
Phase	<0.001	<0.001
Protein source × Phase	NS	NS

[1]Soya bean meal and rapeseed meal were exposed to a two-step *in vitro* assay as described by Walk *et al.* (2012*c*) with slight modifications. Means represent the average of 10 replicates per feed ingredient. [a,b]Means within a column with no common superscript are different ($P < 0.05$).

Table 4. *Interaction of digestion phase, phytase and protein source on gut pH and solubility of Ca (fractional (g/100 g) from soya bean meal (SBM) and rapeseed meal (RSM) based diets subjected to the gastric and small intestine phase of a two-step in vitro assay procedure[1] and in the gizzard and jejunum of 28-d-old broilers[2]*

	Dietary treatment		Method			pH		
Phase	Protein source	Phytase (FTU)	*In vitro*	*In situ*	r^2	*In vitro*	*In situ*	r^2
Gastric	SBM	0	0.68	0.42[b]	0.208	3.75	2.85	0.880
		500	0.52	0.34[c]	0.803	3.98	2.99	0.771
		5000	0.68	0.45[b]	0.862	3.68	2.88	0.301
	RSM	0	0.78	0.55[a]	0.407	3.15	2.43	0.205
		500	0.65	0.40[b]	0.407	3.40	2.45	0.045
		5000	0.81	0.36[c]	0.580	3.14	2.34	0.046
		SEM	0.021	0.019		0.046	0.037	
Small intestine	SBM	0	0.26	0.21[bc]	0.002	7.54	6.00	0.755
		500	0.24	0.17[c]	0.986	7.61	6.14	0.077
		5000	0.28	0.24[bc]	0.156	7.42	6.08	0.313
	RSM	0	0.30	0.22[b]	0.803	7.57	5.91	0.704
		500	0.29	0.19[bc]	0.186	7.60	6.05	0.092
		5000	0.31	0.25[a]	0.130	7.47	5.99	0.474
		SEM	0.014	0.011		0.080	0.030	
Gastric	SBM		0.67[a]	0.40		3.89	2.91	
	RSM		0.75[a]	0.50		3.25	2.41	
Small intestine	SBM		0.26[b]	0.21		7.46	6.07	
	RSM		0.30[a]	0.22		7.55	5.98	
Gastric		0	0.73[a]	0.49		3.45	2.64	
		500	0.59[b]	0.37		3.69	2.72	
		5000	0.73[a]	0.50		3.41	2.53	
Small intestine		0	0.28[c]	0.22		7.48	5.96	
		500	0.27[c]	0.18		7.61	6.09	
		5000	0.30[b]	0.25		7.45	6.04	
Gastric			0.69	0.45		3.52[a]	2.66[a]	
Small intestine			0.28	0.22		7.50[b]	6.05[b]	
P-value:								
Phase × protein source			< 0.001	NS		NS	NS	
Phase × phytase			< 0.001	NS		NS	NS	
Protein source × phytase × phase			NS	< 0.001		NS	NS	
Phase						< 0.001	< 0.001	

[1]Data were exposed to a two-step in vitro assay as described by Walk et al. (2012a) with slight modifications (see the Materials and Methods section). Means represent the average of a minimum of 9 replicates per feed ingredient. [2]Means represent the average response of 32 chicks/ treatment. [3]Strength of the relationship between dry in vitro and in situ readings. [a–c]Means within the same column with no common superscript differ significantly ($P < 0.05$).

studies (Shafey, 1999; Pang and Applegate, 2007). In addition, Ca solubility in SBM has been previously reported as approximately 93% soluble (Zhang and Coon, 1997) and P as approximately 52% soluble (Ciurescu, 2009), which is comparable to the 89% and 45% soluble Ca and P, respectively, observed in this study (Table 3). Rapeseed meal contained slightly more soluble Ca and P than SBM, in both the gastric and SI phases (Table 3). This may be because both gastric and SI pH were lower when the RSM was subjected to the *in vitro* assay, resulting in less precipitation of Ca-phosphate or Ca-phytate compared to the SBM samples (Table 4).

Solubility of Ca and P in SBM- and RSM-based diets supplemented with phytase

The *in vitro* solubility of Ca was lowest in the diets supplemented with 500 FTU/kg phytase compared to the other diets in the gastric and SI phase (Table 4). This resulted in a phase × phytase ($P < 0.05$) interaction: the reduction in Ca solubility between the diets supplemented with 500 FTU/kg phytase and the other diets was greater ($P < 0.05$) in the gastric phase than the SI phase. This effect was overcome when the diets were supplemented with 5000 FTU/kg of phytase and may be associated with the molar Ca concentration of the diets and the Ca-to-P ratio.

