The Effect of High Phytase Inclusion in Laying Hen Diets

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Supplemental phytase is routinely added to monogastric diets to increase the availability of phytate phosphours. Higher phytase inclusion levels (>2,500 FTU kg^{-1}) are attracting interest as extra-phosphoric benefits are seen with increasing phytic acid hydrolysis. Phytic acid hydrolysis has been shown to increase protein, mineral and energy utilisation, reduce endogenous losses and increase the availability of *myo*-inositol. It is through these improvements that phytase has been shown to benefit broilers and pigs, but not layers. Four experiments were conducted to assess the effect of high phytase inclusion in the laying hen. In the first experiment, phytase was shown to increase the solubility of the diet within the gastrointestinal tract, and in particular the solubility of numerous minerals. Notably, calcium and phosphorus solubility were increased from 4 and 7.4 to 12.3 and 15.9 g soluble 100 g^{-1} total respectively within the duodenum. Phytase also increased the concentration of calcium, magnesium, zinc and manganese but not phosphorus within the blood plasma of laying hens. In the second experiment, phytase significantly improved the FCR of laying hens from 2.08 to 2.02 and had an additive effect with glucanase in terms of weight gained. The third experiment showed that higher phytase inclusion could increase egg mass in comparison to a positive control, negative control and standard phytase inclusion diet. Chickens fed a diet containing 4,000 FTU kg^{-1} produced 61.07g/d egg mass whereas birds fed a 250 FTU kg^{-1} produced just 58.62g/d. In the final experiment, phytase reduced laying hen FCR from 2.11 to 2.03 in the absence of betaine, but with betaine included in the diet phytase had a negative effect increasing FCR from 1.99 to 2.07. Throughout the four experiments phytase was shown to inconsistently benefit shell quality, which may be due to elevated dietary calcium levels in the second and third experiments. Finally, in the third and fourth experiments phytase was shown to increase the concentration of hepatic and circulating *myo*-inositol respectively. Birds fed diets containing 16,000 FTU kg^{-1} had hepatic concentration of *myo*-inositol greater than double those fed a diet with 250 FTU kg^{-1}. This thesis has shown that high phytase inclusion can benefit laying hen performance which may allow dietary nutrient dilutions.

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Chapter 1
Review of the Literature on Phytic Acid and Phytase

1.1 Phosphorus

Phosphorus is an essential mineral for all known forms of life, and therefore an important nutrient within the laying hen diet. Exhibiting multiple functions, phosphorus is located within the skeleton, soft tissues and extracellular fluids of the body. The vast majority of phosphorus is found within the skeleton as part of a hydroxyl-apatite mineral complex with calcium (Neuman and Neuman, 1953). Apatite gives the bone its strength which is essential for providing the body with support, physical protection and allowing movement. The phosphorus in bones also acts as a reservoir for the bird that can be made available when needed.

At the cellular level, phosphorus is a major structural component of the both deoxyribonucleic acid and ribonucleic acid (Schneider, 1945). As a part of the phospholipid bilayer, phosphorus defines the cell boundaries helping concentrate nutrients while providing a physical barrier (Robertson, 1959). Phosphorus is also a constituent of adenosine triphosphate, the energy currency of the cell and an important molecule involved in regulatory phosphorylation (Lipmann, 1941).

1.2 Sources of Phosphorus in Laying Hen Diets

Poultry diets are formulated to meet the animal's requirements for maintenance and production by providing a mix of numerous feed ingredients; these include cereal grains, oilseed meals, fats, minerals, vitamins and enzymes in addition to both animal and plant based by-products. When formulating a laying hen diet the nutritionist can provide the bird with phosphorus from three origins: animal, rock and plant. The concentration of available phosphorus in laying hen diets ranges from 0.5% to 0.3% (Lesson and Summers, 2008). Generally, phosphorus levels are reduced with age as egg production falls. Available phosphorus has been

defined differently by a number of author's but is generally accepted to be the phosphorus that could be retained by the bird. Both qualitative and quantitative measurements have been used to assess phosphorus availability (Shastak and Rodehutscord, 2013). Different sources of phosphorus have different quantities of unavailable and available phosphorus.

1.2.1 Animal Phosphorus Sources

Meat and bone meal (MBM) is a by-product of the beef and pork industries utilising discarded carcasses. MBM is used as a source of protein in diets as it has a protein content of approximately 500g kg^{-1} (Parsons et al., 1997). Although somewhat variable, the phosphorus content of MBM is around 60g kg^{-1}(Van der Klis, 1996). The majority of this phosphorus is sourced from the bone and so tends to be readily available. After the epidemic of Bovine Spongiform Encephalopathy (BSE) in the United Kingdom, restrictions were put into place across the European Union to prevent the use of animal products as feed ingredients. This is because the standard rendering processes used do not inactivate the prions which cause BSE (956/2008, of 29 September 2008). New technologies that use high pressure treatments have been reported to be effective at destroying prions. However, there is little appetite within the current European marketplace for MBM as consumers still resist its reintroduction. Fish meal, blood meal and insect meal also contain phosphorus, but as these ingredients are not currently used within laying hen diets, they are of little interest.

1.2.2 Rock Phosphorus Sources

Inorganic phosphate starts as a rock mineral that is mined from the ground; in their natural state many of these phosphates are unavailable to the bird and must be heated during their processing. Solubility in hydrochloric acid at pH4 is a useful but blunt tool used to measure phosphorus availability (Leeson, 2008). After processing, the phosphorus availability of these phosphates can be near 100% which is useful for nutritionists, but as ingredients, they do have their pitfalls. Firstly, some rock phosphates contain trace amounts of contaminants such as vanadium (Romoser et al., 1961) and fluorine (Kick, 1933), which can negatively affect shell quality and

hatchability of breeder eggs (Bressman et al., 2002). The second drawback to inorganic phosphorus is the expense; the availability, extraction, processing and transport of the raw rock make phosphorus one of the most expensive nutrients to supply to the animal after energy and protein (Shastak and Rodehutscord, 2013). This was exacerbated in 2008 when a price spike lead to an increase from approximately £30 t t^{-1} to £260 t^{-1}. The price has since settled down to approximately £110 t^{-1} but is unlikely to return to the pre-2008 price (ICIS, 2012).

The dramatic increase in cost is due partly to the distribution of inorganic phosphorus. Production is limited to few countries with the United States of America, the People's Republic of China and the Kingdom of Morocco being the largest (Cordell et al., 2009). All three sites have production issues however; China is reducing its exports to accommodate an ever increasing internal demand, while the USA is running low of raw rock and so importing more from Morocco (Jasinski, 2008). Morocco has the greatest deposits with some estimates giving it three times more rock phosphorus than the rest of the world combined (IFA, 2006). The main problem with Morocco's supply is that it is geopolitically sensitive as much comes from the Western Sahara. The Western Sahara is an area that is mostly under the control of Morocco, although sovereignty is not universally recognised and is contested by some nations. This has led the Western Sahara Resource Watch to claim Morocco is breaking international law (WSRW, 2007), the UN to condemn the trade and a number of high profile boycotts (Corell, 2002). It is predicted that at present rates, global phosphate rock reserves will run out in around 400 years, and that both China and the USA may have to cease production within 100 years if they continue to increase processing at current rates. However, it is believed that the demand for rock phosphorus fertiliser will continue to increase, shortening these time frames dramatically (Cordell et al., 2009).

The final problem with inorganic phosphorus is its overuse in animal feed. Many nutritionists overestimate the available phosphorus requirements of their animals to ensure that phosphorus does not become a limiting nutrient. In addition to this, inorganic phosphorus is used to supply available phosphorus when a diet contains a high level of unavailable phosphorus.

This gives the diet an adequate available phosphorus but high total phosphorus content. Supplying phosphorus at a level greater than the animal's requirements leads to an increase in the phosphorus excreted in the manure. When excreta is spread as fertiliser, leaching and surface runoff lead to a build-up of the phosphorus in the surface water which can lead to eutrophication and the death of aquatic animals (Sharpley, 1999, Correll, 1999). Phosphorus is the greatest pollutant within freshwater systems and the animal agricultural sector has to accept some responsibility for this. In recent years legislative branches throughout the European Union have introduced limits to the amount of phosphorous that can be spread on fields.

1.2.3 Plant Phosphorus Sources

Laying hens within the United Kingdom are predominately fed a diet based on plants, notably wheat and soybean. In the past, MBM and inorganic phosphorus were used to increase the dietary phosphorus to compensate for the poor availability of the phosphorus in plants. This is because the majority of the phosphorus in plants is present as phytate phosphorous (phytate-P) (Selle and Ravindran, 2007). Phytate is an insoluble, organic complex, made from phytic acid and other nutrients. Phytic acid is made of an inositol ring with six phosphate groups, shown in Figure 1. It is highly charged and so binds to nutrients reducing the availability of the phytate-P and the other nutrients. As a result, much of this phytate-P passes through the bird without being digested or absorbed (Cowieson et al., 2011). Nutritionists can supplement diets with the enzyme phytase to break down phytic acid and release phytate-P, and thereby increasing its availability (Wodzinski and Ullah, 1996b). The Alltech Global Feed Survey estimates that in 2012 approximately 417.8 million tonnes of poultry feed was produced of which 141.1 million tonnes was made for laying hens (Alltech, 2013). Given that the range of phytate-P within a poultry diets falls between 2.5 – 4 g kg^{-1} (Ravidran, 1995), the amount of phytate-P fed to all poultry in 2012 was over one million tonnes, while the World's laying hens were fed just under half a million tonnes. Each year the World uses approximately 0.9 million tonnes of inorganic phosphorus for animal feed additives and 17.5 million tonnes are used in crop fertiliser production (Correll, 1999). Using phytase to help exploit the phytate-P that is already being fed to chickens

can help reduce the poultry industries reliance on inorganic phosphates and MBM, producing cheaper diets that are more socially acceptable, economically stable and environmentally friendly.

1.3 Phytic Acid

1.3.1 Phytic Acid Definitions

The term phytate is often used when referring to any of the phytic acid related compounds; however, the term actually refers to the mixed salt of phytic acid (Selle and Ravindran, 2007). Phytic acid is the free form of phytate that may also be referred to as *myo*-inositol hexaphosphate, or IP_6 (Cheryan, 1980). Phytase can remove phosphate groups from phytic acid to generate the lower esters which are listed: *Myo*-inositol pentaphosphate (IP_5), *Myo*-inositol tetraphosphate (IP_4), *Myo*-inositol triphosphate (IP_3), *Myo*-inositol biphosphate (IP_2) and *Myo*-inositol monophosphate (IP_1). Phytin is a specific complex of phytate found within plants that incorporates potassium, magnesium and calcium (McCance and Widdowson, 1935).

1.3.2 Chemical Structure of Phytic Acid

Figure 1.1 shows the structure of phytic acid, consisting of a *myo*-inositol 6 carbon ring that has 6 phosphate groups, giving it a molecular weight of 660 (Anderson, 1914).

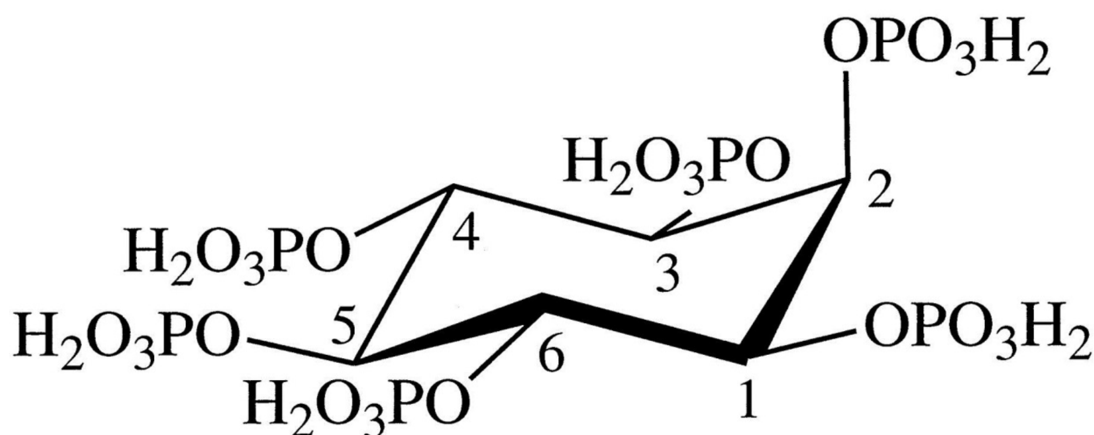

Figure 1.1The Structure of Phytic Acid first proposed by Anderson (1914)

The phosphate moieties are equatorially aligned with the exception of the group at C_2 which is axially aligned. Of the 12 proton dissociation sites, 6 are strongly acidic at pKa of approximately 1.5, 3 have weakly acidic pKa values of 5.7, 6.8 and 7.6 and the final three are very weakly acid, with pKa values greater than 10 (Costello et al., 1976). Within the pH range of the digestive tract all of these sites will dissociate, giving the phytic acid a sizeable negative charge and large chelating capacity of cations. This enables phytic acid to bind to positively charged minerals and form phytate complexes.

Using pH-drop tests, Cheryan (1980) found that phytic acid forms the most stable phytate with Zn^{2+}, followed by Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+} in order of stability. Depending upon the concentration of the ions and the pH, phytate can occur as either soluble chelates or an insoluble precipitate. Precipitation of phytate increases with the concentration of minerals relative to the phytic acid. A rise in pH towards more neutral and basic ranges will also increase precipitation. Using shifts in ^{31}P nuclear magnetic resonance spectra of phytic acid, it has been shown that an individual Zn^{2+} ion can bind to the C_5 phosphate groups on two separate phytic acid molecules to form a cationic bridge (Champagne and Fisher, 1990, Champagne et al., 1990). This would enable the generation of large complexes that would lead to precipitation.

1.3.3 Occurrence of Phytic Acid

Phytate is the principal molecule used to store both *myo*-inositol and phosphorus within plant seeds for germination, with approximately two thirds of the total phosphorus (total-P) in plant seeds found as phytate-P (Cosgrove, 1966, Simons et al., 1990b) Phytic acid can also act as a reservoir for other minerals. During rice seed development, phosphorus is rapidly transported to the aleurone layer; here it is immediately used to generate phytic acid which then attracts metals to form the phytin complexes (Iwai et al., 2012). As discussed previously, phosphorus has numerous essential roles within cells and so is crucial for plant development and growth. *Myo*-inositol also plays a pivotal role as a precursor for many molecules, with critical functions including the formation of cell walls and

plasma membranes, as well as acting as an oxidative stress protectant (Bohnert et al., 1995).

1.3.3.1 Concentration of Phytic Acid in Common Ingredients

Table 1.1 Mean (and range) of total-P and phytate-P concentrations, and the proportion of phytate-P to total P in feed ingredients

Feed Ingredient	Total-P (g kg^{-1})	Phytate-P (g kg^{-1})	Percentage (%)
Maize	2.62 (2.30-2.90)	1.88 (1.70-2.20)	71.6(66-85)
Sorghum	3.01 (2.60-3.09)	2.18 (1.70-2.46)	72.6(65-83)
Wheat	3.07 (2.90-4.09)	2.19 (1.80-2.89)	71.6(55-79)
Rapeseed	9.72 (8.79-11.5)	6.45 (4-70.78)	66.4(36-76)
Cottonseed	10.02 (6.40-11.36)	7.72 (4.90-9.11)	77.1(70-80)
Soybean	6.49 (5.70-6.94)	3.88 (3.54-4.53)	59.9(53-68)
Rice bran	17.82 (13.40-27.19)	14.17 (7.90-24.20)	79.5(42-90)
Wheat bran	10.96 (8.02-13.71)	8.36 (7.00-9.60)	76.3(50-87)

Table adapted from Selle and Ravindran, (2007) using data dervied from studies by Nelson et al. (1968), Kirby and Nelson, (1988), Eeckhout and Depaepe, (1994) Ravindran et al. (1994), Viveros et al. (2000), Selle et al. (2003b) and Godoy et al. (2005)

The concentrations of phytic acid found within different dietary ingredients can vary widely from 5 – 25g kg^{-1} giving the variety if phytate-P concentrations shown in Table 1.1. Selle and Ravidran (2007) summarised several papers to give the mean and range of phytate-P within a number of important ingredients; cereals have approximately 2 g kg^{-1} of phytate-P making up around 70% of their total-P. Grain legumes such as chickpeas and field peas were not included in this review, but have a similar concentration of phytate-P to the cereals. The oilseed meals however, have a much higher level of both total-P and phytate-P, but with similar proportions. Soybean meal is currently the most popular oilseed meal used with laying hen diets and has the lowest proportion of phytate-P of all major ingredients, but also the lowest level of total-P compared to the other oilseed meals. By-products like wheat bran have higher levels of total and phytate-P in comparison with oilseeds and cereals, but in addition to this the proportion of phytate-P is extremely high, reaching 80%. Variation of phytate-P concentration within a feed ingredient is caused by a number of factors including the amount of phosphorus available to the plant during growth,

individual plant genotypes and year of harvest (Reddy et al., 1982, Steiner et al., 2007, Coelho et al., 2002).

1.3.3.2 Form of Phytic Acid in Dietary Ingredients

As discussed previously, phytate-P is poorly available to monogastrics such as the laying hen. This availability can in part be influenced by the location of the phytic acid within the feed ingredient and what other nutrients it is associated with. The location and storage of phytic acid varies between different plants but generally it is found as the potassium, magnesium and calcium salt, phytin, in subcellular membrane bound protein vacuoles that are referred to as protein bodies (Lott et al., 1985, Ockenden et al., 2004). The phytin can form crystal globoid structures that are visible within the protein bodies using an electron microscope. Ogawa et al. (1975) found that the globoid phytin crystals in rice consist of 670 g kg^{-1} phytic acid, 190 g kg^{-1} potassium, 110 g kg^{-1} magnesium and 10 g kg^{-1} calcium on a dry matter basis. The size of the crystals will depend upon the ratio of potassium relative to other cations; larger crystals will form when the level of potassium in the protein bodies decreases (Lott et al., 1985). If there is a large proportion of potassium, phytin will not form crystals but instead interacts directly with protein to create soluble protein-phytin complexes that are distributed consistently within the protein bodies. This is the case in both soybeans and peas where crystals are either smaller in size or not present, as the phytin compounds are completely soluble(Lott and Ockenden, 1986).

Within cereals such as wheat, barley and rice, the protein bodies that store the phytin are concentrated within the aleurone layer and the bran of the grains; this may explain why the by-products of cereals have such high levels of phytate-P (Steiner et al., 2007). Maize, soybean and rapeseed however, contain their protein bodies within their embryonic cells and the endosperm of the seed (de Boland et al., 1975, Odell et al., 1972, Erdman, 1979). It is highly likely that the location and form of the phytin within an ingredient will have an effect on the availability of the phytate-P. For example large insoluble phytin crystals found within the fibrous aleurone layer are likely to be less available to the bird than those soluble phytins found within the endosperm.

1.3.4 Factors Effecting Phytate-P Hydrolysis

It has been stated that poor phytate-P digestion by poultry and other monogastrics is due to the lack of endogenous phytase and phosphatase activity. This is incorrect; the reason for poor phytate-P utilisation is due to the poor availability of the substrate phytic acid (Cowieson et al., 2011). As previously mentioned, phytic acid contains numerous negative charges which will bind to cations to form the insoluble phytate complexes. It is the lack of phytate solubility that prevents the phytase in the brush border of the intestinal membrane breaking down phytic acid to release the phytate-P.

There is undeniably a great deal of variation in the degree of phytate-P digestion, ranging from 0 to 60% in broilers and 8 to 60% in layers (Nelson, 1976, Edwards, 1983, Rutherfurd et al., 2004, Mohammed et al., 1991). The control of this variation is multifactorial and can be influenced by both nutritionist and farmer.

1.3.4.1 Effect of Bird on Phytate-P Hydrolysis

The type of bird (broiler vs layer) will affect the hydrolysis of phytate-P (Peeler, 1972); laying hens have a greater endogenous phytase activity within the small intestine than broilers (Maenz and Classen, 1998). The laying hen also has a slower transit time and so it may be that phytase has longer to act upon the substrate. Any genetic disposition to alter the environment of the gastrointestinal tract will also have an effect, as this can alter the solubility of the phytic acid and hence its availability to phytase. The age of the birds may also affect the breakdown of phytate; Scheideler and Sell (1987) found as laying hens aged from 34 to 50 weeks phytate-P retention fell from 47% to 9%. This may have been because of a reduction in phytic acid hydrolysis or a decrease in phosphorus absorption. The phosphorus requirement of the laying hen falls with age and so without the same physiological pressure, the bird may no longer need phytase to gain adequate phosphorus.

1.3.4.2 Effect of NPP on Phytate-P Hydrolysis

A common feature of many enzymes is a feedback mechanism whereby the product of the reaction inhibits the continued activity of the enzyme. The

hydrolysis of phytic acid produces orthophosphate, which has been shown to reduce the activity of phytase (Greiner et al., 1993, Lei and Stahl, 2000, Konietzny and Greiner, 2002a). The level of dietary inorganic non-phytate phosphorus (NPP) will strongly effect the concentration of orthophosphate present in the gastrointestinal tract, and so logically the hydrolysis of phytate-P is dependent upon the amount of NPP present in the diet.

In the rat, low levels of NPP reduced available phosphorus marginally below the animals requirements which increased the digestibility of both total-P and phytate-P by 12 and 62% respectively. Leytem et al. (2008) showed that an increase in NPP levels within a broiler diet could affect the digestively of phytate phosphorus, but that this was dependent upon the diet's base ingredients.

1.3.4.3 Effect of Calcium on Phytate-P Hydrolysis

The limited availability of phytate-P is due to the formation of insoluble complexes, and so it stands to reason that a change in the concentration of cations that cause these complexes to form will affect the phytate-P hydrolysis (Selle et al., 2009c). Because of the bird's large requirement, calcium is the most prevalent cation within the gastrointestinal tract lumen and so the most important when discussing phytate complex formation. The *de novo* formation of Ca-phytate complexes is influenced by both the pH of the tract and the level of calcium in the diet.

Within an *in vitro* system it was found that calcium reduced phytic acid hydrolysis by 22% in the presence of wheat phytase (Hill and Tyler, 1954). Wise and Gilbert (1982) showed that high levels of dietary calcium reduced phytate hydrolysis within rats. Multiple studies have shown a negative effect of calcium on phytate hydrolysis in poultry (Selle et al., 2009c), and Taylor (1965) showed that very high levels of calcium can halt phytate hydrolysis completely. An increase in calcium concentration from 4.7 to 11.6 g kg^{-1} resulted in a decrease in the apparent ileal digestibility (AID) of phytate-P from 0.201 to 0.059 (Plumstead et al., 2008). In another study, phytate-P AID decreased by 63% when calcium was increased from 2 to 7 g kg^{-1} (Tamim et al., 2004b). Within laying hens this can be problematic, as the calcium levels have to be high because of the large requirements to maintain

bone strength and produce quality egg shells. The calcium concentration in a broiler diet is typically around 10 g kg^{-1}, while the laying hen requires approximately 40 g kg^{-1} dietary calcium. Van der Klis et al. (1997) found that reducing calcium levels from 40 g kg^{-1} to 30 g kg^{-1} increased ileal phytate hydrolysis from 9% to 33%. This shows phytic acid can be broken down within the tract of the laying hen. However, a calcium level of 30 g kg^{-1} should not be recommended as this diet would not meet the birds calcium requirement.

1.3.4.3.1 Limestone

Limestone is the most prevalent calcium source in broiler and layer diets. The acid binding capacity of limestone is very high at 15,044 meq kg^{-1} at pH 3 (Lawlor et al., 2005); this means that an increase in limestone will result in a rise in the pH of the digesta within the gastrointestinal tract lumen. Increase in dietary calcium using limestone from 10.7 to 25.3 g kg^{-1} resulted in an increase in pH in both the crop (4.89 vs 5.32) and ileum (6.62 vs 7.39)(Shafey et al., 1991). The pH of the tract is crucial for the formation of cation-phytate complexes (Maenz et al., 1999). As the pH rises so will the quantity and strength of the bonds between the minerals and phytic acid, making the phytic acid substrate refractory to endogenous intestinal mucosal phytase activity. The pH will also rise above the optimum range of the phytase enzymes (Engelen et al., 1994). This means that limestone addition has a triple effect, increasing the concentration of calcium ions in the lumen in addition to increasing the pH of the tract to favour complex formation and inhibit phytase activity.

Limestone particle size can also have an effect on phytate hydrolysis. An increase in particle size from 28 to 1300 µm improves phytate-P hydrolysis both *in vitro* and *in vivo* (Manangi and Coon, 2006). This is because the smaller limestone particles are more soluble, and so there is an increased likelihood of them being available to bind to the phytic acid to form phytate complexes.

1.3.4.3.2 Calcium Complications

There are additional mechanisms other than increasing pH and calcium-phytate complex formation that could inhibit the hydrolysis of phytate. The

benefits of reducing calcium can be complicated by the relationship between calcium and phosphorus homeostasis. A dietary calcium reduction relative to phosphorus could cause greater uptake of both calcium and phosphorus from the intestines (Tamim et al., 2004b). Therefore, a reduction in dietary calcium could benefit phytate hydrolysis through a reduction of intestinal phosphate concentration. It is important to consider the calcium and phosphorus ratio as well as the overall values when investigating the hydrolysis of phytic acid (Lei et al., 1994). There is also a possibility that calcium may interfere with the endogenous phytase itself and reduce its activity in this way but there is no substantial evidence of this (Applegate et al., 2003).

A 5 g kg^{-1} addition of limestone calcium reduced the digestibility of total-P, phytate-P and NPP by approximately 57%, 63% and 40% respectively (Tamim et al., 2004b). This trial supports the theory that limestone can increase the formation of phytate through increasing luminal calcium concentration and pH.

The effect of calcium on NPP is due to the formation of calcium phosphates, which also have a reduced solubility and therefore availability (Holt et al., 1925). Harms and colleagues (1962) showed that the difference between the utilisation of phytate-P and NPP increased as the calcium phosphorus ratio is increased from 1:1 to 2:1. This supports the work of Gosselin and Coghlan (1953), showing that calcium has a greater affinity to phytic acid than organophosphates.

A reduction in the dietary calcium level or limestone inclusion can help mitigate the formation of complexes somewhat and improve phytic acid hydrolysis, phosphorus digestibility and bird performance (Selle et al., 2009c). However, this is not always possible if the bird’s calcium requirements are going to be met in a long term commercial setting.

1.4.4.4 Effect of Other Minerals on Phytate-P Hydrolysis

In vitro studies have shown that other cations like magnesium, manganese, iron and zinc can all inhibit the hydrolysis of phytic acid (Maenz et al., 1999). The magnitude of their effect is dependent upon the pH of the gastrointestinal tract and molar ratios present. Banks et al. (2004a) showed

that dietary copper could reduce phosphorus retention, presumably though a decrease in the solubility of phytate-P. Another study showed that an increase in copper levels from 100 to 250 mg kg^{-1} had no significant effect, but increasing the copper level above 250 mg kg^{-1} reduced phosphorus retention by 19% (Banks et al., 2004b).

Since the use of antibiotics as growth promoters was banned in the European Union, the use of zinc as zinc oxide has increased in piglet and broiler diets (Hahn and Baker, 1993). For zinc to have a pharmaceutical effect it has to be included within the diet at high concentration, which has been shown to reduce the release of phytate-P by 30% in the broiler (Augspurger et al., 2004).

Once associated with phytic acid, the effect of different minerals will vary. Champagne and Hinojosa (1987) showed that copper-phytate complexes are more soluble than zinc-phytates and that these complexes had a greater solubility.

1.4.4.5 Effect of Vitamin D on Phytate-P Hydrolysis

Multiple studies have shown that vitamin D can increase the hydrolysis of phytic acid and phytate-P utilisation (Deluca, 1988, Mitchell and Edwards, 1996, Carlos and Edwards, 1998, Edwards, 1993). The mechanism behind this could be multifactorial. Firstly, dietary vitamin D can increase the calcium absorption (Bauman, 1983). As the concentration of calcium cations present in the lumen of the gastrointestinal tract decreases, so will the formation of calcium-phytate complexes. An increase in vitamin D will therefore increase the solubility of phytic acid and so increase its susceptibility to phytase activity.

Vitamin D can also increase the transport of phosphorus within the small intestine (Tanaka and Deluca, 1974, Harrison and Harrison, 1961). This will increase the retention of any phosphorus hydrolysed from phytic acid and so increase the retention of phytate-P will be greater.

Vitamin D may interact with phytase more directly as it has been shown to increase the activity of endogenous intestinal mucosa phytase in the rat (Pileggi et al., 1955) and chicken(Davies et al., 1970).

1.4.4.6 Effect of Ingredients on Phytate-P Hydrolysis

Variation in the amount, form and location of the phytin found within different feed ingredients will inevitably affect it's hydrolysis as the solubility and accessibility of the phytin differs. Leytem and colleagues (2008) fed broilers four diets based on different cereal grains. They found that the barley diet had the highest AID coefficients of phytate-P (0.29) followed by wheat (0.26), and oats (0.26) and finally maize (0.10).

As well as the raw material itself, different processing treatments can affect the availability of phytate-P within an ingredient. For example, a decrease in the size of maize particles from coarse (894µm) to fine (484 µm) resulted in a decrease in the retention of calcium, total-P and phytate-P (Kasim and Edwards, 2000). Heuser (1945) showed that larger maize particles cause an increase in digesta retention times, while others have shown that coarser grains can increase gastric secretions and muscular activity(Nir et al., 1994). It is likely that a change in luminal pH and retention time will increase phytase activity and give the phytase longer to hydrolyse phytic acid. Many factors such as dietary fat, fibre and xylanase supplementation are known to affect gastrointestinal tract retention time and luminal environment; it may be possible that these factors can also influence phytic acid hydrolysis (Ouhida et al., 2001).

Plants use phytin within their seeds to accumulate phosphorus and inositol for germination (Loewus and Murthy, 2000). Therefore, plants need phytase activity to break down the phytic acid and utilise the stored nutrients (Konietzny and Greiner, 2002a). The level of phytase activity varies amongst ingredients and consequently different diets will hydrolyse phytic acid to different degrees (Selle and Ravindran, 2007).

On the other hand, within most dietary ingredients phytase activity is quite small, and it is possible that any differences in plant phytase activity do not significantly affect the hydrolysis of phytic acid within the gastrointestinal tract of the hen (Zimmermann et al., 2002). Steam pelleting can also affect phytic acid hydrolysis as it can denature the plant phytase enzymes and so remove any small benefits they may give the bird (Jongbloed and Kemme, 1990). Although phytic acid is a thermostable molecule, it has been shown

that autoclaving can increase phosphorus availability in rice and soybean bran, through the breakdown of phytic acid (O'Dell, 1962, Takemasa and Hijikuro, 1991). It is possible that steam pelleting can have the same effect.

Given the low level of plant phytase activity and the lack of susceptibility of the phytate complexes to the endogenous phytase in the small intestines, increasing phytase activity within the foregut sections of the gastrointestinal tract would benefit phytic acid hydrolysis significantly. The addition of exogenous phytase allows the nutritionist to increase the phytase activity of the diet; this can significantly increase phytic acid hydrolysis and phosphorus availability in both broiler and laying hen diets.

1.3.5 Phytic acid as an Anti-Nutrient

Phytic acid can be considered a nutrient because of the potential phosphorus that can be made available to the bird; it can also be considered an anti-nutritional factor because of the negative effects phytic acid can have on the digestion of other nutrients. Few studies have been conducted to investigate the effects of phytic acid concentration on monogastric performance, but those that have show that an increase in the concentration is detrimental to body weight gain (BWG) of fish, chicks, broilers and piglets as shown in Table 1.2. Only two studies have investigated the effects of phytic acid concentration in laying hens. The first used low phytic acid maize, but failed to find a significant effect on laying hen performance (Ceylan et al., 2003). This is likely because the difference between the two levels of phytic acid within the study was quite small, in comparison with the other experiments. The second trial, although small found that increasing phytic acid to very high levels with the inclusion of sodium phytate decreased egg weight, egg mass and increased feed conversion ratio(Jubarah et al., 2006).

Unfortunately, this study was not powerful enough to find a significant effect on egg production due to insufficient bird numbers and trial duration. However, a large numerical reduction in egg production was seen, from 75.7% to 67.9%.

The unfavourable effects of increasing phytic acid concentration in diets with adequate non-phytate phosphorus (NPP) suggest that phytic acid is not

simply affecting phosphorus availability, but also acting as an anti-nutritional factor.

Table 1.2 The effect of phytic acid concentration on the performance of monogastrics

Study	Animal	Phytic acid g kg^{-1}	Phytic Acid Effect
Usmani and Jafri, 2002	Indian Carp	0 vs 15	27% ↓BWG, 25%↑ FCR
Woyengo et al., 2012	Piglet	0 vs 20	37% ↓BWG
Liu et al., 2009	Broiler	7.8 vs 15.7	3% ↓BWG
Liu et al., 2008a	Broiler	7.8 vs 15.7	7% ↓BWG
Cabahug et al., 1999	Broiler	1.04 vs 1.57	7% ↓BWG
Onyango and Adeola, 2009	Broiler	0 vs 1.65	28% ↓BWG
Shan and Davis, 1994	Layer chicks	0 vs 16.5	44% ↓BWG
Ceylan et al., 2003	Laying hen	5.7 vs 7.1	=
Jubarah et al., 2006	Laying hen	0 vs 9.1	2%↓ EW, 12%↓EM and 26%↑ FCR

Table adapted from Woyengo and Nyachoti (2011), BWG = Body Weight Gain, FCR= Feed Conversion Ratio, EW= Egg Weight, EM= Egg Mass

1.3.5.1 Effect of Phytic acid on Minerals

1.3.5.1.1 Effect of Phytic acid on Calcium

The first non-phosphorus effect to be researched in detail was with calcium (Nelson, 1984). The calcium requirements of monogastric animals are greater than that of any other mineral and so the calcium concentrations in pig and poultry diets are high; this is especially the case in laying hens. This high calcium concentration causes calcium to be the most important mineral

in regard to phytate complex formation. Nelson (1984) proposed an equation for the calcium requirements of broilers dependent upon phytate-P concentration that suggests each phytic acid molecule holds 5.1 atoms of calcium. Nash (1998) showed slightly less calcium was bound phytic acid with a ratio of 4.62 to 1 while others have reported a ratio of 4.93 to 1(Marini et al., 1985). Wise (1983) summarised that, depending upon luminal mineral concentration and pH, a range of 3 – 6 moles of calcium would bind to 1 mole of phytic acid. In broilers, the dietary concentrations of phytic acid and calcium are both approximately 10 g kg^{-1}. If it is assumed that 6 moles of calcium can bind to one mole of phytic acid, then over one third of the dietary calcium could be bound to phytate in broiler and piglet diets (Selle et al., 2009c). Using the same assumptions in laying hen diets, up to 10% of dietary calcium could be bound to phytate.

It has been shown that increases in dietary phytic acid concentrations will increase the amount of calcium bound to phytate, which will decrease calcium digestibility. As phytic acid increased from 10.4 g kg^{-1} to 13.6 g kg^{-1} in broiler diets calcium AID was reduced from 37.3 to 36.0 (Ravindran et al., 2006). In laying hens, Ceylan et al. (2003) failed to find an effect of phytic acid on calcium utilisation, but again, this could be due to the relatively small difference in dietary treatments discussed previously.

1.3.5.1.2 Effect of Phytic acid on Other Minerals

Woyengo et al. (2009)showed that increasing phytic acid concentration could significantly affect the AID of many minerals and not just calcium (Table 3.) The greatest phytic acid effect was on sodium where the AID coefficent actually suggests a net loss of the mineral.

There is some considerable overlap with human nutrition in regard to the anti-nutrient properties of phytic acid; many malnourished people eat plant based diets high in phytic acid which can exacerbate any mineral deficiencies. Kim et al. (2007) discovered that zinc absorbtion was correlated with phytic acid intake in both elderly and young women. Another trial in rats found that addition of 1.3 g kg^{-1} phytic acid to a cereal diet led to a 33% decrease in the absorption of iron. Jubarah et al. (2006) did not look at the digestibility of any minerals, but did find that phytic acid decreased the level

of iron in egg yolks. There was also a numerical but insignificant decrease in the level of zinc in the blood serum and egg yolk.

Table 1.3 The effect of phytic acid concentration on AID coefficients in piglets

Mineral	Dietary Phytic Acid g kg $^{-1}$			
	0	5	10	20
Ca	0.782	0.711	0.534	0.369
Mg	0.291	0.188	0.137	-0.030
P	0.801	0.654	0.505	0.372
Na	0.492	0.427	0.466	-0.182
K	0.876	0.866	0.890	0.811

Adapted from Woyengo et al. (2009)

1.3.5.1.3 Mechanism of Phytic Acid Restricting Mineral Availability

The reduction in mineral digestibility could be a result of the direct binding of minerals to phytic acid to form insoluble phytate. For minerals such as calcium to be absorbed they have to be soluble, so a reduction in their solubility will cause a decrease in their absorption. Reduced mineral solubility through phytate complex formation, however, is just one of the mechanisms that could affect mineral digestibility.

Phytic acid increases endogenous secretion of minerals. Phytic acid forms insoluble complexes within the small intestines as the pH rises towards neutrality by binding to divalent cations (Tamim et al., 2004b). Monovalent cations like sodium will also bind to phytic acid but do not form such strong bonds (Erdman, 1979). In addition, monovalent cations cannot bridge between two individual phytic acid molecules and so their complexes do not precipitate out of solution as easily (Champagne et al., 1990). It is therefore most likely that the decrease in sodium digestibility is actually due to an increase in endogenous sodium secretion and losses, not through complex formation.

Phytic acid has been shown to bind to both proteins and endogenous enzymes for example pepsin within the stomach (Zebrowska et al., 1983). The presence of phytic acid reduced the digestion of bovine serum albumin by pepsin *in vitro* (Knuckles et al., 1985). Liu et al. (2009) showed that the pepsin activity in the proventriculus was reduced by 6.3% with an increase in phytic acid concentration from 2.2 g kg^{-1} to 4.4 g kg^{-1}. Woyengo, (Woyengo, 2011) showed a similar effect in piglets where an increase from 0 to 20g kg^{-1} sodium phytate resulted in a decreased stomach digesta pepsin activity from 265.5 to 142.5 PU/ml .

Within the digestive system feedback mechanisms are in place whereby low enzyme activities are compensated for by increased enzyme secretions. This feedback can be prompted by the presence of undigested feed or inactivated enzymes within the gastrointestinal tract. In the rat, low stomach pepsin activities caused by tannic acid resulted in an increase in the secretion of both hydrochloric acid and pepsin (Mitjavil. et al., 1973). To protect the small intestines from acid erosion and enzyme digestion the animal will secrete intestinal and pancreatic bicarbonates and mucin that are high in sodium (Zebrowska et al., 1983). It seems therefore, that phytic acid increases pancreatic secretions to protect the intestines from the increased hydrochloric acid secretions brought about by reduced enzyme activities and low nutrient availabilities, which results in large sodium losses.

Cowieson et al. (2004) investigated the effect of phytic acid on endogenous losses in broilers by giving them a precision fed diet of 50 ml glucose. The test diet was created by adding 1 g of free phytic acid to that solution. They found that phytic acid led to increases in the excretion of calcium, iron, copper and sulphur, but by far the greatest increase was with sodium.

This was followed by a similar 48 hour precision fed experiment where broilers were fed a casein solution with the same 1 g of IP_6 added to create a test diet. In this experiment calcium, magnesium, manganese and sodium losses were increased by 187%, 39%, 87% and 174% respectively (Cowieson et al., 2006). Liu and Ru (2010) performed a similar experiment using a purified corn starch diet and the addition of sodium phytate to increase the phytate-P concentration from 2 g kg^{-1} to 4 g kg^{-1}. The only

significant effects reported were a reduction in the endogenous secretion of copper and iron. The differences seen between the experiments is probably due to the form of phytic acid used. Liu and Ru (2010) used the sodium salt of phytic acid whereas Cowieson and colleagues used a free form of phytic acid in both of their experiments, (Coweison et al., 2004, 2006). This shows how the form of phytic acid can affect not just its hydrolysis but also the anti-nutritional effects it can have within the gastrointestinal tract. The form of phytic acid has been shown to affect the endogenous mucin secretions with phytin having a greater effect than that of free phytic acid(Onyango et al., 2009).

Table 1.4 The effect of phytic acid addition to endogenous mineral excretion (mg/bird/48h)

Mineral	Control	Control + 1g Phytic Acid	p value
Calcium	72.5	122.5	<0.05
Copper	0.3	0.3	NS
Iron	5.2	6.8	<0.05
Magnesium	30	36.3	NS
Manganese	0.5	0.6	NS
Sodium	38.7	155	<0.001
Sulphur	66.8	98.1	<0.05
Zinc	12	14.9	NS

Adapted from Cowieson et al. (2004)

Phytic acid can also increase endogenous secretions by binding to metalloenous digestive enzymes that are tightly bound to a metallic ion co-factor, such as ɑ-amylase and alkaline phosphatase. ɑ-amylase relies upon calcium as a co-factor while alkaline phosphatase requires zinc (Malmstro and Neilands, 1964). Deshpande and Cheryan (1984) showed that phytic acid can reduce the activity of ɑ-amylase *in vitro*. Liu et al. (2008b) showed that increasing phytic acid concentration from 2.2 g kg^{-1} to 4.4 g kg^{-1} in broiler diets reduced ɑ-amylase activity by over 8%. If phytase leads to an increase in the secretion of the metalloenous enzymes by reducing their

activity, it is probable that there will also be an increase in the secretion of the enzyme cofactors, most notably calcium and zinc (Woyengo and Nyachoti, 2013).

Phytic acid could also increase endogenous losses by directly binding to secreted minerals to form complexes and so preventing them being reabsorbed. It is likely that this action is on multivalent cations rather than monovalent cations as they form stronger bonds and the complexes generated are less soluble (Scheuermann et al., 1988).

Finally, phytic acid may act on mineral absorption through altering the osmotic gradient across the gastrointestinal tract wall. Phytic acid can restrict the digestibility and absorption of nutrients such as sugars and amino acids, thereby reducing their active transport into enterocytes (Selle and Ravindran, 2007). The reduced presence of solutes within the enterocytes will decrease the osmotic flow of water which will in turn reduce the absorption of minerals. Minerals can also be co-transported with other nutrients, for example sodium can be transported with glucose. If phytic acid reduces the absorption of glucose it is possible that sodium transport will also be reduced (Fordtran et al., 1968, Fordtran, 1975).

1.3.5.2 Effect of Phytic acid on Protein

There is still some debate about the ability of phytic acid to impede protein and amino acid digestibility within poultry. The range of results seen by different authors stem from a number of contributing components within the experiments, including the inert marker used, ingredients used, levels of calcium, levels of NPP, levels and source of phytic acid, dietary electrolyte balance, bird genetics, bird age, housing and the inherent digestibility of the amino acids (Selle and Ravindran, 2007).

Liao et al, (2005) reported that increasing phytic acid concentration in piglet diets from 7.8 to 15.6 g kg^{-1} resulted in a decrease in the AID of nitrogen as well as the amino acids: histidine, isoleucine, phenylalanine, threonine with the largest effect on valine. In broilers, an increase in phytic acid from 10.4 to 15.7 g kg^{-1} resulted in significant decreases in the AID of nitrogen by 2.3% and multiple amino acids by an average of 2.0% (Ravindran et al., 2000a).

Threonine digestibility was the most effected by rises in phytic acid concentration.

Phytic acid could inhibit protein digestion through a number of mechanisms. Phytic acid will inhibit the digestion of the plant proteins associated with it within the plant cells. As seeds ripen phytin accumulates and becomes linked with protein (Cosgrove, 1980). These complexes have reduced solubility and may be refractory to pepsin digestion, preventing the amino acids becoming available for absorption (Ravindran et al., 1999a).

Secondly, protein-phytate complexes may also form *de novo* within the gastrointestinal tract, after phytic acid has become dissociated with the minerals and proteins it was bound to within plant cells. Phytic acid is able to bind to protein via strong electrostatic interactions dependant on pH (Selle et al., 2000). When the pH is below the isoelectric point of protein the negatively charged phytic acid will bind to the basic amino acids arginine, lysine, and histidine within the protein (Singh, 2008). This occurs quickly and is followed by the slow accumulation of proteins before eventually precipitating (Cosgrove, 1980). The formation of the phytate-protein complexes reduces protein susceptibility to pepsin because they have reduced solubility and structural differences. These changes in structure and solubility could affect the functions of the proteins. If enzymes become bound to phytate this may affect their level of activity. Due to the low pH required it is likely that in birds any *de novo* formation of phytate-protein complexes will occur in the proventriculus.

As the pH rises towards the isoelectic point of protein, phytase and protein become unbound as the protein charges are neutralised. Above the isoelectric point it is hypothesized that divalent cations such as calcium, magnesium and zinc bound to phytate can act as a bridge to the negatively charged protein carboxyl groups (Selle et al., 2000). This again would lead to proteins accumulating and precipitating out of solution within the gastrointestinal tract.

Another possible way phytic acid may inhibit apparent protein digestion is by increasing the flow and losses of the birds' endogenous proteins and amino acids (Ravindran et al., 1999a). This is supported by changes in threonine

digestibility, an amino acid that exists in high concentrations within endogenous secretions (Siriwan et al., 1994, Angkanaporn et al., 1994). Cowieson and Ravindran (2007) showed that the addition of 14.5 g kg^{-1} sodium phytate salt on top of a phytic acid free broiler diet resulted in an 87% increase in the endogenous flow of nitrogen and amino acids. To date, no published work has looked at the effect of phytic acid of the endogenous losses of amino acids and protein in laying hens.

Finally, phytic acid may also bind with enzymes to make phytate-enzymatic protein complexes. This could reduce enzyme activity and so slow down the rate of digestion. The chelating of cations such as calcium, discussed above, can also be essential for digestive enzymes such as trypsin and α-amylase (Selle et al., 2000). If calcium is restricted, the enzymes that require it will have reduced activity.

1.3.5.3 Effect of Phytic acid on Energy

Phytic acid has been shown to be detrimental to the energy status of a diet. In the piglet, it has been shown that increasing phytic acid concentration from7.8 to 17.0 g kg^{-1} can reduced the AID of energy (Liao et al., 2005). In broilers, an increase from 10.4 to 13.6 g kg^{-1} dietary phytic acid resulted in a decrease in apparent metabolisable energy (AME) from 14.04 to 13.73 MJ kg^{-1} dry matter (Ravindran et al., 2006). Miles and Nelson (1974) created a chick diet with and without the inclusion of dephytinised soybean meal and showed the removal of phytic acid increased energy utilisation.

It can be concluded therefore, that phytic acid can decrease the energy available in the diet to an animal. However, energy itself is not a nutrient, but the result of the metabolism of protein, carbohydrate and lipids. Therefore any effect in energy utilisation will be due to a change in the ileal digestibility of amino acids, carbohydrates and lipids (Camden et al., 2001). To understand the effect of phytic acid on energy the effects of phytic acid on the individual components of energy need to assesed .