The observed difference in *in vitro* Ca solubility between the two protein sources was greater ($P < 0.05$) in the gastric phase than the SI phase. This resulted in a phase × protein source ($P < 0.05$) interaction and may be due to the greater difference in pH ($P < 0.05$) between the gastric phase and the SI phase, and the precipitation of Ca and phytate at higher pH. Interactions among phytase supplementation, protein source and digestion phase ($P < 0.05$) were observed on *in situ* Ca (Table 4) and P solubility, and *in vitro* P

Table 5. *Interaction of phase, phytase and protein source on solubility of P liberated (g/100 g) from soya bean meal (SBM) and rapeseed meal (RSM) based diets subjected to the gastric and small intestine phase of a two-step in vitro assay procedure[1] and in the gizzard and jejunum of 28-d-old broilers[2]*

	Dietary treatment		Method		
Phase	Protein source	Phytase (FTU)	*In vitro*	*In situ*	r^3
Gastric	SBM	0	0.03[h]	0.48[fg]	0.988
		500	0.07[g]	0.49[f]	0.966
		5000	0.71[c]	0.54[e]	0.863
	RSM	0	0.47[d]	0.39[i]	0.544
		500	0.31[f]	0.42[gh]	0.722
		5000	0.69[c]	0.47[f]	0.159
		SEM	0.021	0.011	
Small intestine	SBM	0	0.26[f]	0.24[hi]	0.284
		500	0.29[f]	0.25[h]	0.115
		5000	0.38[e]	0.29[f]	0.604
	RSM	0	0.26[f]	0.23[i]	0.463
		500	0.27[f]	0.25[h]	0.567
		5000	0.58[b]	0.52[e]	0.998
		SEM	0.002	0.009	
P-value					
Protein source × Phytase × Phase			0.037	< 0.001	

[1]Diets were exposed to a two-step in vitro assay as described by Walk et al. (2012a) with slight modifications (see the Materials and Methods section). Means represent the average of a minimum of 9 replicates per feed ingredient. [2]Means represent the average response of 32 chicks/ treatment. [3]Strength of the relationship between the in vitro and in situ readings. [a–i]Means within the same column with no common superscripts differ significantly ($P < 0.05$).

solubility (Table 5). In the gizzard, Ca solubility was reduced in both SBM and RSM diets supplemented with 500 FTU/kg phytase, compared to the other diets and reduced in SBM diets compared with RSM diets. However, there was no effect of diet or protein source on *in situ* Ca solubility in the SI phase, except when RSM was supplemented with 5000 FTU/kg phytase, which was higher than SBM at 500 FTU/kg (Table 4).

The *in vitro* studies indicated that, in the SBM-based diets, variance in P solubility between the two phases was not influenced by phytase inclusion level. Conversely, in the RSM-based diets, as phytase level increased, the difference in P solubility between the gastric and SI phase increased ($P < 0.05$). Additionally, as phytase level increased, the difference in gastric P solubility between the two protein sources decreased ($P < 0.05$) but no differences were observed in the SI phase at any phytase supplementation level (Table 5). This again highlights that mineral solubility in the gastric phase determines subsequent mineral absorption in the SI, and suggests that, within the SI itself, diet composition has no direct effect on mineral solubility.

In situ P solubility in the gastric and intestinal phases increased with increasing phytase supplementation and was higher in the SBM diets than the RSM diets at all phytase inclusion levels. However, in the gastric phase, an increase in P solubility compared with the non-phytase supplemented SBM diet was higher only in the presence of 5000 FTU/kg phytase. Phytase supplementation increased *in situ* P solubility in the RSM diets comparable to the non-phytase-supplemented SBM diets, but only at 5000 FTU/kg (Table 5). This may be partly because the solubility of phytate and its susceptibility to degradation by phytase differs between SBM and RSM; the phytate present in SBM is potentially more soluble and susceptible to the effects of phytase than the phytate present in RSM (Maenz *et al.*, 1999).

Comparison between *In vitro* and *In situ* methodologies

The range of parameters measured revealed many consistencies but also some differences between the *in vitro* and *in situ* methods. There were several strong relationships between the two methods; particularly for Ca and P solubility in the gastric phase ($r = 0.963$ and 0.917, respectively), and for Ca and P solubility in the SI phase ($r = 0.713$ and 0.824). While Tables 4 and 5 show predominantly strong and medium relationships between the two methods in the gastric phase, the SI phase shows a higher proportion of weak relationships between the methods. This suggests that the *in vitro* assay is more comparable to *in situ* measures for mineral solubility in the gastric phase, rather than the SI phase, most likely due to the lack of absorption and secretion of Ca and P in the SI phase *in vitro* compared to *in situ*.