As discussed above, multiple studies have shown that phytic acid can reduce the digestibility of protein and multiple amino acids through a number of possible mechanisms. As well as being an important nutrient within its own right, protein can be used as an energy source and so a reduction in

protein digestibility will affect the AME of a diet. In a broiler diet, the inclusion of sodium phytate, increasing the phytic acid concentration from 8.5 to 14.5 g kg^{-1} , resulted in an increase in the endogenous loss of protein of 6125 mg kg^{-1} (Cowieson et al., 2008).This protein loss is equivalent to 96kJ kg^{-1} DM energy intake (Woyengo and Nyachoti, 2011). However, the phytic acid doesn't have to increase endogenous losses, just secretions, to have an effect on energy. The digestion of feed has an inherent energy cost, increasing endogenous secretions will increase these costs even if the proteins are later recovered.

Although phytic acid can restrict the energy of a diet through manipulation of protein digestion, this is not the only mechanism. Low phytic acid feeds were made by adding 300g kg^{-1} phytase treated rapeseed meal to a basal diet. This was compared to a diet with 300g kg^{-1} placebo treated rapeseed meal and it was found that the low phytic acid diets had an improved apparent metabolisable energy but not an improved ileal protein digestibility. This suggests the phytic acid can inhibit energy utilisation without impeding protein digestion. Therefore, phytic acid must also interfere with the digestion of the other energy providing nutrients, starch and lipids (Newkirk and Classen, 2001).

In vitro starch digestibility was improved by 25% when navy beans were treated to remove all their phytic acid (15 g kg^{-1}); this improvement was reversed by the addition of 10 g kg^{-1} sodium phytate (Thompson et al., 1987). Yoon et al. (1983) showed similar results whereby 20 g kg^{-1} phytic acid addition to a human saliva *in vitro* digestion of wheat starch reduced starch digestibility by 50 %. This was alleviated with the addition of calcium, presumably through the creation of calcium phytate complexes. Lee et al. (2006) showed that the addition of 10 g kg^{-1} phytic acid to a phytate free diet reduces glucose absorption through low blood glucose levels in mice. Onyango et al. (2008) also showed that phytic acid reduced glucose absorption in the jejunum of broilers. Similarly a 6% blood glucose level reduction was seen in broilers when dietary phytic acid concentration was increased from 7.9 to 15.7 g kg^{-1} (Liu et al., 2008b). This study also reported that increasing dietary phytic acid reduced the activity of maltase, ɑ-amylase and sucrase by, 6, 8.3 and 11.4 % respectively. It appears that phytic acid

could therefore reduce the absorption of carbohydrates by inhibiting the activity of the digestive enzymes. This may explain why Yoon et al. (1983) found calcium alleviated their depressed starch digestion as calcium competed with protein to form complexes. Woyengo et al. (2013) also suggested that phytic acid could restrict carbohydrate digestion by binding to proteins that are associated with starch. Finally, it is structurally possible that phytic acid may bind to starch through phosphate linkages and therefore reduce starch solubility in addition to digestibility, but there is little evidence for this (Thompson, 1986).

Studies investigating the effects of phytic acid on lipid digestion are sparse, but some work has been done suggesting there is a phytate effect and to explain possible mechanisms. Lee et al (2005) showed that increasing dietary phytic acid from 0 to 15 g kg^{-1} in mice diets reduced the hepatic total lipids and total cholesterol while also reducing the concentration of cholesterol, and low density lipoproteins in the blood. This could reflect a decrease in lipid digestion, and in 2007 a similar study supported these findings while showing a reduction in the apparent absorption of total lipids and cholesterol (Lee et al., 2007). Camden et al. (2001)also found that a reduction in phytic acid level with the inclusion of exogenous phytase increased the ileal digestibility of fats.

The reduced digestion of lipids could be due to the formation of lipid-peptide-phytin complexes called lipophytins. It is possible that calcium-phytate complexes may form metallic soaps in the gastrointestinal tract. Matyka et al. (1990) found that the addition of beef tallow reduced phytate hydrolysis in chicks and increased the excretion of soap fatty acids. Ravindran et al. (2000b) showed that the energy effect of phytate was more pronounced when calcium was increased in the diet, suggesting calcium-phytate complexes are important in energy utilisation. It is also very likely that phytic acid inhibits the digestion of lipids by interacting with the enzymes of fat digestion. Phytic acid has been shown to reduce the activity of pancreatic lipase *in vitro* (Knuckles et al., 1989) and *in vivo* (Liu et al., 2009). Finally, there is the possibity that phtyic acid can, using multivalent cation bridges, bind to bile acids and form mineral-bile-phytic acid complexes. This would

reduce the digestion of fat but this possible mechanism has not yet been tested experimentally.

1.3.5.4 Conclusion on the Anti-nutritional Effects of Phytic Acid

Phytic acid is a polyanionic negatively charged molecule within the gastrointestinal tract, which enables it to bind to a multitude of nutrients and endogenous secretions. This causes the formation of insoluble phytate complexes which are refractory to phytase activity. The phytate-P bound within the complex are less available to the bird, as are the other nutrients bound to phytate. This could be through a drop in the solubility of nutrients within the complex or through increasing endogenous secretion as enzymes activities are reduced, Figure 1.2. Whatever the mechanisms, it is clear that phytic acid can be considered both a potential nutrient and an important anti-nutrient. Some have tried to solve this problem by producing plants low in phytic acid. This will remove the anti-nutritional effect of phytic acid, but also removes an important source of phosphorus and inositol from the animal. The use of exogenous phytase is a better solution to the phytic acid problem. The increased hydrolysis of phytic acid can remove the anti-nutritional effects while increasing the availability of the phytate-P.

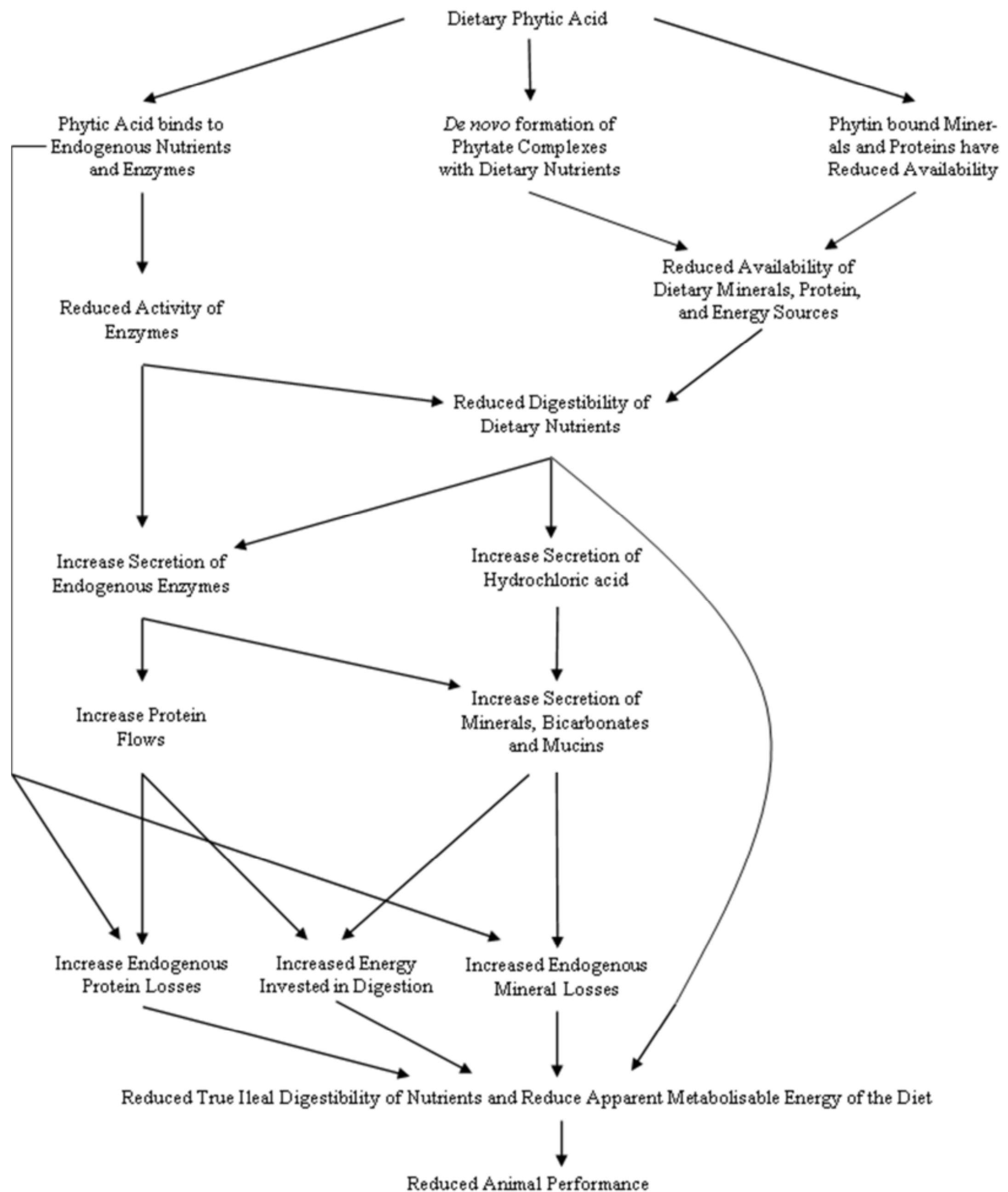

Figure 1.2 Summary of the anti-nutritional effects of phytic acid, adapted from Woyengo et al. (2013)

1.4 Phytase

1.4.1 Introduction to phytase

Improving phytate-P availability is an ecologically sustainable and cost effective alternative to increasing dietary phosphorus. For the phytate-P to be accessible to the chicken, it is necessary that phytic acid is hydrolysed to *myo*-inositol and inorganic phosphorus. Phytase (*myo*-inositol

hexaphosphate phosphohydrolase) is the name given to an enzyme that breaks down phytate (Singh, 2008).

Some believe the poor phytate-P utilisation is due to a lack of phytase within the gastrointestinal tract, however, this is inaccurate (Cowieson et al., 2011). There is phytase and phosphatase activity within the intestinal mucosa, blood and liver of the chicken capable of hydrolysing phytate (Oshima et al., 1964, Birge and Avioli, 1981, Maenz and Classen, 1998). The lack of phytate digestion is actually caused by poor substrate solubility within the intestine, caused by the formation of cation-phytate complexes previously mentioned (Wise and Gilburt, 1981).

The poor phytate-P digestibility can be resolved with the addition of supplemental exogenous phytases within monogastric diets. These phytases are active in the crop, proventriculus and gizzard where the pH is lower. Phytate complexes become insoluble upon entering the small intestines. Therefore exogenous phytases are capable of hydrolysing phytic acid before complexes become insoluble (Simon and Igbasan, 2002). Problems could occur within the crop where the pH is variable. A rise in crop pH could allow the formation of complexes before supplemental phytase can act.

1.4.2 Classification of Phytase

Phytases are a subgroup of phosphomonoesterases that use progressive steps to hydrolyse phytic acid through sequentially lower inositol polyphosphates ($IP_6 \rightarrow IP_5 \rightarrow IP_4 \rightarrow IP_3 \rightarrow IP_2 \rightarrow IP_1$), removing phosphorus to produce inorganic phosphorus moieties and a *myo*-inositol ring. Some phytases cannot hydrolyse phytic acid completely and only take the molecule to the lower esters. The term phytase is used to describe any enzyme that hydrolyses polyphosphate inositol to potentially release phosphate groups, and so encompasses a diverse group of enzymes with a broad range of sizes, structures and mechanisms (Bedford and Partridge, 2001). This has led to a number of classification practices. There are 4 distinct catalytic classes of phytase: Class I are the histidine acid phytases (HAPhy), class II are the β-propeller phytases (BPPhy), Class III are the cysteine phytases (CPhy) and finally Class IV, the purple acid phytases (PAPhy) (Mullaney and Ullah, 2003, Greiner, 2007). Phytases are also

defined as either acidic or alkaline phytases based upon their pH optimum. Finally, phytases can be categorised depending upon which *myo*-inositol carbon number they dephosphorylate first. There are three known starting positions giving the groups 3-phytase (EC 3.1.3.8), 5-phytase (E.C.3.1.3.72) and 6-phytase (EC 3.1.3.26)(Bedford and Partridge, 2001).

The majority of phytases found in nature belong to the HAPhy class and have no need for a metallic co-factor. Almost all HAPhy enzymes are acid phytases and have been found in bacteria, fungi, plants and animals (Wodzinski and Ullah, 1996a, Mullaney et al., 2000, Greiner, 2007). The active sites of the HAPhy enzymes are located between a conserved α/β-domain and a variable α-domain (Kostrewa et al., 1999). It is thought that the variability of the α-domain leads to differences in substrate binding. The final product of HAPhy activity is *myo*-inositol monophosphate. The phosphorus at C_2 position is refractory to hydrolysis and so phytic acid is rarely hydrolysed completely. Subsequently the products are normally 5 inorganic phosphorus moieties and one *myo*-inositol monophosphate (IP_1)(Wodzinski and Ullah, 1996b). The HAPhy Class contains both 3-phytases and 6-phytases.

BPPhy enzymes were first discovered in *Bacillus spp.,* but have now also been found in *Xanthomus oryzae* and *Shemanella oneidensis* (Kerovuo et al., 1998, Chatterjee et al., 2003, Cheng and Lim, 2006). They are a 3-phytase that share no homology with other types of phosphatases. Their structure consists of a folded propeller with 6 blades, each with a calcium binding site (Shin et al., 2001). The presence of 3 calcium ions on the high affinity sites increases the thermostability of the enzyme. The addition of another 3 calcium ions activates the enzyme by removing the negative charge from the active site making it suitable for phytate. They are an alkaline phytase capable of hydrolysing calcium phytate complexes between pH 7 and 8 (Oh et al., 2001). Alkaline phytases exhibit strict substrate specificity for calcium–phytate complexes and produces *myo*-inositol trisphosphate as the final product (Oh et al., 2004).

CPhy and PAPhy like HAPhy are both acidic phytases. PAPhy are a type of acid phosphatases given this name because of the purple colour of their

oxidised form. Purple acid phosphatases have been found in bacteria, fungi, animals and plants, but it is only in plants that they show phytase activity. They were first discovered in the cotyledons of germinating soybean seeds, but have since been found in wheat and barley (Hegeman and Grabau, 2001). PAPhy contain a binuclear centre with at least one Fe^{3+} ion and either a Mn^{2+}, Zn^{2+}, Mg^{2+} or another Fe^{3+} ion (Bedford and Partridge, 2001).

CPhy were first discovered in *Selenomonas ruminantium,* an anaerobic ruminal bacterium. They are a cysteine phosphatase which is related to the tyrosine phosphatases that do not require a co-factor. The active site of CPhy consists of a loop that creates a pocket to hold the substrate. This is a similar mechanism to that of the trypsin phosphatases. The loop is cable of holding a phytic acid molecule that is fully phosphorylated to IP_6 (Yanke et al., 1999).

1.4.3 Phytase Discovery and Development

Phytase activity was first discovered in rice bran over 100 years ago ((Suzuki et al., 1907), 50 years after the discovery of phytate itself (Hartig, 1855, Cowieson et al., 2011). In the 1960’s Nelson and colleagues started the first in-depth research into the negative effect of phytate on the availability of phosphorus and calcium (Nelson, 1967). This coincided with the first attempts to produce a phytase feed additive (Wodzinski and Ullah, 1996b). However, phytase didn’t take off at that time as production on a large scale was difficult and expensive. This would have made phytase uneconomical compared to the inorganic phosphorus sources that were relatively cheap compared to today’s prices.

After developments in genetic engineering, it was in 1991 that the first commercially viable phytase products became available (Bedford and Partridge, 2001). The use of phytase was encouraged by legislation brought into force in the Netherlands that produced substantial pressure to reduce phosphorus pollution on agricultural land (Chesson, 1993). A simple way to reduce phosphorus pollution is to use phytase in the diet to improve plant phytate-P availability and reduce the level of NPP within animal diets thereby increasing total-P retention and reducing phosphorus excretion.

1.4.4 Defining phytase activity

There is no single universally recognised international standard for phytase activity which can cause confusion within the nutrition industry and academia. However, *fytase* units (FTU) are the most common measurement of phytase activity within the literature. One FTU is defined as the amount of enzyme that liberates 1 µmol of inorganic phosphorus from 0.0051 mol L^{-1} sodium phytate at pH 5.5 and 37°C per minute (Engelen et al., 1994). When included within a diet the amount of phytase will be expressed as FTU kg^{-1}.The use of FTU provides an easy and simple system to measure the activity of phytase, but is limited as different phytase enzymes will have different pH optima and Michaelis constants. This has led to other assays being used for activity measurements using pH ranges more suitable to the phytase being analysed. In practice, the activity of phytase will also be affected by the conditions the digestive tract such as the temperature, retention time and pH in addition to the concentrations of different nutrients within the diet (Selle and Ravindran, 2007). Another problem with the FTU measurement is the substrate sodium phytate. Sodium phytate does not accurately represent the “naturally” occurring phytin which is less soluble and harder to degrade because it has different and multiple minerals, most commonly potassium, magnesium and calcium (Onyango et al., 2009).

1.4.5 Sources of Phytase

There are four sources of phytase in the diet of the laying hen (Selle and Ravindran, 2007, Bedford and Partridge, 2001).

- Endogenous intestinal phytase in the digestive secretions
- Gastrointestinal tract microflora phytase
- Intrinsic plant ingredient phytase
- Supplemental microbial phytase

1.4.5.1 Intrinsic Plant phytase

Plants need to use phytases to utilise the *myo*-inositol, phosphorus and other minerals stored within the phytin complexes. These are 6-phytases with relatively low levels of activity and little effect on the hydrolysis of phytic acid but they have been shown to break down phytic acid within feed

(Temperton and Cassidy, 1964, Scheuermann et al., 1988). The level of phytase activity within an ingredient will be dependent upon its cultivation, genetics, age, drying and storage. Plant phytases are vulnerable to pH, temperature and proteolysis, and so do not survive high temperature pelleting processes or for very long in the gastrointestinal tract (Konietzny and Greiner, 2002b). To overcome this, transgenic plants have been developed using the phytase genes from fungi and bacteria to create high levels of stable phytase enzymes. These sources of phytase could become important in the future if they provide cheap phytate hydrolysis, but currently are not widely used.

1.4.5.2 Endogenous and Microfloral Phytase

McCollum and Hart (1908) were the first to show phytase activity in any animal tissue. Since this pioneering work, other studies have shown that phytate can be hydrolysed by the intestinal mucosa of the human, rat, calf, and chicken (Dias et al., 2010, Bitar and Reinhold, 1972). The level of phytic acid present in a chick’s blood increases dramatically in the first three weeks after hatch indicating that phytic acid can be digested (Oshima et al., 1964). Maenz (1998) demonstrated that there was substantial phytase activity within the small intestines of the chicken that reduced distally through the jejunum and ileum. The activity was also found to be greater in the mature laying hen relative to the younger broiler chick. Lopez et al. (2000) found that in the rat the greatest phytase activity within the small intestines was present in the duodenum (Lopez et al., 2000). The production of endogenous phytase increases with dietary phytic acid concentration and decreases as the concentration of NPP rises suggesting that animals can react to the form and availability of dietary phosphorous (Lopez et al., 2000, Hu et al., 1996).

Kerr et al. (2010) suggested that micro-organisms in the crop may also help hydrolyse phytate. However, it has been shown that germ-free rats can break down phytic acid as easily as conventional rats; this suggests it is the endogenous and not microbial phytase that is more important in the hydrolysis of phytate within the gastrointestinal tract (Wise and Gilburt, 1982).

Although the bird has substantial phytase activity it is not utilised because the phytic acid is bound within phytate complexes and so not available for hydrolysis within the intestinal mucosa (Cowieson et al., 2011). At very high calcium concentrations it has been reported that the amount of phytic acid hydrolysed is negligible (Taylor, 1965).

1.4.5.3 Exogenous Supplemental Phytase

Nelson et al. (1971) showed microbial phytase could break down the phytic acid of plant feed ingredients. There is now a range of supplemental phytases available on the market that are stable at high temperatures, active over a wide pH range, refractory to proteolysis and will readily hydrolyse phytic acid (Usmani and Jafri, 2002).

Phytases available on the commercial market are most commonly HAPhy and come from *Aspergillus niger* , *Peniophora lycii* and *Escherichia coli* (Bedford and Partridge, 2001). *A. niger is* a type 3-phytase while both *P lycii* and *E. coli* phytases are type 6. The phytases are produced by genetically engineered fungi that have cloned vectors inserted into their DNA, which are then overexpressed to produce large quantities of the enzyme. The fungi are housed in a batch-fed submerged pure culture fermentation container (Bedford and Partridge, 2001). The fermentation broth is collected and the phytase is captured by multiple processes. The first step of the purification process is to centrifuge the broth removing the majority of the fungi cells. Filtration will remove the remainder of the cells while ultrafiltration and diafiltration will remove any insoluble non-phytase compounds of low molecular weight. The final product will be subject to a number of tests to ensure it has the required phytase activity, purity and chemical composition.

Phytase can be added to animal feed as dust/granules or in a liquid form. If the feed is pelleted the phytase is often added in liquid form after the pelleting process, which involves temperatures exceeding 80°C, to overcome heat stability problems. Heat stable varieties are now available that provide a more reliable enzyme that can be added to the feed before pelleting and reduce manufacturing costs (Garrett et al., 2004). *E. coli* phytase enzymes are reported to be more heat stable compared to others (Igbasan et al., 2000).

1.4.6 Sites of Phytase Activity

Within the chicken, exogenous phytase is mainly active in the first half of the gastrointestinal tract; the crop, proventriculus and gizzard. This is because the pH is lower and so more favourable for phytase activity. Phytase from *E. coli* has been shown to maintain activity later in the tract compared to *P. lycii* phytase, which suggests it may be more resistant to proteolytic enzymes active in the latter parts of the tract (Table 1.5; Onyango et al., 2005b). *E. coli* phytases have also retained the highest residual activity (93%) after incubation with proventriculus and gizzard digesta supernatants for 1 h at 40°C (Igbasan et al., 2000). Phytase activity within a section of the gastrointestinal tract, however, does not guarantee phytate hydrolysis. Many factors can affect the efficacy of exogenous phytase within the gastrointestinal tract.

Table 1.5 Phytase activity (FTU kg^{-1} DM) along the gastrointestinal tract when a control diet is supplemented with different phytases

	Control	Control + 1000 FTU Kg^{-1} *E. coli*	Control + 1000 FTU Kg^{-1} *P. lycii*
Crop	67^{c}	649^{a}	404^{b}
Proventriculus	28^{b}	406^{a}	63^{b}
Jejunum	29^{b}	554^{a}	25^{b}
Ileum	16^{b}	91^{a}	6^{b}

Adapted from Onyango et al. (2005b)

$^{a-b}$ Means within columns with no common superscript differ significantly (P<0.05)

1.4.7 Factors Affecting the Efficacy of phytase

The efficacy of phytase will be affected by all of the factors that affect the hydrolysis of phytic acid without exogenous phytase supplementation that have already been discussed in Section 1.4.4.

The form of the phytic acid substrate will effect it's hydrolysis, both with and without exogenous phytase. Leske and Coon (1999) showed the effect of 600 FTU kg^{-1} phytase on the hydrolysis of phytic acid from different ingredients to vary widely from 6.3% in rapeseed meal to soybean meal at 37.5 %. This difference is seen despite the fact that rapeseed meal has a

higher level of phytic acid and so should have greater concentration of substrate available for hydrolysis.

Of particular importance when discussing phytase efficacy is the availability of the phytic acid to the exogenous phytase. The increase in dietary mineral levels will allow a greater formation of phytate complexes, and so reduce phytic acid solubility and the efficacy of phytase. Minerals themselves can bind to phytase and reduce phytase activity, but there is little evidence of this. The inclusion of limestone especially can have a large effect on phytase efficacy as it can result in a dramatic rise in gastrointestinal tract pH, notably in the crop (Shafey et al., 1991). A rise in crop pH can force the early formation of phytate complexes before the phytase can significantly hydrolyse the phytic acid.

Different commercially available phytase enzymes will have different levels of activity per kilogram, but even if they are equated in terms of FTU kg^{-1}, differences in Km values, pH optimum ranges, thermostability and resistance to pepsin degradation will all affect the amount of phytic acid they hydrolyse. Onyango et al. (2005b) added 1000 FTU kg^{-1} of *E. coli* or *P. lycii* phytase to a diet with a low level of NPP and found that the *E. coli* phytase had a higher level of activity in all sections of the gastrointestinal tract.

The addition of other exogenous enzymes can also affect the activity of phytase. The use of xylanase in wheat diets can help phytase access the phytic acid substrate and improve the utilisation of nutrients released by phytase (Ravindran et al., 1999b). The addition of proteinase enzymes may also affect phytase activity (Cowieson and Adeola, 2005). It could possibly benefit phytase by reducing the formation of protein-phytate complexes, or inhibit phytase activity through increased proteolysis.

Finally, as shown in Section 1.342, we know that free phosphorus can inhibit phytase activity and so a increases in dietary NPP will inevitably reduce the activity of phytase. There is also a possibility that plant, endogenous and gut microbial activity could affect the activity of endogenous phytase through competition for substrate and an increase in the presence of free phosphorus. However, given their low levels of activity this effect is probably negligible.

1.4.8 Benefits of Dietary Phytase Inclusion

There have been many studies that have shown the supplementation of exogenous phytase in chicken diets can improve performance. For example, the growth performance of broilers fed a diet with 4.5 g kg^{-1} total phosphorus improved by 54% when 1500 FTU kg^{-1} was included in the diet (Simons et al., 1990a). The benefits of phytase are more pronounced when the diets have reduced levels of dietary phosphorus, indicating that increased phosphorus digestion may be the potential mechanism. However, phytase has also improved the growth of animals fed diets with adequate levels of phosphorus. Selle et al. (1999) showed that phytase could improve the weight gain of birds fed a diluted diet with lower levels of phosphorus, calcium, protein and energy. The treatments did not significantly affect the toe ash and so it was suggested that the phytase benefits are extra-phosphoric as phosphorus was not limiting.

Diets with high nutrient specifications are less likely to show phytase performance responses. This is because the anti-nutritional effects of phytic acid on nutrient availability will not be as significant, as the birds' requirements can still be met by the base diet. As a result, the hydrolysis of phytic acid by exogenous phytase will benefit the bird less. The effect of phytase on performance will be related to the underlying level of phytic acid within the diet. Selle et al. (2003a) showed that exogenous phytase allows a diet dilution without reducing the desired production level and that this nutritional strategy can be more economical. In laying hens, the supplementation of phytase reversed the increase in FCR caused by an increase in phytic acid (Jubarah et al., 2006).

1.4.8.1 Effect of Phytase on Phosphorus

Numerous studies have shown that the addition of phytase to a poultry diet can improve the availability of phytate-P and increase phosphorus retention through the hydrolysis of phytic acid (Denbow et al., 1995, Ravindran et al., 2000b, Boling-Frankenbach et al., 2001, Augspurger and Baker, 2004, Onyango et al., 2005a)

The improvement in phytate-P utilisation allows nutritionists to decrease the amount of NPP or TP within the diet. Phytase has been shown to reverse

negative effects caused by low phosphorus diets in pigs, broilers and laying hens (Denbow et al., 1995, Harper et al., 1997, Van der Klis et al., 1997, Gordon and Roland, 1998, Jalal and Scheideler, 2001). The decrease of NPP within diets and improvement of phytate-P absorption can be used to reduce excreted phosphorus and therefore phosphorus pollution.

The ability to replace NPP with phytase has led to the search for equivalence values. In order to formulate diets correctly, nutritionists require knowledge of how much phytate-P phytase releases *in vivo*. The fytase units given to phytase enzymes are based on *in vitro* experiments and so they are not appropriate for dietary formulations; equivalencies are generated with bioassay experiments based on performance data. However, these can be flawed because phytase can have additional beneficial effects unrelated to phosphorus. Many have studied the phosphorus phytase equivalences in broilers, and have produced results varying from 400 FTU kg^{-1} = 1 g phosphorus to 1146 FTU kg^{-1} = 1g phosphorus (Augspurger et al., 2003, Yi et al., 1996, Selle and Ravindran, 2007). In laying hen, Van der Klis et al. (1997) reported a phosphorus equivalence of 190 FTU kg^{-1} = 1 g phosphorus, but also found this varied dependant on NPP dose. This variance in equivalences stems from the differences in response criteria, basal diet, level of NPP, level of phytic acid, calcium phosphorus ratios and all the other factors that can affect phytase efficacy (Denbow et al., 1995). These factors should be taken into account when reducing the level of NPP in the diet in favour of phytase.

1.4.8.2 Effect of Phytase on Minerals

As phytase breaks down the phytic acid it will also reduce the amount of calcium and other minerals that become bound within the insoluble phytate complexes. Phytase is not as effective at hydrolysing phytic acid when it is already bound to calcium (Plumstead et al., 2008). That said, exogenous phytase acts in the foregut and so can hydrolyse phytate before mineral-phytate complexes can form within the small intestines unless the pH of the crop rises.

Laying hens have a high calcium demand for eggshell production. This will increase the amount of calcium available for Ca-phytate complex formation

and can increase the pH of the digestive tract, more specifically the crop, towards an environment more suitable for complex formation; this may inhibit the effect exogenous phytase can have on breaking down phytate. However, 1000 FTU kg^{-1} did hydrolyse phytate to lower esters in layer hen diets with high calcium concentrations of ~45g kg^{-1} DM (Agbede et al., 2009b). Shirley and Edwards (2003) showed that increasing phytase inclusion increased the retention of dietary calcium in broiler diets. Augspurger and Baker (2004) took a basal diet deficient in calcium and added either phytase at 500 FTU kg^{-1} or one of five graded doses of Ca (0-4g kg^{-1}) to calculate equivalence values. They found that their phytase released the equivalence of 0.72 g Ca kg^{-1}.

1.4.8.3 Effect of Phytase on Protein

As previously discussed phytic acid can be detrimental to protein and amino acid digestion through a number of mechanisms. It stands to reason therefore that phytase addition would hydrolyse phytic acid and prevent these negative effects. Shirley and Edwards (2003) showed that increasing phytase inclusion increased the retention of nitrogen. Meanwhile, multiple studies have shown that phytase can improve amino acid digestibility(Selle et al., 2000). Rutherfurd et al. (1997) showed that phytase benefitted the true ileal digestibility coefficient of 16 amino acids in maize, wheat, rapeseed meal and soyabean meal by 3.90, 12.94, 9.31 and 6.39% respectively when fed to broilers. In laying hens, Van der Klis and Versteegh (1991) showed the addition of 250-300 FTU kg^{-1} gave significant, if small, improvements in the apparent ileal absorption of nitrogen indicating a protein effect of phytase. A more recent trial showed that diluting a diet to reduce the level of crude protein (17 vs 15%), metabolisable energy (2,900 vs 2800kcal ME kg^{-1}), calcium (4.2 vs 3.8%) and phosphorus (0.375 vs 0.28%) had no negative effect on performance if supplemented with phytase at 600 FTU kg^{-1} (Lima et al., 2010) .

Snow et al. (2003) added 300 FTU kg^{-1} phytase to three different laying hen diets. The control was a maize-soybean diet, the treatment diets had either 75 g kg^{-1} meat and bone meal or 100g kg^{-1} wheat middling added. Although numerical increases between 2-3% were seen there was no significant

phytase effect on the digestibility of amino acid. Liu et al. (2007) showed that the addition of phytase to a calcium and phosphorus deficient diet improved the nitrogen and amino acid digestibility in comparison with a negative control diet. Agbede et al. (2009a,b) have published two similar trials where supplemental phytase was added to the diets of caectomised laying hens. In the first trial they found phytase could significantly improve the utilisation of protein, energy and amino acids in diets with low levels of calcium (Agbede et al., 2009a). This effect was reduced somewhat when the calcium concentration was higher. In their second trial, phytase failed to significantly affect the digestibility of nitrogen or amino acids across a range of dietary phosphorus levels.

It appears that phytase does have the capacity to improve protein and amino acid utilisation in laying hens, however, this is by no means consistent. It may be that the high calcium level required in laying hen diets goes some way to explain the inconsistencies seen between trials. Regardless, a number of factors need further investigation before it can be concluded that the protein effects of phytic acid and phytase are significant in explaining performance differences seen.

1.4.8.4 Effect of Phytase on Energy

In broiler diets, the inclusion of phytase consistently increases the apparent metabolisable energy of a diet (Selle and Ravindran, 2007). Selle et al. (2005) showed that including phytase at 600 FTU kg^{-1} in a wheat based diet increased the AME from 14.22MJ kg^{-1} to 14.56MJ kg^{-1}. Scott et al. (2001) showed that 1000 FTU kg^{-1} phytase inclusion in layer diets improved the AME in both maize or wheat based diets by 3.5 and 3.6% respectively. Liebert et al. (2005) found that 300 FTU kg^{-1} phytase had no effect on AME in layer diets. It is probably that the difference seen between these studies is due to the difference in phytase dose. A reduction in metabolisable energy from 12.14 to 11.72 MJ ME kg^{-1} had no negative effect with the inclusion of 600 FTU kg^{-1}(Lima et al., 2010). Camden et al. (2001) found that phytase improved the AME of a broiler diet by 1.1% and that this was due to an improved ileal digestibility of fat, protein and starch by 3.5, 2.6 and 1.4%. To conclude, phytase can affect the apparent metabolisable energy (AMEn) of a

diet, but it appears that higher doses are required for the improvements to become significant.

1.4.9 High Phytase inclusion

The standard inclusion of exogenous phytase within poultry diets is 250 – 1000 FTU kg^{-1}. Recently there has been increased interest in “super-dosing” or including phytase at levels higher than those of the standard inclusion. A phytase dose is considered to be above the standard inclusion level when the phytase activity is at or greater than 2500 FTU kg^{-1}. A number of studies have now shown that the inclusion of high levels of phytase within diets can benefit animal performance (Cowieson et al., 2011).

The aim of the high inclusion levels is to hydrolyse all phytic acid in the foregut down to the lowest esters. Within the small intestines a rise in pH will strengthen the association between the higher phytic acid ester and charged nutrients resulting in precipatiation. Hydrolsying phytic acid into the lower esters before it enters the duodenum reduces the formation of phytate complexes, as the lower esters are more soluble and have less ability to bind cations (Persson et al., 1998). This means they are more available to endogenous phytase which can further hydrolyse the phytic acid to *myo*-inositol, IP_1 and phosphorus. These can then be absorbed and utilised by the bird.

A number of mechanisms have been suggested to explain how a high phytase diet can improve performance (Cowieson et al., 2011); the first is through the release of additional phosphorus. The complete hydrolysis of phytic acid will release almost all the phytate-P(Nelson et al., 1971). This is a substantial amount of phosphorus, however, this will only improve the performance if phosphorus is a limiting nutrient, and so is unlikely to be the main mechanism seen in the trials with adequate phosphorus. Excessive phosphorus will just be excreted in the manure (Angel and Applegate, (2002)). Although this is probably not the mechanism that improves performance, the release of all of the phytate-P will allow further reduction in NPP and therefore could reduce the cost and environmental impact of monogastric diets.

Phytase targets the higher esters of phytic acid (Wyss et al., 1999), IP6 and IP5; this is important because these phytic acid molecules have a greater calcium binding capacity than the lesser esters, IP4 and IP3. For example, IP3 has approximately 10% of the calcium binding capacity of IP6 (Persson et al., 1998). Therefore, initial phytase activity will release disproportionately high amounts of calcium compared to phosphorus. The use of high phytase doses can deliver a more balanced release of phosphorus and calcium, closer to the 1.1 calcium phosphorus ratio normally used for phytase matrices. It may be that the benefits seen in the higher inclusion trials are due to a restoration of the calcium phosphorus ratio (Cowieson et al., 2011).

The complete hydrolysis of phytic acid within the gastrointestinal tract will also have a benefit through the removal of the extra-phosphoric effects of phytate. As previously mentioned, phytate complex formation hinders mineral, protein and energy nutrition. Dephosphorylation of phytic acid earlier within the tract has the potential to remove these negative extra-phosphoric effects by preventing the formation of phytate complexes.

Finally, there is the benefit of increased *myo*-inositol availability. After phytate has been dephosphorylated to the lower esters, they can be absorbed and further broken down into free phosphorus and *myo*-inositol. *Myo*-inositol is known to be involved with the transport of fats and fat soluble nutrients, and it has been shown to increase growth in rats and decrease fatty liver syndrome (Katayama, 1997). It is also worth noting that *myo*-inositol is the precursor of multiple compounds that are important for maintaining normal cellular function. For example IP_3 can be used as a secondary messenger during cell signalling or in the control of intracellular calcium ion concentration (Berridge, 1993). Little work has been conducted on animal requirements of *myo*-inositol, but Hegsted (1941b) showed that it improved chick growth. It may be that phytase provides the bird with a large amount of *myo*-inositol which can improve animal performance.

1.5 Concluding Remarks

Phytic acid is an integral part of plant seeds, feed ingredients and the laying hen diet and can be thought of as both a potential nutrient and anti-nutrient.

Phytic acid contains a large proportion of dietary phosphorus but unfortunately it is not readily available. This is because the phosphate groups on the phytic acid molecule make it very negatively charged. These negative charges attract a large number of cations within the tract to form insoluble phytate complexes. The insolubility of these complexes make them refractory to endogenous phytase activity and so the phosphorus is largely unavailable. The formation of these complexes also restricts the digestion of the other nutrients, these are known as the extra-phosphoric effects of phytate.

Exogenous phytase can be included in the diet to hydrolyse phytic acid before phytate complexes form, and so release more of the valuable phytate-P. In doing this, the number of phytate complexes formed are reduced. The reduction in phytate formation reduces the adverse effects of phytic acid on calcium, other minerals, protein and energy of the diet.

The effects and main mechanisms of phytic acid and phytase are well understood in broiler nutrition,n but little research has been done on laying hens due to the difficulty and expense of running layer trials. While much of the broiler research can be transferred to laying hens, the fundamental differences between the birds and their diets requires experiments focused on layers. For example, the difference in dietary calcium concentration indicates the need for layer trials, as the concentration of calcium can have such an adverse effect on phytase efficacy. Finally, the use of high levels of phytase beyond the standard inclusion levels have shown to benefit both broilers and piglets, but this has not yet been shown in the laying hens and the underlying mechanisms are not well understood.

1.6 Study Aims

This thesis aims to investigate if using higher than standard phytase inclusion levels can benefit laying hen performance and to investigate the mechanisms behind such improvements.

Chapter 2
Materials and Methods

2.1 Introduction to General Methods

To avoid repetition, a general methods chapter has been included to detail methodologies that were similar throughout the different experiments. All experiments were reviewed by the University of Leeds animal ethics committee and given approval before taking place. The animal housing and husbandry was in accordance with current agricultural regulations, Council Directive 99/74/EC and the Welfare of Farmed Animals (England) Regulations 2007. In addition to this, all scientific animal procedures were in line with the Home Office guidelines (Home Office, 2014) and Animals (Scientific Procedures) Act 1986. The animals were monitored by poultry vet Jim Morris from Haven Veterinary Group.

2.2 Animal Husbandry

Throughout the different experiments the birds were kept either individually or in small groups. The cages of the birds differed depending upon whether the birds were housed individually or not.

2.2.1 Individual housing

The birds were kept in wire mesh cages that measured 51cm wide by 52 cm deep. The floor had an 8° slope to remove eggs from the cage giving a minimum height of 69cm and maximum height of 76 cm. The feed was presented to the bird in a 36cm long trough that hooked onto the outside of the door at the front of the cage. The birds accessed the trough through two 15 cm holes in the front door. The trough had a lip at the front to prevent feed wastage and side baffles to stop birds eating their neighbour’s diets. The cages were held in stacks of 6 with 2 tiers and kept in two rooms. Each

cage had 3 water nipples, an abrasive claw shortening device and a tray under the mesh floor to collect excreta.

2.2.2 Group Housing

The chickens were housed within one animal room in cages across 3 tiers, 20 cages per tier. These were enriched, colony style cages that were inspected by an Animal Health officer after the ban on conventional barren cages. The cages measured 72cm wide and 50cm deep. The floor of the cage had a 8° slope to remove eggs. The height at the rear and front of the cage were 56cm and 62cm, respectively. Each cage was equipped as standard, with two 32cm feed troughs, a nest area, 60cm of perch space, a scratching litter area, an abrasive claw shortening device and at least 3 water nipples at all times. An electric wire was located under the egg baffle to prevent birds egg eating and a tray was placed under the cage to collect excreta. The cages were used to house either 3 or 4 birds depending upon the trial.

2.2.3 Birds

The laying hen breed used throughout all experiments was the Bovan Brown layer. The breeding company's husbandry recommendations were used as a guide during bird management. The age of the birds used was dependent upon the experiment. In experiments using start of lay birds the pullets were purchased from a commercial floor rearing facility. The birds were selected and brought to the University of Leeds farm before the onset of lay and went onto trial at 21 weeks of age. Experiments on mid-lay birds started at 36 weeks of age. These birds were purchased from a commercial enriched cage laying hen farm a few weeks before the trials commenced. Any birds that reacted badly to their move during lay were not put onto trial. Numbered wing bands were used to identify individual birds.

Health checks were undertaken on birds before being brought onto the farm; these included inspections of the pubic bone, vent, comb, wattle, and feather cover of the birds.

2.2.3 Lighting Temperature and Health

The health status of the birds was checked daily; any birds showing signs of sickness or disease were noted and if deemed necessary were euthanized. Any natural deaths and euthanized birds were recorded on the day they occured. The rooms were kept at 21°C using 4 ventilation inlets, two extracting fans and heaters when necessary. The hours of light provided each day were dependant on the age of the birds and followed the breeders' recommendations; 15 hours of light at 21 weeks of age and 16 hours of light thereafter. The light intensity was kept between 6-10 lux, but increased to 50 lux for daily inspections.

2.2.4 Feed and Water

Both feed and water were available *ad lib,* to the birds at all times. The feed was regularly topped up to ensure it was fresh and the water lines were flushed once a week to prevent the build-up of stale water or microbial contamination. Daily checks were carried out on water line and feed level. Throughout all of the trials the feeds used were mash diets. All the experimental diets were formulated with help from the industry sponsors on Format International feed formulation software. The diets were then produced by Target Feeds Ltd, a specialist trial feed mill, in single batches a week before the experiment began. Feed was stored in 25 kg coloured bags in a cold room and opened only when needed to ensure it was kept fresh. Each diet had a different coloured bag and a lettered label that corresponded to a particular treatment. The identity of the treatments was unknown throughout the duration of the experimental trials to ensure all measurements were unbiased. Each week a 250 g sample of feed was taken and frozen at -20°C. At the end of the experiment equal weighted sub-samples of the feed from each week were mixed and sent for analysis. All of the feed analysis was done by Sciantec Analytical. The diets were analysed for moisture, crude protein, crude fibre, oil A (ether extract), ash, calcium, phosphorus, sodium and phytate phosphorus.

2.3 Animal Performance Measurements

2.3.1 Feed Intake

During all of the trials several production criteria were measured and recorded. The amount of feed presented to the birds and the feed refused by birds each week were recorded and used to calculate the average daily feed intake. When individually housed, the chickens were fed daily and were given 5 grams more than their previous day's feed intake. This was to ensure the birds ate the complete diet and prevent the birds selectively feeding. This was not a problem in the group housed birds.

2.3.2 Bird Weight

The chickens were weighed at the start and end of every trial. This was used to calculate the average weight gained during the trial. The birds were always weighed in the afternoon to ensure they had all laid their eggs for that day.

2.3.3 Egg Production and Weight

Eggs were collected and counted throughout the trial, facilitating the generation of hen-day egg production data. The eggs were then weighed to give an average egg weight and daily egg mass production. Accurate egg weights cannot be obtained from wet cracks and dropped eggs so these eggs were excluded from the average egg weight and egg mass production data sets. An adjusted egg mass (Adj Egg Mass) was calculated making the assumption that the weight of any dropped or cracked egg was that of the average egg for that cage and week.

2.3.4 Feed Conversion Ratio

The egg mass production and the average daily feed intake were used to calculate the Feed Conversion Ratio (FCR) of the laying hens. An adjusted FCR (AdjFCR) was also calculated using the adjusted egg mass production.

2.3.5 Egg Quality Analysis

On specific days during the trials detailed egg quality analysis was done. On these days the eggs were individually marked for identification, weighed to 0.01 g accuracy and then candled. Eggs were classified as whole, with a hairline crack, gross crack or wet crack. The eggs then had their midline drawn on for future measurements. The whole eggs were then strength tested using a ORKA Egg Force Reader to an accuracy of 0.001 kgf. Immediately after the strength test the eggs were cracked open onto a level glass breakout unit with an angled mirror beneath. When the eggs were broken open the midline of the shell was kept intact. The breakout unit and mirror were used to inspect the egg for blood and meat spots. The eggs were given a spot score with 0 indicating no spots, 1 representing a few spots only noticeable under detailed inspection and 2 signifying a large amount of spots clearly noticeable without detailed inspection. The albumen height was then measured using a BAXLO tripod based dial micrometre to an accuracy of 0.1 mm. The albumen height was measured at three points 1cm from the egg yolk. The average albumen height was used with the egg weight to generate a Haugh Unit measurement using the equation (Haugh, 1937):

$$HU = 100 \times \log(AH - 1.7EW^{0.37} + 7.57)$$

Where

HU = Haugh units

AH =Albumen Height

EW = Egg Weight

The yolk colour was then recorded using a DSM yolk colour fan chart. Larger scores indicate a deeper colour becoming more orange and less pale. Finally, the eggshells were cleaned with gentle flowing distilled water above a sieve to catch any small detached fragments of shell. These were carefully cleaned, retained and the complete shell was dried in an oven set at 100°C for 24 hours. Once dry the shell was weighed to 0.01g and the shell thickness was measured at three points on the midline drawn previously on the egg to an accuracy of 0.001mm. In some of the trials the yolks were then separated and weighed to 0.01g after their colour had been recorded.

2.4 Laboratory analysis

2.4.1 Mineral Analysis of Feed and Digesta

2.4.1.1 Cleaning of Glassware

To remove any mineral contamination all glassware used during these procedures were acid washed before use. Briefly, after the standard laboratory glass wash 10 ml 5M hydrochloric acid was added to each piece of glassware and brought to the boil using a hot plate. The acid was then disposed of, this process was repeated and finally the glassware was rinsed with distilled water three times. The glassware was then allowed to dry in a clean drying oven set at 100°C for at least 24 hours and then covered with parafilm and stored within a clean cupboard until used.