Both the *in vitro* and *in situ* assays detected that, across both gastric and SI phases, Ca solubility was lowest in the diets supplemented with 500 FTU/kg and was highest in the diets supplemented with 5000 FTU/kg, and that P solubility increased with increasing phytase supplementation level. Both methods also identified that Ca solubility was higher in the RSM-based diets compared to the SBM diets (Table 4), and that P solubility was higher in the SBM-based diets (Table 5). A phase × protein source × phytase inclusion level interaction on P solubility (Table 5) and phase effect on pH (Table 4) was also detected by both methods. These findings indicate that this *in vitro* assay could be a proficient tool to indicate the efficacy of phytase at increasing Ca and P solubility.

However, there were some contrasting findings between the *in vitro* and *in situ* analysis, which are likely due to the closed and dynamic systems respectively with *in vitro* and *in situ* methods. The *in vitro* assay is a closed system where the hydrolysis of phytate results in increased P and Ca ion concentrations in the supernatant, as minerals are not absorbed and are therefore not removed from the system (Walk *et al.*, 2012a). The likelihood of Ca-phosphate precipitation is higher *in vitro* than *in situ* because high accumulated Ca levels *in vitro*

promote both precipitation and increased pH, causing a reduction in mineral solubility (Selle *et al.*, 2000). This implies that minor phytase and protein source effects are likely to appear to have more influence on mineral solubility when observed *in vitro* than would occur *in situ*. This may explain why interactions between protein source and phytase inclusion level were observed on Ca solubility in the gastric phase (Table 4) and P solubility in the SI phase (Table 5) when measured *in situ*, but were not identified when measured *in vitro*. A methodological limitation in the *in situ* study may also have lessened its sensitivity; as no sedation was applied prior to killing, it is possible that some digesta mixing between phases may have occurred through *post mortem* peristalsis, but the differences identified in the study suggest that minimal interference occurred. No relationship was observed between the two methods for the measurement of Ca solubility in the SBM control diets in the SI phase (Table 4).

The relationships between the two methods for the measurement of P solubility in the SI phase were stronger in the RSM-based diets compared to the SBM diets (Table 5). This may be attributable to the higher Ca to available P ratio (2.39:1) of the SBM-based diets, which caused phosphate to be readily precipitated and reduced release of free P. In the *in vitro* system, the release of these small amounts of free P is detectable, as there is accumulation, whereas *in situ* the free P would have been readily absorbed. Phytases are most active at low pH, so the hydrolysis of phytate–calcium and phytate–phosphorus complexes were likely to be less when determined *in vitro* than *in situ*, due to the higher pH in the *in vitro* samples. This would result in greater Ca-phosphate formation and precipitation in the SI, and hence lower mineral solubility *in vitro* compared to *in situ*. In future studies, pH may need to be manipulated in the *in vitro* assay, based on the properties (namely Ca, P and phytate content) of the diet being analysed, in order to accurately identify phytase efficacy. There is also variation in retention time between the two methods, which may explain why a phase × protein source × phytase supplementation level interaction was found when Ca solubility was measured *in situ*, but was not when detected *in vitro* (Table 4). In the *in situ* samples there are minor amounts of endogenous phytase in the SI phase that contribute towards mineral release from phytate, which may go some way towards explaining why protein source and phytase supplementation had an effect on SI phase pH when determined *in situ*, but not when measured *in vitro* (Table 4). This indicates that the *in vitro* assay is more proficient at measuring mineral solubility in the gastric phase than the SI phase.

The two-step *in vitro* assay in this study was successful at identifying the effects of phytase supplementation on Ca and P solubility in the gastric and SI phase, signifying that it can be used as a tool to indicate dietary effects on the GIT environment. This assay is, however, unable to measure digestibility as it is a closed system so cannot completely mimic the dynamic bird GIT conditions, suggesting *in situ* analysis is still required to fully distinguish phytase effects on mineral solubility. Dietary levels of Ca, P and phytase dictate gastric phase precipitation of soluble Ca and P and hence availability of these minerals. Therefore, calcium phosphate precipitation occurs if the Ca:P ratio is not balanced and if the pH is high. The effects of phytase on dietary free Ca and P and phytate are positive *in situ*, but potentially cause increased calcium phosphate precipitation *in vitro*, which reduces the reliability of this assay.

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