2.4.1.2 Dry Matter and Ash Weight

Clean thermostable glass tubes identified with a thermostable ink were oven dried at 100°C until the tube reached a stable weight (approximately 24 hours) and allowed to cool in a desiccator. The tube was weighed to the nearest 0.0001g before a known weight of approximately 1 g of sample was added to a tube. This weight was recorded as the sample wet weight. The sample was then placed in the same oven for 24 hours. The tube was allowed to cool in a desiccator and reweighed to give the sample dry weight. The sample was then put in a muffle furnace at 550°C for 16 hours. The furnace was allowed to cool to 100°C before the tubes were placed into a

desiccator and allowed to cool. The tube was then weighed to give the sample ash weight.

2.4.1.3 Dissolution of Minerals

An acid rinsed watch glass was placed onto the sample tube before 10 ml 5M hydrochloric acid was added to the tube. This was added as two lots of 5ml to avoid loss by effervescence. The sample tube was placed on a cold hot plate and the acid was brought to the boil by slowly increasing the temperature of the hot plate. The sample was then allowed to simmer for 5 minutes. The tube was then removed from the hot plate and allowed to cool. Once cool the solution was rinsed through an ashless filter paper into a 100ml volumetric flask. The tube was then washed into the filter paper with hot distilled water three times and the filter was rinsed with hot distilled water. The washings were sealed to prevent contamination and allowed to cool to room temperature. Once cool, the solution was brought up to 100ml with distilled water, resealed and mixed by inverting 10 times. This solution was stored in acid washed glass tubes for determination of mineral concentration using inductively coupled plasma mass spectroscopy (ICP-MS).

2.4.1.4 Inductively Coupled Plasma Mass Spectroscopy

When ready for analysis the solutions were diluted to bring the concentration of minerals into the correct range (approximately 1000 µg/l). The solution was diluted with a 2% nitric acid solution that was prepared fresh from ICP grade 70% concentrated nitric acid and ultrapure water with a resistance of 18 megohms or greater. An ICP-MS mineral standard was purchased and diluted with the same 2% nitric acid to create a five point standard curve including a blank. The standards and samples were run on a Perkin Elmer Elan DRCe ICP/MS with a control solution after every 25 samples to ensure the machine did not need recalibrating. The ICP/MS analyser used the standards to generate a standard curve and reported the concentration of the minerals within each sample solution. The minerals analysed were

sodium, potassium, calcium, phosphorus, magnesium, zinc, iron, copper and manganese.

2.4.2 Egg Yolk Analysis

2.4.2.1 Egg Yolk Fatty Acid Analysis

During the third experiment the fatty acid composition of egg yolks was analysed. This was done through a one-step fatty acid extraction and methylation process to create fatty acid methyl esters which were then run on a gas chromatographer. This method was adapted from Park and Goins (1994).

2.4.2.1.1 Solubilising the lipid fraction of the yolk

The yolks were collected during egg quality analysis and frozen at -20°C in a zip lock plastic bag with the air removed. Upon analysis the yolks were removed from the freezer and allowed to thaw. Once liquid, the yolk sample was mixed within the bag and then a weighed sample of approximately 1g was taken into a glass tube using yolk from three different parts of the bag. One gram of glass beads were added to the yolk sample in addition to 3 ml 2% NaCl solution. The tube was flushed with nitrogen gas and capped with a Teflon lined lid. The tube was vortexed three times for 15 seconds. After this the tube was placed in an orbital shaker for 10 hours at 250 rpm per minute and 20°C.

2.4.2.1.2 Methylation and extraction of fatty acids

After shaking, 1 ml of the sample solution was taken into a fresh tube. To this tube 200 μl methylated chloride and 2ml 0.5 N NaOH in methanol was added. The tube was then flushed with nitrogen gas and capped with a Teflon lined lid and vortexed three times for 15 seconds. The sample was then placed into a water bath set at 90°C for 10 minutes. The tubes were then vortexed once more and allowed to cool to room temperature. Once cool, 2ml 14% boron trifluoride in methanol was added to the tube before being flushed with nitrogen and capped again. The tube was vortexed three times for 15 seconds and then placed in the 90°C water bath for 10 minutes. The sample was allowed to cool to room temperature once more and then

2ml distilled water was added to the sample as well as 1ml hexane. The tube was vortexed three times for 30 seconds before being centrifuged at 3000 rpm for 10 minutes. Immediately after centrifugation, 800 µl of the hexane proportion of the solution containing the fatty acid methyl esters was carefully pipetted into a 2ml gas chromatography vial.

2.4.2.1.3 Gas Chromatography

The samples were run on a Varian 3400 with the Varian 8200CX autosampler. The column used was a 25m long BPX70 capillary column from SGE with an internal diameter of 0.22mm and 0.25µm film thickness.

The column starting temperature of 70°C was held for 30 seconds after sample injection before increasing at a rate of 10°C min^{-1} to 170°C. The temperature was then held for 10 minutes at 170°C before increasing to 220°C at a rate of 5°C min^{-1}. The injector and flame-ionisation detector temperatures were set at 230°C and 240°C respectively. Helium was used as the carrier gas. The fatty acid percentage was calculated by means of direct normalisation of the peak areas that were quantified by the Hewlett Packard 3395 Integrator. All samples were run in duplicate.

2.4.2.2 Colorimetric Cholesterol Analysis

For analysis the yolks were removed from the freezer and allowed to thaw. Once liquid, the yolk sample was mixed within the bag and then a weighed sample of approximately 1g was taken into a glass tube using yolk from three different parts of the bag. One gram of glass beads were added to the yolk sample in addition to 10 ml 2% NaCl solution. The tube was flushed with nitrogen gas capped with a Teflon lined lid. The tube was vortexed three times for 15 seconds. After this the tube was placed in an orbital shaker for 10 hours at 250 rpm per minute and 20°C.

The cholesterol assay kit MAK043 from Sigma Aldrich was used to determine the concentration of total cholesterol in the egg yolk solution. A 2 µg/µl cholesterol stock solution was used to create 0, 2, 4, 6, 8, 10 ng/µl cholesterol standards. Fifty microlitre of the standard or sample solution was added to a flat bottomed 96 well plate. Forty four microlitres of the assay

buffer was then added followed by 2µl of the cholesterol probe, the enzyme mix and the cholesterol esterase. Each well was then mixed by pipetting before incubation at 37°C for 60 minutes. During incubation the plate was protected from light. The absorbance of the assay was measured on a Thermo Scientific Multiskan FC plate reader at 570nm. All samples were analysed in duplicate.

2.4.3 Blood Analysis

2.4.3.1 Collection

Blood samples were collected during dissections immediately after death from the common carotid artery during dissections. The blood was collected into a vacuum container that contained heparin. This was then gently inverted to mix the blood and heparin before being stored at room temperature for 30 minutes. The tube was then centrifuged at room temperature for 10 minutes at 6000 × g. After centrifugation aliquots of the blood plasma were taken and frozen at -20°C.

2.4.3.2 Mineral

Three hundred microlitres of the blood plasma was added to 1200 µl 2% nitric acid within an eppendorf. The mixture was then vortexed twice for 15 seconds before being centrifuged at 15,000 × g for 10 minutes. The supernatant was gently removed into a fresh eppendorf. This solution was then used for ICP-MS analysis in line with the methods set out in section 2.4.1.4. All samples were analysed in duplicate.

2.4.3.3 Blood Urea Nitrogen

For the analysis of blood urea nitrogen the STA-382 kit from Cell Biolabs Inc. was used. To begin, 500µl blood plasma sample was added to 500µl distilled water to bring the urea concentration into the correct range.

Urea standards were made using serial dilutions of the stock provided to create a range of concentrations from 50 mg dl^{-1} to 0.78 mg dl^{-1}. A blank distilled water sample was also analysed. 10µl of the diluted samples and standards were added to wells on a 96 well plate. 100 µl of a urease enzyme and ammonia reagent solution were then added to each well. The wells were

then mixed using a pipette, being careful not to cause bubbles or foaming. The wells were then incubated at 37°C for 10 minutes. 100 µl of the developing solution was then added to each well and again the solutions were mixed carefully. The plate was then incubated for 30 minutes at 37°C. Finally, the colour intensity was measured on a Thermo Scientific Multiskan FC plate reader at 620 nm for 10 seconds. The known concentrations of urea were used to make a standard curve, and from this the concentration of urea in the blood plasma was calculated taking the dilution into account. The standards were analysed in triplicate and samples were analysed in duplicate.

2.4.3.4 *Myo*-inositol

The analysis of *myo*-inositol was performed using the K-INOSL kit from Megazyme with some minor adaptions. To begin, 500µl of blood plasma was deproteinised using 500 µl 1M perchloric acid. This solution was vortexed twice for 15 seconds before being centrifuged at 10,000 g for 10 minutes. 500 µl of the supernatant was then carefully removed and added to 500 µl 1M potassium hydroxide. This solution was vortexed twice for 15 seconds before being centrifuged at 15,000 × g for 10 minutes. The supernatant of this solution was then carefully removed and used for the *myo*-inositol analysis. Serial dilutions were performed on the 0.25 mg ml^{-1} *myo*-inositol stock solution to create a set of 5 standards including a blank. Within an eppendorf, 50 µl of the blood sample was added to 200 µl of distilled water, 50 µl pH 7.5 buffer, 50 µl ATP solution and 10 µl hexokinase solution. This was then incubated at room temperature for 15 minutes. 500 µl of pH 9.5 buffer was then added with 250 µl of a nicotinamide-adenine dinucleotide iodonitrotetrazolium chloride solution and 10 µl of the diaphorase solution. This was then incubated at room temperature for 3 minutes, protected from light. After the three minutes 200 µl sample was pipetted from the eppendorf into a well on a 96 well plate to give a before reaction absorbance value (A_1).10 µl of the *myo* -inositol dehydrogenase solution was then added to the eppendorf, mixed and incubated for 10 minutes at room temperature, protected from light. Another 200 µl was then pipetted from the eppendorf into the well plate to give an after reaction absorbance value (A_2). The absorbance values of both A_1 and A_2 were then measured at 490nm on a Thermo Scientific Multiskan FC plate reader. The absorbance of A_1 was then subtracted from A_2 and this value was compared to the standards to give the concentration of *myo*-inositol.

2.4.4 Phytate-P Analysis

The analsyis of phytate-phosphorus used the kit K-Phyt from Megazyme. This was done by determining the total and non-phytate phosphorus contents of the sample. Phytate phosphorus content was determined by difference between the total and non-phytate phosphorus contents.

2.4.4.1 Sample Extraction

To begin, 0.5 g of sample was accurately weighed into a 15 ml Falcon tube. 10 ml of 0.5M hydrochloric acid was then added to the sample to extract the free and phytate-phosphorus. This tube was then vortexed for 15 seconds four times. After vortexing the tube was placed in a rotatory incubator for 12 hours at room temperature during which it was shaken at 250rpm.

After the phosphorus was extracted, 1.5ml of the acid extract was centrifuged at 15,000 × g. 0.5 ml of the supernatant was then transferred into an eppendorf with 0.5ml 0.75M sodium hydroxide. This solution was then used to determine the phytate-P concentration.

2.4.4.2 Enzymatic Dephosphorylating Reaction

The sample underwent two procedures at the same time to determine the total and non-phytate phosphorus content. For each procedure 50 µl of the sample was added to 600 µl distilled water and 200 µl pH5.5 buffer within an eppendorf. To the total eppendorf, 20 µl phytase solution was added while the non-phytate eppendorf had another 20 µl of distilled water added. Both tubes were mixed by vortexing and incubating in a 40°C water bath for 10 minutes. After incubation both eppendorfs had 200 µl pH10.4 buffer added to them. The total phosphorus tube also had 20 µl alkaline phosphatase suspension added, while the non-phytate eppendorf had another 20 µl of distilled water added to it. Both tubes were mixed again by vortexing and then incubated at 40°C for 15 minutes. After 15 minutes the eppendorfs were place in a water bath at 100°C for 10 minutes to stop the reaction. The tubes were then centrifuged at 15,000 × g for 10 minutes and the supernatant was removed for colorimetric phosphorus analysis.

2.4.4.3 Colorimetric Determination of Phosphorus

A 10% ascrobic acid, 1M sulphuric acid solution was created fresh on the day in addition to a 5% ammonium molybdate solution. One millilitre of the ammonium molybdate solution was then added to 5ml of the ascorbic acid solution to create a yellow colour reagent.

Using the stock solution provided in the kit, 5 phosphorus standards including a blank were created (7.5µg/ml-0.5 µg/ml.) One hundred microlitres of the standards and samples were added to wells in a 96 well plate. To each of these wells 50 µl of the colour reagent was added. The plate was then incubated in an oven at 40°C for one hour before the absorbance of the wells was read at 655nm on a Thermo Scientific Multiskan FC plate reader. The absorbance of the sample and the standard curve were used to give the phosphorus concentration of the total phosphorus solution and the non-phytate phosphorus solution. These were used to calculate the concentration of total and non-phytate phosphorus in the sample on a weight/weight basis. These concentrations were then used to calculate the g kg^{-1} of phytate phosphorus in the sample.

Chapter 3
The Effect of Phytase and Rapeseed Meal Inclusion on Laying Hens

3.1 Abstract

The experiment was conducted to assess the effects of including 175 g kg^{-1} rapeseed meal and 2,500 FTU kg^{-1} phytase on the solubility of minerals within the gastrointestinal tract of the laying hen in mid-lay. In addition to this the effects on short term performance and blood mineral concentrations were also studied.

The birds were individually housed and fed their respective diets for four weeks during which their performance was recorded, in the final week the strength of the egg shells was also recorded. At the end of the four weeks the birds were killed via cervical dislocation. A peripheral blood sample was taken in addition to the empty weight of the crop, proventriculus and gizzard, duodenum, jejunum and ileum. A total digesta and soluble digesta sample from each section of the gastrointestinal tract were collected and weighed. Each digesta sample had its pH recorded before being frozen at -20°C to await mineral analysis.

The inclusion of phytase had no effect on the performance of the birds throughout the four weeks of the trial, but did increase the egg shell strength in the final week from 3.49 to 3.93kgf. Rapeseed meal had no significant effect on the performance of the laying hens, however, there was a trend for the rapeseed meal diet to increase weight gain from 107 to 158g.

The inclusion of rapeseed meal had no effect on the weight of any of the sections of the gastrointestinal tract, however, phytase significantly reduced the weight of the proventriculus and gizzard from 41.9 to 39.0g; this suggests the phytase supplemented diet needed less mechanical or gastric digestion. Rapeseed meal had no effect on the weight or pH of the digesta from any part of the tract. Including phytase in diets increased the weight of

duodenal digesta from 2.74 to 3.20 g and decreased its pH from 5.87 to 5.60.

Rapeseed meal increased the solubility of the diet within the duodenum in comparison to the control diet while phytase supplemented diets were significantly more soluble than unsupplemented diets in the gizzard and duodenum. There was a trend for the ash content of the phytase diets to be more soluble within the crop and gizzard; this effect became significant within the duodenum. Within the crop there was an interaction between the two treatment factors whereby phytase increased the control diet solubility, but had no effect on the rapeseed meal diet. The solubility of minerals within the crop varied greatly and was not affected by either treatment. Within the gizzard rapeseed meal decreased the solubility of zinc from 13.1 to 5.6 g soluble 100 g^{-1} total digesta, while phytase inclusion increased the solubility of calcium, iron, copper and manganese. Phytase also increased the solubility of calcium, phosphorus, magnesium, copper and manganese in the duodenum and phosphorus in the jejunum. Rapeseed meal had no effect on the solubility of any minerals within the digesta of the duodenum or jejunum. Within the ileum rapeseed meal increased the solubility of sodium, calcium and phosphorus while phytase increased the solubility of calcium, phosphorus, iron and manganese from 0.5, 0.5, 7.7 and 1.8 to 1.3, 1.6, 19.2 and 3.2 respectively. The concentration of minerals in the blood was not affected by rapeseed meal inclusion, however, phytase increased the circulating concentrations of calcium, magnesium, zinc and manganese from 194.7, 33.7, 0.707 and 0.025 to 219.4, 37.1 0.897 and 0.032 respectively. The inclusion of phytase increased the solubility of some minerals along the gastrointestinal tract making them more available to the bird. This appeared to increase the absorption of several minerals, which could affect performance or egg quality.

3.2 Introduction

Modern commercial poultry rations utilise cereals to give a dietary base providing a low cost energy source, but unfortunately they provide insufficient protein. By-products of animal production such as meat and bone meal or fish meal can be used to provide concentrated protein. However, fish meal is usually avoided due to the egg taint it can produce (Pearson et al., 1983) while the European prohibition of processed animal

protein (956/2008, of 29 September 2008) prevents the use of meat and bone meal in animal feeds.

As a consequence, oilseed meals have become the main source of protein, the most common of which is soya bean meal. Rapeseed meal is a cheap, U.K. grown alternative that could significantly reduce the cost of feed production (Leeson et al., 1987). Rapeseed meal has the potential to replace soya bean meal as both meals have similar amino acid profiles; rapeseed meal contains more sulphur amino acids, but lower levels of lysine, tryptophan, leucine and isoleucine (Bell, 1984). SBM also has a higher crude protein concentration (460 vs 340 g kg^{-1}) and apparent metabolisable energy (10 vs 7 MJ kg^{-1}.) Rapeseed meal also has a higher level of Crude Fibre (120 vs 50 g kg^{-1}) as well as calcium and phosphorus (Bell, 1984, Nutrition, 2010). Unfortunately, rapeseed meal is also associated with a number of anti-nutritional factors that have previously restricted its use.

Rapeseed meal historically had high levels of erucic acid, but this was reduced through selective breeding in the 1960s to give 0-rapeseed. In the 1970s plant breeders reduced the levels of glucosinolates to produce a rapeseed referred to as 00-rapeseed (Mawson et al., 1993). The negative association with the word rape in some western countries has also led to the use of the name canola which takes its name from CANadian Oil Low Acid. Rapeseed also has relatively high levels of tannins which are currently being reduced to produce a 000-rapeseed. Another factor which has restricted the inclusion of rapeseed in commercial layer diets is the fish taint it can produce in the eggs. Rapeseed contains high levels of choline, which is fermented in the gastrointestinal tract to trimethylamine (TMA) (March and MacMillian, 1979). Fish taint is caused by an accumulation of TMA in the ovarian follicles which go on to form eggs. Some brown egg strains of layers have a defective flavin-containing monooxygenase 3 (*FMO3*) gene which is responsible for oxidising the TMA into an odourless product (Honkatukia et al., 2005). Recently, poultry breeders have used genetic markers to select for laying hens that have a functional *FMO3* gene and so rapeseed meal can be fed without a detrimental effect on egg taste. The final anti-nutrient that causes a problem with the use of rapeseed as a protein source is phytic acid.

Rapeseed contains a higher concentration of phytic acid than soya bean meal (6.45 vs 3.88g kg^{-1})(Selle and Ravindran, 2007) . The form the phytic acid takes within rapeseed meal may increase the potency of its anti-nutritional effects. It is possible that the phytic acid in soyabean meal is more soluble than that of rapeseed meal (Maenz et al., 1999). It is also known that the phytin within soyabeans is very soluble due to the amount of potassium bound within their salts (Lott and Ockenden, 1986). Leske and Coon (1999) found the hydrolysis of phytic acid was almost 6 times greater in soya bean meal than rapeseed meal when subjected to 600 FTU kg^{-1} phytase.

Earlier trials showed that replacing soya bean meal with moderate levels of rapeseed meal (110g kg^{-1}) reduced feed intake which led to a decrease in egg production and body weight (Leeson et al.,1976). The new varieties of rapeseed meal could potentially be included in layer diets at higher concentrations since the high inclusion of 300 g kg^{-1} in broilers rations had no detrimental effect on bird health or performance (Ramesh et al., 2006). In layers, the inclusion of 80g kg^{-1} expelled RSM did not affect egg production, weight, feed intake, fed conversion ratio or egg quality, but did reduce calcium and phosphorus retention presumably because of the high phytic acid concentration (Swiatkiewicz et al., 2010). However, when included at higher levels negative effects on performance have been seen. Jeroch et al. (2009) found that the inclusion of rapeseed meal at 300 g kg^{-1} reduced egg weight and weight gain of laying hens. Other work has shown that 100g kg^{-1} rapeseed meal inclusion can actually benefit bird performance with an increase in egg weight (Riyazi et al., 2008b). It is noteworthy that this change in egg weight was accompanied by a 15% increase in feed intake. Work with broiler breeders supports this showing that including rapeseed meal at 0, 5, 10, 15 and 20% had no effect on any performance characteristics (Gheisari et al., 2011). It seems likely that rapeseed meal can be included within layer diets at moderate levels, but perhaps not as high as those potentially used in broilers, and that they would benefit from supplemental phytase because of the high phytic acid content. Phytase inclusion at 1,000 FTU g kg^{-1} can improve the performance of broilers fed a diet with 150g kg^{-1} rapeseed meal (Smulikowska et al., 2010). In laying hens, 450FTU kg^{-1} improved the availability of calcium and phosphorus in a diet

with 150g kg^{-1} RSM (Sasyte et al., 2007). Um and Paik (1999) also showed that phytase addition to a diet with a low level of rapeseed meal (30g kg^{-1}) reduced phosphorus excretion.

Phytic acid is considered to be an anti-nutrient because of the negative effect it can have on other nutrients such as minerals within the diet. The six phosphate groups located on phytic acid make it a highly negatively charged molecule within the pH range of the gastrointestinal tract of the chicken. This allows phytic acid to chelate cations and form a phytate salt. The strength of the bonds between the mineral cations and phytic acid varies as follows; $Zn^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+} > Fe^{2+}$ (Cheryan, 1980). Although minerals such as zinc and copper have a greater affinity for phytate it should be noted that calcium is more important and abundant within poultry feed and so it has been studied more.

For minerals to be absorbed from the gut lumen they need to be soluble; it follows that the solubility of minerals is a good indicator of their availability to the bird (Shafey et al., 1991, Tamim et al., 2004a). Cheng and Coon (1990) showed that the quality of egg shells was closely related to the solubility of calcium within the gizzard. Guinotte and colleagues (1995) found that coarser calcium particles resulted in an increase in insoluble gizzard calcium, an increase in the calcium excreted and a decrease in the weight gain of broilers. Zhang and Coon (1997) showed that larger limestone particles increased calcium solubility and retention when fed to layers. Both these studies agree that increasing mineral solubility increases its retention, but differ when comparing feed particle size. This difference between broilers and layers is probably due to the different dietary inclusions and bird requirements. In broilers, smaller particles of limestone will have a greater surface area, allowing more calcium to be solubilised. In layers, larger particles of limestone will be held back in the gizzard for longer; slowly releasing the calcium over time will allow an increase in calcium solubility, retention and an improvement in shell quality. Smaller limestone particles will not be retained in the gizzard and pass through the digestive tract insolubilized.

A number of factors are known to affect mineral solubility. Walk et al. (2012a) showed within an *in vitro* digestion model that the solubility of minerals was affected by the pH of the system. Guinotte et al. (1995) used the proton pump inhibitor omeprazole to show that inhibiting gastric secretions increased gizzard pH and reduced calcium solubility. Calcium solubility can also be affected by dietary calcium levels and gastrointestinal tract and retention time (Zhang and Coon, 1997.) The relationship between minerals within the gastrointestinal tract may be important. A reduction in the calcium: phosphorus ratio from 2:1 to 1:1 increased the solubility of phosphorus in sheep small intestines (Yano et al., 1979). Pontoppidan et al. (2007a) showed that increasing dietary calcium decreased the amount of soluble zinc, manganese and iron *in vitro*. An increase in dietary copper from 8 to 200ppm increased copper solubility, but decreased the solubility of zinc (Pang and Applegate, 2007). Finally, dietary phytic acid can affect the solubility of minerals (Woyengo et al., 2010b). Wise (1983) showed that increasing phytic acid concentration reduced calcium solubility. The inclusion of phytase should be able to increase the solubility of minerals in the tract by negating the effect phytic acid has on solubility.

The solubility of minerals in the gizzard and proximal small intestines is of particular importance in relation to mineral absorption because of phytic acid and the formation of phytate salts. Phytic acid is most soluble and susceptible to phytase within the acidic gizzard (Campbell and Bedford, 1992). Supplemental phytase activity also reaches its peak in more acidic environments and so is most active in the proximal sections of the gastrointestinal tract (Igbasan et al., 2000). The endogenous phytase within the small intestines is poor at hydrolysing phytic acid; this means that as the pH rises in the small intestine additional release of minerals from phytate is limited and so there is no further increase in mineral solubility.

It has been shown *in vitro* that phytase can affect the solubility of minerals (Pontoppidan et al., 2007b, Walk et al., 2012a). In comparison, Woyengo et al. (2010) showed that phytase could numerically increase mineral solubility *in vivo* but failed to find a significant difference. Walk and collegues (2012b) found that the phytase solubility effect was dependant on the ingredients in the diet during *in vitro* incubations. Recently, a comparison between *in vivo*

and *in vitro* work showed similar results with phytase inceasing phosphorus solubility. This study also showed that the use of rapeseed meal in the place of SBM also reduced phosphorus mineral solubity in broilers (Morgan et al., 2014).

The use of diets with high phytase inclusion levels above the standard dose has been shown to benefit both broilers and piglets, but this has not yet been shown in laying hens. Augspurger et al. (2007) found no significant benefits between the inclusion of 300 and 10,000 FTU kg^{-1}, however, there was a numerical increase in egg production numbers in both sections of the trial. Increasing the standard phytase inclusion up to 1000 FTU kg^{-1} further increased the retention of phosphorus in laying hens, suggesting a greater hydrolysis of phytic acid. It seems logical that increasing the phytase inclusion above standard levels will cause a greater breakdown in dietary phytic acid and therefore reduce the formation of insoluble phytate complexes. This would lead to an increase in the solubility of minerals within the gastrointestinal tract and could improve animal performance or allow a dietary mineral dilution.

3.2.1 Objectives

The objective of this study was to investigate the effects of a high phytase inclusion level on laying hens when fed a diet with or without the inclusion of rapeseed meal, specifically looking at the solubility of a wide range of minerals within the digesta in different sections of the gastrointestinal tract.

3.2.2 Hypotheses

Phytase supplementation will increase the solubility of the digesta and minerals within the gastrointestinal tract of the laying hen

Rapeseed meal inclusion will reduce the solubility of the digesta and minerals within the gastrointestinal tract of the laying hen

Phytase supplementation will increase the solubility of the digesta less when birds are fed the rapeseed meal diet

Phytase will improve shell quality

Phytase will increase the concentration of minerals within the blood plasma

It is not expected that phytase or rapeseed meal will affect performance due to the short length of the trial.

3.3 Materials and Methods

An experiment was conducted to investigate the effect of rapeseed meal inclusion at 175 g kg^{-1} and phytase inclusion at 2500 FTU kg^{-1} within laying hen diets on bird performance, digesta mineral solubility, gut weight, digesta weight and pH in addition to blood mineral concentration.

3.3.1 Experimental Design and Treatments

The experiment was a 2×2 factorial design, with or without the inclusion of rapeseed meal at 175 g kg^{-1} and the addition of 0 or 2,500 FTU kg^{-1} Quantum XT™ phytase giving four dietary treatments as described in Table 3.1. Soya bean meal was reduced to accommodate the addition of the rapeseed meal, however, in order to achieve the birds requirements soya bean meal could not be excluded completely from the treatment diets. Wheatfeed was also reduced in the RSM treatment diets. All diets were formulated to have adequate calcium and phosphorus. The phytase was given conservative matrix values during the feed formulation normally used for the inclusion of 300 FTU kg^{-1} Quantum XT™ (0.143% digestible calcium, 0.13% available phosphorus and 0.03% digestible sodium). These matrix values remove any benefit phytase has at a normal inclusion level, therefore any improvement seen in egg production will be due to the higher than standard inclusion of phytase.

3.3.2 Animal Housing

A total of 150 Bovan Brown chickens were weighed and health checked at 34 weeks old at a commercial farm. Fifty healthy chickens with weights closest to the mean were initially selected for transportation to the University of Leeds, Spen Farm. For two weeks before the start of the trial the birds were housed individually and their health status, body weight, feed intake, egg production, egg weight and egg mass were recorded. 40 of these birds were then selected to go onto the trial based upon their weight and health. They were then randomly allocated to treatments, balancing within replicate for body weight and production criteria recorded during the previous two weeks. This resulted in 10 replicates split between two rooms.

Table 3.1 Ingredients and calculated nutrient composition of experimental diets

Ingredient (g kg^{-1})	Control Diet		RSM Diet	
	Without Phytase	With Phytase	Without Phytase	With Phytase
Barley	75.0	75.0	75.0	75.0
Wheat	466.0	456.2	489.0	501.9
Wheatfeed	133.4	153.2	00.0	00.0
Hipro Soya Ext	181.4	179.0	109.1	107.0
Rapeseed Ext	00.0	00.0	175.0	175.0
Layer Premix	02.5	02.5	02.5	02.5
L-Lysine HCl	0.88	0.87	0.95	0.98
DL-Methionine	1.6	1.6	1.1	1.1
Thr Microweigh	0.03	0.02	0.00	0.00
Dry Nutri-Gold	0.13	0.13	0.13	0.13
Citran Pigment	1.50	1.50	1.50	1.50
Roxazyme	0.08	0.08	0.08	0.08
Limestone Granules	90.18	89.07	88.08	86.96
MCP	9.98	4.14	9.24	3.47
Salt PDV	2.78	2.78	2.79	2.77
Sodium Bicarbonate	2.11	1.00	1.96	0.88
Soya Oil GM	32.44	31.92	43.50	39.72
Quantum Dry Phytase	0.00	1.00	0.00	1.00
Nutrient Composition (g kg^{-1})				
Dry Matter	883	882	885	884
Crude Oil	49	49	59	56
Crude Protein	168	169	175	176
Crude Fibre	31	32	42	42
Crude Ash	130	124	129	122
Crude Sugar	44	45	45	45
Calcium (Total)	40	39	40	39
Calcium (Digestible)	40	40	40	40
Phosphorus (Total)	5.6	4.4	5.8	4.5
Phosphorus (Available)	3.3	3.3	3.2	3.2
Sodium (Total)	1.8	1.5	1.8	1.5
AMEn (MJ kg^{-1})	11.4	11.4	11.4	11.4

3.3.3 Feeding Regime and Performance Recording

The birds were single housed and fed their respective treatment diets for four weeks during which their performance was recorded. In the fourth week the shell strength of the eggs was also recorded.

3.3.4 Dissections and Digesta Solubility

After four weeks the birds were killed by cervical dislocation over three consecutive days. Immediately after slaughter a peripheral blood plasma sample was taken for mineral analysis. The gut was then liberated from the bird and divided into crop, proventriculus and gizzard, duodenum, jejunum and ileum. Each section of the gastrointestinal tract was clamped and then weighed while full of digesta. The digesta was then removed from the tract and mixed before the pH was recorded from three points. The empty weight of the tract segment was then recorded. The total weight of the digesta for each gastrointestinal tract section was calculated using the equation:

$$Total\ Digesta\ Weight = \ Full\ GIT\ Weight - Empty\ GIT\ Weight$$

After mixing a digesta sample, hence forth known as the “complete digesta,” was then taken. Another digesta sample was then weighed, centrifuged at 6,466 × g at 4°C for 10 minutes, seperating the digesta into the soluble and insoluble proportions. The soluble fraction was then weighed and used to give the digesta solubility on a wet weight basis using the equation below:

$$Wet\ Digesta\ Solubility = \ 100\ \times \frac{Soluble\ Digesta\ Wet\ Weight}{Complete\ Digesta\ Wet\ Weight}$$

The wet digesta solubility and the total digesta weight were used to generate the weight of the total soluble digesta within each section of the tract using the equation:

$$Soluble\ Digesta\ Weight = \frac{Wet\ Digesta\ Solublilty \times Total\ Digesta\ Weight}{100}$$

The complete and soluble digesta fractions were all frozen at -20°C. They were then analysed for mineral concentration using the methods described in Section 2.4.1.

The wet, dry, ash and individual mineral weights of the analysed digesta samples were used in conjunction with the Total and Soluble Digesta Weights to generate the dry weight, ash weight and individual mineral weights for the complete and soluble fractions of the digesta. For example, the soluble ash weight would be found with the following equation:

$$Soluble\ Ash\ Weight = Soluble\ Digesta\ Weight\ \times \frac{Sample\ Ash\ Weight}{Sample\ Wet\ Weight}$$

The weight of the total and soluble digesta on a dry, ash and individual mineral basis was then used to generate the solubility of the digesta on a DM basis in addition to the solubility of the digesta ash and minerals using the equation:

$$Mineral\ Solubility = 100 \times \frac{Soluble\ Digesta\ Mineral\ Weight}{Complete\ Digesta\ Mineral\ Weight}$$

3.3.5 Statistical Analysis

All data was tested for normality using the Kolmogorov-Smirnov test and transformed when necessary. The egg production data was not normal and so Box-Cow transformations were used to normalise the data before analysis. The homogenity of variance of data was also analysed within the model. Two factor univariate general linear model (GLM) analysis of variance (ANOVA) tests within the statistical programme IBM SPSS Statistic 21 were used to analyse the performance data. Two factor univariate GLM ANOVA tests were also used to analyse the pH, digesta weight, gastrointestinal tract weight and blood mineral concentration with the time of death included in the model as an independent variable. When significant

post-hoc bonferroni tests were performed for multiple comparisons. Significance was assigned at P< 0.05.

3.4 Results

3.4.1 Diet Analysis

The analysis of the diets shows that they were generally true to their formulation. The crude protein and oil contents of all four diets was lower than the calculated levels. The crude protein and oil were higher in the rapeseed meal diet than the control. The fibre content of the rapeseed meal without phytase was also slightly higher than the formulation calculated and the other three diets. The ash, calcium, phosphorus and sodium were all satisfactory, however, there was a slightly reduced level of ash and calcium in the rapeseed meal diet with phytase.

Table 3.2 Analysed nutrient composition of experimental diets

	Control Diet		Rapeseed Meal Diet	
Nutrient Composition ($g\ kg^{-1}$)	Without Phytase	With Phytase	Without Phytase	With Phytase
Dry Matter	895	893	897	893
Oil A	52	51	62	53
Crude Protein	168	164	176	168
Crude Fibre	31	32	49	42
Ash	141	132	152	123
Calcium	45.9	42.8	47.0	35.9
Phosphorus	5.7	4.5	5.8	4.2
Av Phosphorus	2.7	1.6	1.9	2.0
Phytate-P	3.0	3.0	3.8	3.3
Sodium	1.9	1.4	1.9	1.4
Potassium	8.1	8.5	7.4	7.8
Iron	5.3	4.3	3.3	3.5
Magnesium	1.6	1.7	1.8	1.8
Zinc	0.12	0.12	0.10	0.12
Manganese	0.12	0.10	0.09	0.09
Copper	0.03	0.03	0.02	0.02

3.4.2 The Effect of Phytase and Rapeseed Meal on Laying Hen Performance

The inclusion of rapeseed meal had no detrimental effect on performance of the birds throughout this trial, with numerical but insignificant increases in average egg weight and daily egg mass production (Table 3.3). The feed intake and FCR were both higher, but again this difference was not significant. The largest dietary effect on performance seen in this trial was a non-significant trend for an increase in body weight gain of the birds fed the rapeseed meal diets. The hen-day egg production shows no dietary effect with a small decrease in egg numbers with the inclusion of 175 g kg^{-1} rapeseed meal . Hen-housed egg production is not included in the analysis as no mortalities occurred throughout the trial. No difference in egg shell quality was seen between the control and rapeseed meal diet with both having similar egg shell weights and egg shell strengths.

The addition of 2,500 FTU kg^{-1} phytase had no significant effect on the performance of the laying hens. The weight gained by the birds on the phytase diet was 30% greater than those on the control diet, however, this was not significant. Finally, high phytase inclusion benefited shell quality with a numerical increase in shell weight and a significant increase in egg shell strength from 3.49 to 3.93 kgf. No significant interactions were seen between the diet and the inclusion of phytase.

Table 3.3 The effect of phytase and rapeseed meal on laying hen performance

Diet	Phytase	FI	HDEP	Egg Weight	Egg Mass	FCR	Shell Weight	Shell Strength	BWG
	(FTU kg^{-1})	(g)	(%)	(g)	(g)		(g)	(kgf)	(g)
Control	0	114	97	61	58.8	1.93	5.86	3.53	92
	2,500	112	96	61.7	59.1	1.91	5.91	3.92	121
Rapeseed Meal	0	117	96	62.7	60.5	1.93	5.84	3.46	138
	2,500	118	94	63.3	59.2	2	6.08	3.94	178
Control		113	96	61.3	59	1.92	5.89	3.73	107
Rapeseed Meal		117	95	63	59.9	1.97	5.96	3.7	158
	0	115	97	61.8	59.6	1.93	5.85	3.49	115
	2,500	115	95	62.5	59.2	1.95	6	3.93	150
SEM		1.9	0.9	0.55	0.65	0.03	0.06	0.08	14.9
P-Value									
Diet		NS	NS	NS	NS	NS	0.083	NS	0.090
Phytase		NS	NS	NS	NS	NS	NS	< 0.005	NS
Diet × Phytase		NS	NS	NS	NS	NS	NS	NS	NS

FI = Daily deed intake, HDEP = Hen day egg Production, FCR = Feed Conversion Ratio, BWG = Body weight gain throughout trial

3.4.3 The Effect of Phytase and Rapeseed Meal on the Gastrointestinal Tract Weight in the Laying Hen

The weights of the sections of the gastrointestinal tract are shown in Table 3.4. The addition of rapeseed meal had no significant effect on weight of any of the segments of the gastrointestinal tract. However, with the exception of the crop the tract segments were numerically higher when birds were fed a rapeseed meal diet.

Table 3.4 The effect of phytase and rapeseed meal on the gastrointestinal tract weight (g) in laying hens

Dietary treatment		Gastrointestinal Tract Section				
Diet	Phytase FTU kg^{-1}	Crop	Giz[1]	Duo[2]	Jej[3]	Ileum
Control	0	6.61	41.55	18.08	24.61	17.47
	2500	6.96	38.53	16.23	24.07	17.08
Rapeseed Meal	0	6.62	42.22	17.64	25.15	18.47
	2500	6.46	39.40	17.78	26.37	19.23
Control		6.79	40.04	17.16	24.34	17.28
Rapeseed Meal		6.54	40.81	17.71	25.76	18.85
	0	6.62	41.89	17.86	24.88	17.97
	2500	6.71	38.97	17.01	25.22	18.16
SEM		0.328	1.478	0.703	0.935	0.815
P-Value						
Diet		NS	NS	NS	NS	NS
Phytase		NS	<0.05	NS	NS	NS
Diet × Phytase		NS	NS	NS	NS	NS

[1]Proventriculus and Gizzard

[2]Duodenum

[3]Jejunum

The addition of 2500 FTU kg^{-1} to the diet significantly reduced the combined weight of the gizzard and proventriculus from 41.89 to 38.97g (P<0.05). There was also a large decrease in the weight of the duodenum with the addition of phytase to the control diet. This weight difference was not seen in the rapeseed meal diet and therefore phytase failed to significantly affect duodenum weight. The P-value for the interaction between phytase and diet was low but not significant due to the level of variation in duodenum weight. Phytase had no effect on the weight of any of the other sections of the gastrointestinal tract and no significant interactions between diet and phytase were found.

3.4.4 The Effect of Phytase and Rapeseed Meal on the Digesta pH and Weight within the Gastrointestinal Tract of the Laying Hen

Rapeseed Meal had no significant effect on the pH of the digesta in any of the gastrointestinal tract sections while phytase significantly decreased the pH of the digesta in the duodenum from pH 5.87 to pH 5.60 (P<0.01; Table 3.5). There were no significant interactions between phytase and diet. However, the pH of the gizzard and jejunum did show trends towards significance. The addition of phytase to the control diet made the digesta in the gizzard and jejunum more acidic. In the rapeseed meal diets phytase increased the pH of the digesta in the gizzard and jejunum.

The addition of rapeseed meal to the laying hen diet had no effect on the weight of the digesta in the gastrointestinal tract (Table 3.6). Phytase supplementation significantly (P<0.05) increased the weight of the digesta within the duodenum from 2.74 to 3.20 g. Although no significant interaction was found between diet and phytase, the increase in digesta weight is only seen in the rapeseed meal diets. Within the control diet there is a small decrease in digesta weight with dietary phytase addition. No other significant phytase effects or interactions between the treatments were found.

Table 3.5 The effect of phytase and rapeseed meal on digesta pH within the gastrointestinal tract of the laying hen

Dietary treatment		Gastrointestinal Tract Section				
Diet	Phytase FTU kg^{-1}	Crop	Giz[1]	Duo[2]	Jej[3]	Ileum
Control	0	3.98	3.14	5.80	5.91	7.36
	2500	4.07	2.72	5.46	5.63	6.93
Rapeseed Meal	0	4.34	2.71	5.93	5.63	7.16
	2500	4.15	2.97	5.74	5.77	7.28
Control		4.03	2.93	5.63	5.77	7.15
Rapeseed Meal		4.25	2.84	5.84	5.70	7.22
	0	4.16	2.93	5.87	5.77	7.26
	2500	4.11	2.85	5.60	5.70	7.11
SEM		0.338	0.333	0.165	0.130	0.150
P-Value						
Diet		NS	NS	NS	NS	NS
Phytase		NS	NS	<0.01	NS	NS
Diet × Phytase		NS	0.056	NS	0.092	NS

[1]Proventriculus and Gizzard

[2]Duodenum

[3]Jejunum

Table 3.6 The effect of phytase and rapeseed meal on digesta weight (g) within the gastrointestinal tract of the laying hen

Dietary treatment		Gastrointestinal Tract Section				
Diet	Phytase FTU kg^{-1}	Crop	Giz[1]	Duo[2]	Jej[3]	Ileum
Control	0	51.29	18.89	2.89	15.00	11.94
	2500	34.72	17.72	2.76	14.62	11.54
Rapeseed Meal	0	33.57	18.43	2.58	15.20	14.30
	2500	37.45	17.07	3.64	14.97	12.54
Control		43.01	18.31	2.83	14.81	11.74
Rapeseed Meal		35.51	17.75	3.11	15.09	13.42
	0	42.43	18.66	2.74	15.10	13.12
	2500	36.09	17.40	3.20	14.80	12.04
SEM		11.108	1.513	0.318	0.970	1.233
P-Value						
Diet		NS	NS	NS	NS	0.144
Phytase		NS	NS	<0.05	NS	NS
Diet × Phytase		NS	NS	NS	NS	NS

[1]Proventriculus and Gizzard

[2]Duodenum

[3]Jejunum

3.4.5 The Effect of Phytase and Rapeseed Meal on the Solubility of the Digesta within the Gastrointestinal Tract of the Laying Hen

Rapeseed meal significantly increased the solubility of the diet in the duodenum and the ileum on a wet weight basis (Table 3.7). Phytase supplementation also significantly affected digesta solubility within the duodenum with an increase from29.67 to 49.28 g soluble 100 g^{-1} total digesta . Within the crop a significant interaction was seen between the diet and phytase addition. Phytase increased the solubility of the digesta in the crop from 29.01 to 39.12 g soluble 100 g^{-1} total when the birds were fed the control diet. In the rapeseed meal diet, however, phytase supplementation gave a non-significant reduction in solubility from 37.66 to 33.20 g soluble

100 g^{-1} total . No other interactions were seen between phytase and diet in relation to wet digesta solubility.

Table 3.7 The effect of phytase and rapeseed meal on digesta wet weight solubility (g soluble 100 g^{-1} total) within the gastrointestinal tract of the laying hen

Dietary treatment		Gastrointestinal Tract Section				
Diet	Phytase FTU kg^{-1}	Crop	Giz[1]	Duo[2]	Jej[3]	Ileum
Control	0	29.01^{b}	16.27	26.39	36.16	13.01
	2500	39.12^{a}	19.53	39.52	36.92	12.38
Rapeseed Meal	0	37.66^{a}	16.45	32.95	36.03	21.11
	2500	33.20^{ab}	16.28	59.05	37.43	17.81
Control		34.06	17.90	32.95	36.54	12.70
Rapeseed Meal		35.43	16.37	46.00	36.73	19.46
	0	33.33	16.36	29.67	36.09	17.06
	2500	36.16	17.90	49.28	37.18	15.10
SEM		1.796	1.337	4.047	1.555	1.678
P-Value						
Diet		NS	NS	<0.05	NS	<0.05
Phytase		NS	NS	<0.05	NS	NS
Diet × Phytase		<0.05	NS	NS	NS	NS

$^{a-b}$ Means within columns with no common superscript differ significantly (P<0.05)

[1]Proventriculus and Gizzard

[2]Duodenum

[3]Jejunum

Analysis of digesta solubility on a dry weight basis showed that phytase increased the solubility of the digesta in the gizzard and proventriculus from 2.63 to 3.79 g soluble 100 g^{-1} total digesta (Table 3.8). In agreement with the wet weight solubility results rapeseed meal increased the solubility of the digesta in the duodenum from 6.55 to 25.25 g soluble 100 g^{-1} total . There was also a trend for the rapeseed meal diets to have a more soluble ileum digesta than the control diets.

Within the crop there was a trend for the digesta ash to be increased by phytase supplementation and the addition of 175 g kg^{-1} rapeseed meal (Table 3.9). The solubility of ash in the gizzard and duodenum digesta was significantly increased by the inclusion of phytase from 13.05 and 15.94 to 21.46 and 27.34 g soluble 100 g^{-1} total. Both phytase and rapeseed meal significantly increased the solubility of the digesta ash in the duodenum. The was also a near significant trend for rapeseed meal inclusion to increase the solubility of the ash in the ileum.

Table 3.8 The effect of phytase and rapeseed meal on digesta dry weight solubility (g soluble 100 g^{-1} total) within the gastrointestinal tract of the laying hen

Dietary treatment		Gastrointestinal Tract Section				
Diet	Phytase FTU kg^{-1}	Crop	Giz[1]	Duo[2]	Jej[3]	Ileum
Control	0	6.36	2.87	5.60	14.89	3.75
	2500	6.51	4.25	7.50	17.54	4.18
Rapeseed Meal	0	5.32	2.40	21.01	15.21	6.91
	2500	4.64	3.34	29.48	17.18	5.54
Control		6.44	3.56	6.55	16.22	3.96
Rapeseed Meal		4.98	2.87	25.25	16.19	6.22
	0	5.84	2.63	13.31	15.05	5.33
	2500	5.57	3.79	18.49	17.36	4.86
SEM		0.520	0.301	2.336	0.818	0.592
P-Value						
Diet		NS	NS	<0.05	NS	0.059
Phytase		NS	<0.05	NS	NS	NS
Diet × Phytase		NS	NS	NS	NS	NS

[1]Proventriculus and Gizzard

[2]Duodenum

[3]Jejunum

Table 3.9 The effect of phytase and rapeseed meal on digesta ash weight solubility (g soluble 100 g^{-1} total) within the gastrointestinal tract of the laying hen

Dietary treatment		Gastrointestinal Tract Section				
Diet	Phytase FTU kg^{-1}	Crop	Giz[1]	Duo[2]	Jej[3]	Ileum
Control	0	11.78	12.81	9.76	14.01	3.81
	2500	18.34	26.59	17.27	19.41	4.12
Rapeseed Meal	0	9.54	13.30	22.12	16.30	6.50
	2500	11.60	16.33	37.41	15.92	7.22
Control		15.06	19.70	13.52	16.71	3.97
Rapeseed Meal		10.57	14.81	29.76	16.11	6.86
	0	10.66	13.05	15.94	15.16	5.16
	2500	14.97	21.46	27.34	17.66	5.67
SEM		1.240	2.290	3.108	1.720	0.727
P-Value						
Diet		0.080	NS	<0.01	NS	0.052
Phytase		0.067	<0.05	<0.05	NS	NS
Diet × Phytase		NS	NS	NS	NS	NS

[1]Proventriculus and Gizzard
[2]Duodenum
[3]Jejunum

3.4.6 The Effect of Phytase and Rapeseed Meal on the Solubility of Minerals within the Gastrointestinal Tract of the Laying Hen

3.4.6.1The Effect of Phytase and Rapeseed Meal on the Solubility of Minerals within the Crop

There was tendency for 2500 FTU kg^{-1} phytase supplementation to increase the solubility of zinc in the crop from 5.9 to 9 g soluble 100 g^{-1} total (Table 3.10). In addition to this there were also large numerical increases in the solubility of sodium, calcium, magnesium, iron, and copper with phytase addition. These differences were not significant as there was a large degree of variation in crop mineral solubility and some birds dissected earlier in the day failed to contribute a sample.

Rapeseed meal did not significantly affect the solubility of any minerals in the crop and no large numerical differences were seen. Similarly, no significant interactions were seen between phytase and rapeseed meal addition.

3.4.6.2 The Effect of Phytase and Rapeseed Meal on the Solubility of Minerals within the Proventriculus and Gizzard

The solubility of minerals within the proventriculus and gizzard were significantly affected by the treatment diets (Table 3.11). Rapeseed meal significantly decreased the solubility of zinc from 13.1 to 5.6 g soluble 100 g^{-1} total. There was also a trend for rapeseed meal to decrease the solubility of sodium and iron. Rapeseed meal failed to affect the solubility of potassium, calcium, phosphorus, magnesium, copper or manganese.

Phytase increased the solubility of calcium, iron, copper and manganese from 5.1, 27.0, 18.7 and 14.5 to 7.9, 53.7, 41.1 and 26.8 g soluble 100 g^{-1} total respectively.

No significant interactions were seen between phytase and rapeseed meal in regard to gastric mineral solubility.

3.4.6.3 The Effect of Phytase and Rapeseed Meal on the Solubility of Minerals within the Duodenum

Within the duodenum the addition of rapeseed meal had no significant effect on the solubility of minerals, whereas phytase resulted in numerical increases in the solubility of all minerals except potassium (Table 3.12). The increases were significant for calcium, phosphorus, magnesium, copper and manganese. Calcium solubility was increased 3 fold from 4.0 to 12.3 g soluble 100 g^{-1} total while the phosphorus solubility doubled from 7.4 to 15.9 g soluble 100 g^{-1} total. The increases in copper and manganese solubility were equally large while the increase in magnesium solubility was not as great from 21.8 to 36.4 g soluble 100 g^{-1} total.

No significant interactions were found, however, there was a trend for an interaction between rapeseed meal and phytase in relation to the solubility of

copper. The effect of phytase on the solubility of copper was much greater in the rapeseed meal diet compared to the control diet. A similar effect was seen in relation to solubility of magnesium.

3.4.6.4 The Effect of Phytase and Rapeseed Meal on the Solubility of Minerals within the Jejunum

Phytase had less effect on the solubility of minerals within the jejunum with the only significant effect being an increase in phosphorus solubility from 5.9 to 14.8 g soluble 100 g^{-1} total (Table 3.13). The increase in phosphorus solubility was larger than that seen in the duodenum. There were two trends seen from phytase supplementation with increases in calcium and zinc solubility.

Rapeseed meal had no effect on the solubility of minerals within the jejunum and no discernible patterns or differences can be seen in the data.

There was a significant interaction in relation to copper solubility whereby rapeseed meal increased copper solubility from 20.4 to 36.2 g soluble 100 g^{-1} total when birds were fed a diet without phytase. When fed phytase rapeseed meal inclusion had no effect on the copper solubility. Phytase significantly decreased the solubility of copper in the rapeseed meal diet from 36.2 to 23.4 g soluble 100 g^{-1} total. In the control diet phytase gave a near significant increase in solubility ($P=0.060$).

3.4.6.5 The Effect of Phytase and Rapeseed Meal on the Solubility of Minerals within the Ileum

Within the ileum the addition of rapeseed meal had its greatest effect relative to the other sections of the gastrointestinal tract (Table 3.14). Rapeseed meal significantly increased the solubility of sodium and calcium from 10.7 and 0.4 to 21.4 and 1.4 g soluble 100 g^{-1} total respectively. Rapeseed meal also gave trends for increases in the solubility of phosphorus, magnesium, iron and copper. Phytase supplementation significantly increased the solubility of calcium, phosphorus, iron and manganese.

No significant interactions were found between phytase and rapeseed meal.

Table 3.10 The effect of phytase and rapeseed meal on the solubility (g soluble 100 g^{-1} total) of minerals within the crop

Diet	Phytase FTU kg^{-1}	Na	K	Ca	P	Mg	Zn	Fe	Cu	Mn
Control	0	62.1	23.3	1.7	27.1	27.1	7.8	7.6	39.5	28.6
	2,500	90.0	23.8	3.0	28.1	35.5	10.2	11.8	42.8	33.2
Rapeseed Meal	0	81.0	20.0	2.2	25.2	32.4	3.9	8.1	27.4	20.3
	2,500	81.7	17.4	3.0	23.1	31.0	7.8	7.1	39.8	21.6
Control		76.1	23.5	2.3	27.6	31.3	9.0	9.7	41.2	30.9
Rapeseed Meal		81.3	18.7	2.6	24.2	31.7	5.8	7.6	33.6	21.0
	0	71.5	21.6	1.9	26.1	29.8	5.9	7.8	33.4	24.5
	2,500	85.9	20.6	3.0	25.6	33.3	9.0	9.4	41.3	27.4
SEM		4.9	2.4	0.4	2.6	2.1	1.0	1.3	5.1	3.4
P-Value										
Diet		NS	NS	NS	NS	NS	NS	NS	NS	NS
Phytase		NS	NS	NS	NS	NS	0.074	NS	NS	NS
Diet × Phytase		NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 3.11 The effect of phytase and rapeseed meal on the solubility (g soluble 100 g^{-1} total) of minerals within the proventriculus and gizzard

Diet	Phytase FTU kg^{-1}	Na	K	Ca	P	Mg	Zn	Fe	Cu	Mn
Control	0	37.0	3.5	5.0	7.1	15.0	12.7	33.8	23.9	14.9
	2,500	39.6	4.2	7.7	7.7	16.7	13.6	68.4	41.5	29.4
Rapeseed Meal	0	22.7	4.4	5.1	6.8	13.4	6.2	20.2	13.6	14.0
	2,500	23.9	4.7	8.2	7.2	12.8	5.0	39.0	40.7	24.2
Control		38.3	3.9	6.3	7.4	15.9	13.1	51.1	32.7	22.1
Rapeseed Meal		23.3	4.6	6.6	7.0	13.1	5.6	29.6	27.1	19.1
	0	29.8	4.0	5.1	7.0	14.2	9.4	27.0	18.7	14.5
	2,500	31.7	4.4	7.9	7.5	14.8	9.3	53.7	41.1	26.8
SEM		3.9	0.7	1.2	0.8	1.4	1.7	6.6	4.5	2.5
P-Value										
Diet		0.061	NS	NS	NS	NS	< 0.05	0.086	NS	NS
Phytase		NS	NS	< 0.05	NS	NS	NS	< 0.05	< 0.05	< 0.05
Diet × Phytase		NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 3.12 The effect of phytase and rapeseed meal on the solubility (g soluble 100 g^{-1} total) of minerals within the duodenum

Diet	Phytase FTU kg^{-1}	Na	K	Ca	P	Mg	Zn	Fe	Cu	Mn
Control	0	39.0	9.9	4.7	9.2	27.0	38.4	22.9	17.7	8.7
	2,500	41.3	6.1	9.0	13.6	30.8	25.4	28.8	19.7	19.5
Rapeseed Meal	0	32.1	6.4	3.3	5.6	16.6	15.6	14.7	9.3	5.4
	2,500	48.8	8.8	15.5	18.2	42.0	35.5	20.2	40.3	16.8
Control		40.1	8.0	6.9	11.4	28.9	31.9	25.9	18.7	14.1
Rapeseed Meal		40.5	7.6	9.4	11.9	29.3	25.5	17.4	24.8	11.1
	0	35.6	8.1	4.0	7.4	21.8	27.0	18.8	13.5	7.1
	2,500	45.0	7.5	12.3	15.9	36.4	30.4	24.5	30.0	18.2
SEM		4.5	1.1	2.0	2.0	3.4	6.8	4.5	4.3	2.7
P-Value										
Diet		NS	NS	NS	NS	NS	NS	NS	NS	NS
Phytase		NS	NS	< 0.05	< 0.05	< 0.05	NS	NS	< 0.05	< 0.05
Diet × Phytase		NS	0.17	NS	NS	0.102	NS	NS	0.078	NS

Table 3.13 The effect of phytase and rapeseed meal on the solubility (g soluble 100 g^{-1} total) of minerals within the jejunum

Diet	Phytase FTu kg^{-1}	Na	K	Ca	P	Mg	Zn	Fe	Cu	Mn
Control	0	39.1	14.9	1.7	5.3	23.2	2.8	10.2	20.4^{b}	4.5
	2,500	36.7	14.3	5.7	19.4	27.1	8.2	15.1	26.5^{ab}	9.3
Rapeseed Meal	0	46.8	17.6	3.0	6.6	26.1	2.0	19.2	36.2^{a}	5.0
	2,500	37.1	11.7	4.0	10.2	26.2	3.5	13.9	23.4^{b}	5.2
Control		37.9	14.6	3.7	12.4	25.1	5.5	12.7	23.5	6.9
Rapeseed Meal		42.0	14.6	3.5	8.4	26.1	2.8	16.5	29.8	5.1
	0	43.0	16.3	2.4	5.9	24.6	2.4	14.7	28.3	4.7
	2,500	36.9	13.0	4.9	14.8	26.7	5.9	14.5	24.9	7.2
SEM		2.3	1.7	0.7	1.8	1.4	1.0	3.2	2.4	1.1
P-Value										
Diet		NS	NS	NS	NS	NS	NS	NS	NS	NS
Phytase		NS	NS	0.065	< 0.05	NS	0.092	NS	NS	NS
Diet × Phytase		NS	NS	NS	0.12	NS	NS	NS	< 0.05	NS

[a-b] Means within columns with no common superscript differ significantly (P<0.05)

Table 3.14 The effect of phytase and rapeseed meal on the solubility (g soluble 100 g^{-1} total) of minerals within the ileum

Diet	Phytase FTU kg^{-1}	Na	K	Ca	P	Mg	Zn	Fe	Cu	Mn
Control	0	9.5	2.5	0.2	0.3	4.0	1.4	6.0	6.2	1.8
	2,500	12.0	1.7	0.6	0.9	5.3	2.9	11.1	7.8	2.7
Rapeseed Meal	0	22.9	3.5	0.8	0.8	7.0	2.7	9.3	11.4	1.9
	2,500	19.8	1.8	2.0	2.3	7.4	8.2	27.2	10.0	3.7
Control		10.7	2.1	0.4	0.6	4.7	2.1	8.6	7.0	2.3
Rapeseed Meal		21.4	2.6	1.4	1.5	7.2	5.4	18.3	10.7	2.8
	0	16.2	3.0	0.5	0.5	5.5	2.0	7.7	8.8	1.8
	2,500	15.9	1.7	1.3	1.6	6.3	5.5	19.2	8.9	3.2
SEM		2.1	0.5	0.2	0.3	0.7	1.3	2.6	1.0	0.3
P-Value										
Diet		< 0.05	NS	< 0.05	0.071	0.082	NS	0.058	0.08	NS
Phytase		NS	NS	< 0.05	< 0.05	NS	0.1	< 0.05	NS	< 0.05
Diet × Phytase		NS	NS	NS	NS	NS	NS	NS	NS	NS

3.4.7 The Effect of Phytase and Rapeseed Meal on the Concentration of Minerals in Blood Plasma

The addition of rapeseed meal to the diets of laying hens had no significant effect on the blood plasma mineral concentration compared to birds which had received, the control diets (Table 3.15).

Phytase significantly increased the concentration of blood calcium in both the control and rapeseed meal diets from 191.7 and 197.8 to 211.8 and 227.0 µg ml^{-1} respectively. Phytase also increased the concentration of magnesium and manganese within the blood. The greatest significance was seen in relation to zinc, with an increase in the blood concentration of phytase supplemented birds from 0.707 to 0.897 µg ml^{-1}. Trends were also seen for increases in the concentration of iron and copper in the blood when diets were supplemented with 2500 FTU kg^{-1} phytase.

No significant interactions were seen between diet and phytase addition in relation to concentration of minerals in blood plasma.

Table 3.15 The effect of phytase and rapeseed meal on the concentration of minerals (µg ml^{-1}) within the blood plasma of laying hens

Diet	Phytase FTU kg^{-1}	Na	K	Ca	P	Mg	Zn	Fe	Cu	Mn
Control	0	2615.2	264.3	191.7	103.2	33.7	0.692	0.460	0.166	0.024
	2,500	2691.6	265.2	211.8	103.2	37.9	0.880	0.529	0.188	0.031
Rapeseed Meal	0	2637.2	266.2	197.8	109.2	33.7	0.721	0.413	0.183	0.027
	2,500	2651.4	271.8	227.0	103.1	36.2	0.914	0.526	0.190	0.033
Control		2653.4	264.7	201.7	103.2	35.8	0.786	0.494	0.177	0.027
Rapeseed Meal		2644.3	269.0	212.4	106.2	35.0	0.818	0.469	0.187	0.030
	0	2626.2	265.2	194.7	106.2	33.7	0.707	0.437	0.174	0.025
	2,500	2671.5	268.5	219.4	103.2	37.1	0.897	0.527	0.189	0.032
SEM		33.6	7.0	6.4	3.1	0.8	0.037	0.002	0.005	0.002
P-Value										
Diet		NS	NS	NS	NS	NS	NS	NS	NS	NS
Phytase		NS	NS	<0.05	NS	<0.05	<0.01	0.09	0.06	<0.05
Diet × Phytase		NS	NS	NS	NS	NS	NS	NS	NS	NS

3.5 Discussion

3.5.1 Comparison to Breeder Expected Performance

The hens used in this study conformed to the breeders expectation regarding feed intake, daily egg mass and feed conversion ratio (Joice and Hill, 2014). The birds did have a slightly elevated hen day egg production and somewhat lower egg weight in comparison to published expectations, (96% vs 93% and 62g vs 64g, respectively). The birds were also underweight at the start of the trial in comparison with the breeders expectations (1.67kg vs 1.85kg, respectively). Throughout the trial the weight gained by the birds was high compared to the breeder recommendations (130g vs 10g) and brought the bird weight within reach of the breeder standards (1.81 vs 1.85kg, respectively).

3.5.2 The Effect of Rapeseed Meal on Laying Hen Performance

Within this experiment, the addition of 175g kg^{-1} rapeseed meal to a laying hen diet had no negative effect on performance compared to the control diet. The trend for higher weight gain in the rapeseed meal diets and the numerical increase in egg weights was probably due to the higher levels of protein found in the diets (Keshavarz and Nakajima, 1995). While this data indicates that rapeseed meal could be used in commercial situations, it should be noted that this trial was only a few weeks in length and other work has shown some complications with rapeseed meal addition when fed for a longer period of time (Kaminska, 2003, Najib and Al-Khateeb, 2004, Jeroch et al., 2009). The inclusion of rapeseed meal to 300g kg^{-1} negatively affected the weight gain and egg weight of laying hens in one trial (Jeroch et al., 2009). Kaminska, (2003) also found that replacing soybean meal with rapeseed meal decreased egg weight, however, rapeseed meal had no detrimental effect on egg numbers, or egg quality. Najib and Al-Khateeb (2004) found that the inclusion of rapeseed meal up to 100g kg^{-1} had no effect on egg production, egg weight or FCR, however, a further increase to 200 and 300g kg^{-1} significantly deteriorated bird performance. Smulikowska et al. (2006) also found that 150g kg^{-1} increased FCR. A number of longer trials have shown that smaller inclusion levels of rapeseed meal in layer

diets can be used without causing negative effects in egg performance or quality (Casartelli et al., 2007, Riyazi et al., 2008a, Swiatkiewicz et al., 2010). It can be concluded therefore that the inclusion of rapeseed meal up to 100 g kg^{-1} is not detrimental to performance but that higher inclusion levels and the complete replacement of soya bean meal cannot currently to be recommended. The work of Shen et al. (1983) expanded upon this and found that rapeseed meal could be included up to 200 g kg^{-1} if the meal was ground fine and then steam pelleted. This negative effect seen with rapeseed meal inclusion may be due to high levels of tannins and phytic acid. It is possible that the grinding and pelleting process reduced the negative effects of the tannins and phytic acid, as autoclaving has been shown to increase phytic acid hydrolysis (O'Dell, 1962, Takemasa and Hijikuro, 1991).

3.5.3 The Effect of Phytase Dose on Laying Hen Performance

In the present study high phytase inclusion failed to affect the feed intake of laying hens, which may be because the birds already had an adequate diet. In the past, normal levels of phytase have been shown to increase the feed intake of laying hens with phosphorus deficient diets, but not that of those that are nutritionally adequate (Van der Klis et al., 1997). Hughes et al. (2009) found that doubling phytase dose did not increase the feed intake of layer hens more than regular levels of phytase. In another experiment, 10,000 FTU kg^{-1} had no greater effect than 150 FTU kg^{-1} and 300 FTU kg^{-1} on the feed intake of pigs and laying hens (Augspurger et al., 2007).

Ahmandi et al. (2008) found that adding 150 FTU kg^{-1} phytase decreased the feed conversion ratio of laying hens. However, increasing the phytase to 300 FTU kg^{-1} had no greater effect. This suggests the effect of phytase on improving the FCR is limited, which would explain why 2,500 FTU kg^{-1} had no effect in this experiment. Other work has found that phytase inclusion at 300 and 500 FTU kg^{-1} did not affect the feed conversion ratio of a deficient laying hen diet (Liu et al., 2007).

Although it was statistically insignificant the inclusion of phytase did numerically increase the weight gained by birds across the experiment.

Many studies have shown that phytase can improve the weight gain of laying hens fed deficient diets. However, 1,200 FTU kg^{-1} did not improve the body weight gain of chickens fed diets that were nutritionally adequate (Boling-Frankenbach et al., 2001).

Phytase has previously been seen to increase egg production of layers fed deficient diets by releasing phytate-P. Augspurger et al. (2007) found that 10,000 FTU kg^{-1} was efficacious to egg production, but no more so than 300 or 150 FTU kg^{-1}. This supports the results from this experiment as superdosing phytase failed to affect egg production.

Phytase has been shown to increase the egg weight when layers are fed calcium and phosphorus deficient diets, but has had no effect on layers fed nutritionally sufficient diets where releasing excess calcium and phosphorus had no benefit (van der Klis et al., 1997, Gordon and Roland, 1998). Within this experiment egg weight was not significantly increased by phytase. Despite phytase numerically increasing egg weight in both the control and RSM diets across all four weeks of the trial, this increase was not significant.

Within the literature, it is clear that benefits can be seen when above standard phytase doses are used in piglet and broiler diets (Cowieson et al., 2011). This trial as well as others have failed to find significant performance benefits with above standard phytase inclusion. It may be that the phytase does exhibit the same benefits in layers but that the beneficial effects are hidden by the amount of variation within the data and the short length of the trials. It is also noteworthy that this trial was very short in length. Laying hens may need longer to express the phytase benefits because they are not growing animals and so not harmed as much by the anti-nutrient effects of phytate. It may also be that the benefits of “superdosing” are due to the greater bioavailability of the *myo*-inositol at the end of phytic acid hydrolysis. It is possible growing animals have a previously unknown requirement for *myo*-inositol that is fulfilled by high phytase inclusion but that mature animals have no such need as they can synthesis sufficient *myo*-inositol from glucose (Hooper, 1997). Finally, it may be that the high level of calcium in the laying hen diet prevents the near complete breakdown of phytic acid that generates the benefits of “superdosing.” The higher calcium concentration

does not completely stop phytic acid hydrolysis, but may reduce the rate of hydrolysis. To investigate whether higher phytase inclusion can benefit laying hen performance longer trials need to be run with higher levels of phytase activity.

3.5.4 The Effect of Phytase and Rapeseed Meal on the Gastrointestinal Tract

In this trial phytase decreased the combined weight of the proventriculus and gizzard. The weight of the gizzard is known to be related to the digestibility of the diet; less digestible diets that need greater muscular trituration will increase the muscle mass of the gizzard. It is well documented that increasing fibre or dietary particle size can affect the weight of the gizzard (Jiménez-Moreno et al., 2009; Pacheco et al., 2013) .The reduction in gizzard weight seen in this trial could be due to phytase making the diet more digestible and so reducing the workload of the gizzard and proventriculus. It is also possible however, that the difference is due to the reduction in limestone in the phytase diets to give the equivalence values used during the formulation. Amer (2014) found that phytase addition in broiler diets up to 2000 FTU kg^{-1} had no effect on gizzard weight.

Phytase also increased the weight and decreased the pH of the digesta within the duodenum. It is possible that this is as a result of greater digesta flow through the gizzard as a result of improved digestion. It may also be due to a decrease in the secretion from the duodenum, liver and pancreas as phytate is known to increase the secretion of bicarbonates and mucin (Cowieson et al., 2009). It is possible that the reduction in digesta pH may also be due to the reduction in limestone which has a high acid binding capacity.

3.5.5 The Effect of Phytase and Rapeseed Meal on Diet Solubility

In the crop, phytase increased the digesta solubility when birds were fed the control diet, but had no effect in the rapeseed meal diet. It is logical that the difference seen is due to the susceptibility and concentration of the phytate. A greater concentration of phytic acid will be present in the crop of birds fed

the rapeseed meal diet. This is because rapeseed meal has a higher concentration of phytic acid (Table 1.1), and the phytin within rapeseed meal is known to be less susceptible to phytase activity than that of soyabean meal (Leske and Coon, 1999). As a result phytase had less affect on the solubility of the digesta in the crop.This is backed by the trend for a decrease in ash solubility of the crop digesta in the rapeseed meal diets.

Phytase increased digesta solubility in the gizzard and duodenum. As phytase breaks down more of the phytate along the gastrointestinal tract, the solubility of the digesta ash increases. In the distal regions of the gastrointestinal tract rapeseed meal increased digesta ash. In these areas of the tract the majority of the phytic acid will have been hydrolysed (Walk et al., 2014). The higher ash solubility in the rapeseed meal diets may be a result of the slightly lower levels of minerals in the diet or due to the differences between the dietary ingredients.

It was found that the addition of rapeseed meal increased the solubility of digesta in the duodenum and ileum. This is in spite of the high tannin content of rapeseed meal in addition to the higher phytate and crude fibre concentration seen in the diet analysis.

The increase in duodenal digesta solubility is likely to be the result of greater phytic acid hydrolysis. The breakdown of phytic acid reduces the formation of insoluble phytate complexes (Nolan et al., 1987). The complex formation will reduce the solubility of the bound nutrients and the overall diet.

3.5.6 The Effect of Phytase and Rapeseed Meal on Minerals

Phytase increased the solubility of calcium and phosphorus in the gastrointestinal tract; this is in line with the findings of other authors. Woyengo et al. (2010) found that 20 g kg^{-1} additional phytate decreased mineral solubility, while 500 FTU kg^{-1} supplementation numerically increased the amount of soluble calcium and magnesium in the jejunum, however, this increase was not significant. Pontoppidan et al. (2007b) conducted an *in vitro* study that looked at how calcium concentration and phytase affected mineral solubility. It was found that calcium concentration had a strong effect on the solubility of minerals; increasing calcium concentration decreased the

amount of soluble zinc, manganese and iron at the end of the digestion. The inclusion of 750 FTU kg^{-1} phytase increased the amount of soluble zinc across all calcium levels. The concentration of soluble manganese was also increased by phytase when calcium was included at 1.2 g kg^{-1} DM, however, as calcium inclusion increased phytase had no effect. Phytase did increase the level of soluble iron at all calcium levels. This highlights how minerals can interact within the digesta and affect each other's solubility. This may explain why phytase reduced the solubility of copper in the rapeseed meal diet. Increasing the solubility of one mineral can have a negative effect on the solubility of another (Pang and Applegate, 2007). Walk et al. (2012b) found that 1000 FTU kg^{-1} phytase increased the solubility of calcium and phosphorus in diets at and below broiler phosphorus and calcium requirements. Increasing the time the diets were kept in the gastric phase of the *in vitro* incubation increased the solubility of phosphorus as phytase continued to break down phytic acid. The solubility of calcium did not increase with time in the gastric phase. Further increase in phytase activity from 1000 to 5000 FTU kg^{-1} increased the solubility of phosphorus from 66% to 75%, but had no effect on the calcium solubility which plateaued around 92% soluble. The difference seen between calcium and phosphorus may be due to the nature of phytic acid breakdown. Phytase targets IP_6 esters of phytic acid preferentially over the lesser esters such as IP_3 (Wyss et al., 1999). The cation binding capacity of IP_6 is greater than double that of IP_3 (Persson et al., 1998). This means the initial breakdown of phytic acid will result in a large release of calcium relative to phosphorus in comparison to the overall ratio when phytic acid is completely dephosphorylated (Cowieson et al., 2011). This may explain why phytase increases the solubility of calcium in the gizzard, but phosphorus solubility only increases in the duodenum after a greater amount of phytic acid hydrolysis.

Within the gastric phases of the tract the solubility of minerals was lower in the rapeseed meal diets, however, in the ileum rapeseed meal increased the solubility of sodium, calcium and possibly phosphorus, magnesium, iron and copper. Similar results were found by Morgan et al. (2014) who showed that in broilers, the solubility of calcium during the gastric and intestinal phases was higher in a rapeseed meal diet when compared to a soyabean meal

diet. They attributed this effect to a more acidic pH, but that was not found in this study.

This experiment is the first to measure how using high doses of phytase affects a wide range of minerals *in vivo* . Previously, most others had focused on just phosphorus and calcium. While improving phosphorus and calcium solubility and therefore availability is important, it is unlikely that a surplus of these minerals is responsible for the “superdosing” effects seen in other trials. Walk et al. (2014) showed that within broilers increasing phytase dose beyond the standard inclusion would decrease the FCR, but adding additional dicalcium phosphate numerically increased FCR. This shows that increasing phytase beyond standard doses does not benefit bird performance through extra calcium and phosphorus release from phytate. However, it is possible that one of the benefits of higher than standard phytase inclusion is a greater release of the other minerals from phytate which is shown in this trial through greater mineral solubility.

One possible mechanism is that greater mineral solubility will increase the potential antimicrobial properties of those minerals. Copper and zinc are both known to be potential anti-microbials affecting intestinal microflora (Højberg et al., 2005, Hedemann et al., 2006). The inclusion of zinc has been shown to benefit piglet performance while 500 FTU kg^{-1} allowed zinc concentration to be halved while still maintaining the same pharmacological benefits (Martínez et al., 2004). It is plausible that using very high levels of phytase may allow a further decrease in the concentration of minerals like copper and zinc while still gaining an antimicrobial benefit.

This study also showed that phytase increases the blood concentration of calcium, magnesium, zinc and manganese and potentially iron and copper. Other studies have also found that phytase increased the bioavailability of trace minerals like zinc, iron, manganese and magnesium (Czech and Grela, 2008, Silva et al., 2004). These are all minerals whose solubility was increased with the gastrointestinal tract in this trial. An increase in the blood concentration of these minerals may indicate that the birds requirements were not be being met in relation to these elements when the diets were fed diets without phytase. Therefore using high phytase inclusions may benefit

the bird by increasing mineral availability. It is notable that although phosphorus solubility was increased by phytase, its concentration did not increase in the blood. This supports the theory that high phytase inclusion does not benefit the animal through greater phosphorus availability (Walk et al., 2014).

The increase in calcium availability with a high phytase dose was not responsible for the improvements seen in broiler trials where additional calcium did not give benefits the birds (Walk et al., 2014). However, increasing calcium availability with high phytase doses in this experiment did benefit birds, improving egg shell quality. Using high doses of phytase may be able to improve shell quality and reduce the occurrence of cracks which could provide an economic benefit (Abdallah et al., 1993). The increase in the bioavailability of minerals like magnesium, zinc, manganese, iron and copper may be of interest as they are all essential nutrients required for animal growth and maintenance.

3.5.7 Conclusions

This trial failed to find any significant performance effects from the inclusion of 175 g kg^{-1} rapeseed meal or 2,500 FTU kg^{-1} alone or in combination, however, this is likely due to the length of the trial.

However, phytase did improve shell quality presumably through greater calcium availability. It is probable that this is a result of phytase hydrolysing phytic acid and releasing calcium bound within phytate complexes.

The inclusion of phytase did increase the solubility of calcium in addition to a number of minerals. With the exception of phosphorus, an increase in mineral solubility was translated into an increase in blood plasma mineral concentration. It is possible that using higher than standard phytase inclusion levels increases the availability of these minerals. This may explain somewhat the improvements seen in other monogastric trials.

Phytase increased the solubility of the whole diet, indicating that phytase does not just benefit the minerals of the diet. Within the foregut phytase had little effect on rapeseed meal compared to the control diet, which could be

due to rapeseed meal's high concentrations of phytic acid and its reduced susceptibility to phytase activity. Within the ileum, rapeseed meal improved mineral solubility. The inclusion of phytase also significantly affected the weight of the gizzard and pH of the duodenum; this could be due to phytase improving diet digestibility or because of the difference in dietary ingredients.

Chapter 4
The Effect of Glucanase and Phytase Inclusion on Laying Hens

4.1 Abstract

This experiment was conducted to assess the effects of including glucanase at 0, 10,000, 20,000 and 40,000 GU kg^{-1} with and without the inclusion of 2,500 FTU kg^{-1} phytase in a laying hen diet that met the birds requirements.

The birds were group housed and fed their respective diets for 12 weeks during which their performance was recorded. In addition to this egg quality measurements were analysed every three weeks. At the end of the trial, excreta samples were collected and phytate-phosphorus concentration was determined to assess how the diets had affected the hydrolysis of phytic acid. Glucanase addition to the diet was found to increase bird weight gain to 198g when included at 10,000 GU kg^{-1} comparison to the control diet at 135g. However, increasing the dose of glucanase further reversed these benefits. 2,500 FTU kg^{-1} phytase improved the FCR of birds from 20.6 to 2.01, showing that above standard inclusion of phytase can give extra-phosphoric benefits to the bird. Glucanase and phytase appeared to show greater than additive effects in relation to the weight gained by birds, indicating they can be used together within commercial laying hen rations. Glucanase improved egg shell quality while phytase had no significant effect. Differences in egg yolk fatty acid profiles and cholesterol levels suggests that both glucanase and phytase were affecting fat metabolism and digestion.

Finally, phytase significantly decreased the phytate-phosphorus concentration in the excreta from 0.71 to 0.49 g kg^{-1} suggesting substantial, but not complete, phytic acid hydrolysis. Glucanase supplementation did not increase phytic acid hydrolysis in the phytase supplemented diet. However, in the phytase free diet there was a trend for glucanase to increase total tract hydrolysis of phytic acid.

4.2 Introduction

The base ingredients of all monogastric diets are cereal grains; unfortunately these grains contain a number of anti-nutritional factors that impede digestion and absorption of nutrients within the gastrointestinal tract (Choct, 1997). Barley, rye, oats, wheat and triticale are all classified as viscous cereals as they contain high levels of soluble non-starch polysaccharides (NSPs) (Bedford and Partridge, 2001). NSPs are complex carbohydrates that are components of the cell walls of cereals (Selvendran and Verne, 1990). NSPs are considered to be anti-nutrients because they can act as a nutrient diluter and impede digestion through a number of mechanisms. This decreases the overall apparent metabolisable energy of the diet (Coon et al., 1988). Xylans and β-glucans are the main two NSPs in cereals that cause problems in poultry nutrition (Bedford and Partridge, 2001). All ingredients contain varying combinations of these NSPs. In wheat the xylan to β-glucan ratio is 10:1 whereas in barley the ratio is closer to 1:3 (Henry, 1985). β-glucans are found within the cell wall of the starchy endosperm and in the aleurone layer. β-glucans consist of a β1-4 glucose backbone that can vary in length as well as the degree and pattern of β1-3 linkages (Bielecki and Galas, 1991). These β1-3 linkages increase β-glucan solubility and flexibility. Typically β-glucans contain 70% β-1-4 linkages and 30% β-1-3 linkages (Hesselman, 1983). Fifty four percent of the β-glucans present in barley are soluble although this does vary between cultivars (Aaman and Graham, 1987). It is known that the presence of soluble NSPs can reduce animal performance. Burnett (1966) was the first to identify that β-glucans were an anti-nutrient and suggested that an increase in digesta viscosity may be cause. Wyatt and Goodman (1993) showed that feeding a barley based diet high in β-glucans decreased the feed efficiency of laying hens. Carbohydrases or fibrolytic enzymes that hydrolyse NSP to oligosaccharides are not produced by vertebrates, therefore poultry cannot break down β-

glucans. The addition of exogenous fibrolytic enzymes such as β-glucanase have been shown to benefit animal performance and nutrient digestibility in poultry and have been widely adopted since the 1980s (Newman and Newman, 1987). Glucanases break down β-glucans by hydrolysis of the glucosidic bonds. There are a number of mechanisms by which β-glucans may act as an anti-nutritional factor and glucanase may benefit animal performance.

Soluble β-glucans within the digestive tract hold water and increase digesta viscosity (Bedford and Morgan, 1995). This will slow digestion and absorption as it decreases the rate of diffusion of digestive substrates, enzymes and products. The increase in digesta viscosity has been shown to be detrimental to bird performance and reduce nutrient intake (Burnett, 1966; Choct and Annison, 1990; Choct and Annison, 1992; Danicke et al., 1999). Almirall and Esteve-Garcia (1995) showed that increases in viscosity, caused by β-glucans, reduced enzyme activities in addition to decreasing nutrient digestibility and broiler performance. Glucanase supplementation reversed these effects. Without glucanase supplementation, the birds may compensate for a lack of nutrient digestibility by increasing endogenous secretions, which will cause an increase in endogenous losses and the apparent metabolisable energy of the diet.

Glucanase can also benefit animals without high concentration of viscous grains through releasing nutrients locked within cells. The β-glucans within cell walls act as a physical barrier against digestion, encapsulating nutrients within the endosperm and aleurone layer of the feedstuffs (Bedford, 2002). Glucanase can break open these walls by hydrolysing glucosidic bonds and releasing the nutrients within (Choct, 2006).

Glucanase can be considered a prebiotic because of the effect it can have on the microflora within the intestine (Choct et al., 2001). Microbes do not easily digest large NSPs, but can utilise oligosaccharides. Glucanase supplementation releases oligosaccharides between 3 and 10 sugars in length after the hydrolysis of glucans. These oligosaccharides have been shown to be hydrolysed by *Bifidobacterium* and *Lactobacillus spp.* (Thammarutwasik et al., 2009). Glucanase inclusion can therefore promote

the proliferation of certain microbes by providing an enery source. Glucanase has previously been shown to suppress *Salmonella, Campylobacter* and *Escherchia coli* while increasing the proliferation of *Bifdobacterium* and *Lactobacillius spp* (Choct et al., 2001). The products of microbial fermentation are volatile fatty acids which can benefit energy metabolism (Taylor, 2002).This glucanase probiotic effect can increase the level of microbial fermentation and so the production of volatile fatty acids (Wang et al., 2005).

Finally, glucanase may benefit bird performance through the release of glucose from the β-glucan chain. It is unlikely that this is a key mechanism, however, as glucanase enzymes target the mid sections of chains to create oligosaccharides and rarely release monomeric sugars (Bhat and Hazlewood, 2001).

The addition of glucanase to barley based diets has been shown to overcome the anti-nutrient effects of β-glucans thus improving egg production, egg weight, weight gain, feed intake and feed conversion ratio(Francesch et al., 1995; Wyatt and Goodman, 1993; Brenes et al., 1993; Mathlouthi et al., 2003). However, these effects are inconsistent and will vary depending upon the glucanase used and the bird's physiology (eg. breed, age, weight) in addition to the composition of the diet including the levels of β-glucans, other NSPs and additional anti-nutrients.

In commercial situations, enzymes are most commonly used in combination to gain maximal benefits in animal performance by reducing all potential anti-nutritional affects. The most common enzymes in the monogastric feed industry are NSP degrading enzymes such as glucanase and the phytic acid degrading enzyme phytase. The addition of glucanase, xylanase and phytase in combination can correct a dilution of all the major nutrients within a broiler diet (Lu et al., 2013). Woyengo et al. (2010a) found that the addition of a mix of carbohydrases and phytase gave a greater body weight gain in broilers when compared to phytase supplementaion alone. In contrast, Leslie et al. (2007) found benefits in diet digestibility when diets were supplemented with 500 GU kg^{-1} glucanase but found little benefit from 500 FTU^{-1} phytase addition in a broiler diet. In another study glucanase and

phytase in combination improved the digestibility of dietary energy (Kiarie et al., 2010). Phytase inclusion at 500 FTU kg^{-1} and 1000U kg^{-1} xylanase both failed to improve AME when supplemented alone, but together they significantly increased the AME of the diet suggesting the enzymes had additive effects (Wu et al., 2004). The addition of a glucanase, xylanase cocktail or phytase alone did not improve nitrogen digestibility, but in combination the enzymes and phytase significantly increased nitrogen digestibility (Oryschak et al., 2002). Selle et al. (2009a) found additive amino acid digestibility benefits when broilers were fed xylanase and phytase. Juanpere et al. (2005) observed that the combination of 3150 GU kg^{-1} glucanase and 500 FTU kg^{-1} phytase to a barley based broiler diet improved phosphorus retention by 15%. The addition of phytase alone gave a 1.6% increase in phosphorus retention while glucanase alone gave no benefits. Nortey et al. (2007) found that supplementation of either 500 FTU kg^{-1} phytase or 4375 XU^{-1} xylanase both benefited phosphorus digestibility by 4.8 and 1.9 respectively when added alone to a piglet diet. However, when added in combination the increase in phosphorus digestibility was much greater (12.8%). This suggests there is a synergistic relationship between phytase and glucanase as the effect of the two enzymes combined is greater than the cumulative effect of each alone.

This synergism may result from cell wall breakdown by glucanase, making the phytic acid more available to the exogenous phytase (Woyengo and Nyachoti, 2011). A glucanase induced reduction in viscosity is also likely to increase phytic acid hydrolysis by endogenous phytase in addition to increasing the absorption of nutrients released from phytate. Glucanase could also increase the digesta retention time which would allow phytase longer to hydrolyse phytic acid. Finally, phytic acid may restrict nutrients released by glucanase by binding them into insoluble phytate complexes. The inclusion of phytase would prevent this and give a greater response to dietary glucanase inclusion.

4.2.1 Objectives

The benefits of using a higher than standard phytase doses in laying hen diets have not yet been conclusively shown. The main objective of this study

was to assess the effect of a higher than standard phytase inclusion on laying hen performance over a long term in comparison to a positive control diet without phytase supplementation. A secondary objective of this study was to assess the effect of a new glucanase enzyme on laying hen performance. As high phytase inclusion levels are becoming more common, it is of interest to assess effects of a high phytase dose and glucanase supplementation alone or in combination to better understand the interactions of the anti-nutrients β-glucan and phytate.

4.2.2 Hypotheses

Phytase supplementation will improve performance in comparison to the positive control diet

Increasing glucanase inclusion will improve performance

Phytase will improve egg shell quality

Phytase will increase total tract phytic acid hydrolysis

Phytase and glucanase will give additive improvements in performance and phytic acid hydrolysis when supplemented in combination

4.3 Materials and Methods

4.3.1 Experimental Design and Treatments

The effect of glucanase and phytase enzyme supplementation was assessed in a laying hen performance trial. The trial was designed as a 4 × 2 factorial experiment with 4 levels of glucanase inclusion (0, 10,000, 20,000 and 40,000 GU kg^{-1}) and two levels of phytase inclusion (0 and 2,500 FTU kg^{-1}). The activity of glucanase is defined by an *in vitro* assay where one glucanase unit (GU) is defined as the amount of enzyme that liberates 1 μmol of glucose at pH 5 and 30°C per minute from barley β-glucans. Similarly, one phytase unit (FTU) is defined as the amount of enzyme that liberates 1 μmol of inorganic P from 0.0051 mol l^{-1} sodium phytate at pH 5.5 and 37°C per minute (Engelen et al., 1994). The exogenous enzymes used in this study were provided by AB Vista Feed Ingredients and commercially known as Econase GT and Quantum Blue phytase.

The experimental diets were formulated to meet the nutritional requirements of the laying hen and contain at least 500 g kg^{-1} barley and 100 g kg^{-1} rapeseed meal to provide a diet high in β-glucans and phytic acid. The phytase was given conservative matrix values during the feed formulation normally used for the inclusion of 300 FTU kg^{-1} to give the two base diets in Table 4.1 (0.143% digestible calcium, 0.13% available phosphorus and 0.03% digestible sodium). These matrix values remove any benefit phytase has at a normal inclusion level, therefore any improvement seen in performance will be due to the higher than standard inclusion of phytase. During the milling and mixing of the diets each of the base rations was made in a single batch. They were then split into four equal parts and had the glucanase enzyme added as required to give the 8 experimental diets.

Table 4.1. Ingredients and calculated nutrient composition of experimental diets

Ingredient (g kg^{-1})	Without Phytase	With Phytase
Barley	505.1	535.3
Corn	75.9	53.2
Rapeseed Solv Ext	100.0	100.0
Soybean meal 48	149.1	146.2
Soy oil	55.0	55.0
Salt	03.0	03.0
Sodium Bicarbonate	01.0	01.0
DL Methionine	01.3	01.3
Limestone	92.3	89.8
Dicalcium Phos	12.3	10.2
Vitamin premix	04.9	04.9
Quantum Dry	00.0	01.0
Nutrient Composition (g kg^{-1})		
Crude Oil	69	68
Crude Protein	170	170
Crude Fiber	44	45
Calcium (Total)	40	39
Calcium (Digestible)	40	40
Phosphorus (Total)	07	06
Phosphorus (Available)	04	04
Sodium (Total)	02	02
AMEn (MJ kg^{-1})	11.3	11.3

4.3.2 Animal Housing

A total of 240 Bovan Brown chickens were purchased at 15 weeks old from a commercial pullet rearer. The birds were group housed with 4 birds per cage across 60 cages within one animal house at the University of Leeds, Spen Farm. Forty eight of these cages were allocated to the experimental diets when the birds reached 21 weeks of age, balancing for average bird weight and the previous weeks' performance, giving 6 replicates of 4 birds. All birds put onto trial had come into lay.

4.3.3 Feeding Regime and Performance Recording

The birds were fed their experimental diets for 12 weeks during which the performance criteria were recorded. Every three weeks, one day's eggs were collected and analysed for egg quality as described in Section 2.3.5.

4.3.4 Egg Fatty Acid Profile

The yolks from the eggs analysed for quality in the twelfth week of the trial were collected and frozen at -20°C. These were then analysed for their fatty acid profile and cholesterol content as described in Section 2.4.2.1.

4.3.5 Excreta Phytate-P Analysis

In the final week of the trial an excreta sample was collected from each cage. All feathers, scale and feed were removed and this sample was frozen at -20°C. This sample was then analysed for phytate-P concentration as described in Section 2.4.4.

4.3.6 Statistical Analysis

Two factors univariate general linear model (GLM) analysis of variance (ANOVA) tests within the statistical programme IBM SPSS Statistic 21 were used to analyse the performance and egg quality data. All data was tested for normality using the Kolmogorov-Smirnov test. The homogenity of variance of data was also analysed within the model.

Polynomial contrasts were used within the model to assess glucanase treatment for linear, quadratic and cubic effects. When significant post-hoc

bonferroni tests were performed for multiple comparisons. Significance was assigned at P< 0.05.

4.4 Results

4.4.1 Diet Analysis

The analysis of the diets is presented in Table 4.2. Generally they match the calculated composition of the diets shown in Table 4.1. The calcium concentration was slightly elevated across all diets in comparison with the calculated composition. The level of phosphorus, calcium and sodium were reduced in the diets supplemented with phytase as expected.

Table 4.2 Analysed nutrient composition of diets (g kg^{-1})

Phytase (FTU kg^{-1})	0				2,500			
Glucanase (GU kg^{-1})	0	10,000	20,000	40,000	0	10,000	20,000	40,000
Dry Matter	894	901	899	897	899	900	893	894
Oil	73.4	83.4	71.3	76.0	69.6	77.8	72.1	71.6
Crude Protein	166	160	165	167	162	161	159	161
Crude Fibre	42	39	43	45	45	46	43	46
Ash	149	157	154	156	142	143	141	134
Calcium	48.2	51.3	52.6	54.4	45.2	46.6	46.2	42.5
Phosphorus	5.5	5.7	6.0	5.7	5.2	5.3	5.1	5.0
Available Phosphorus	3.5	3.6	3.9	3.6	2.8	3.0	3.1	3.0
Phytate-P	2.0	2.1	2.1	2.1	2.4	2.3	2.0	2.0
Sodium	1.9	2.2	2.0	2.0	1.8	2.0	1.7	1.7

4.4.2 The Effect of Glucanase and Phytase Inclusion on Laying Hen Performance

The addition of glucanase to the laying hen diet had no significant effect on egg production, feed intake, egg mass or FCR (Table 4.3). The supplementation of glucanase did however, affect the weight gained by the birds across the 12 weeks with a significant increase from 134.9g to 197.5g with the inclusion of 0 and 10,000GU kg^{-1} respectively (P<0.005). The weight gained then fell as the glucanase dose increased, giving no significant difference between 0 and 40,000GU kg^{-1}. There was a trend for glucanase to affect egg weight in a cubic manner with the 10,000 and 40,000 GU kg^{-1} doses giving larger eggs. When the average performance of the last six

weeks of the trial was analysed alone, the glucanase dose did significantly affect the weight of eggs with hens receiving 10,000 GU kg^{-1} giving significantly heavier eggs ($P<0.05$) than those receiving 0 GU kg^{-1}. The weight of the eggs then decreased for hens which received additional dietary glucanase activity of 20,000 and 4000 GU kg^{-1} to give egg weights that were not significantly different from either the 0 or 10,000 GU kg^{-1} dose. The addition of the higher than standard phytase dose increased the FCR of the laying hens. This was seen for both the standard FCR and adjustedFCR as a near significant trend ($P=0.056$) and significant difference respectively ($P<0.05$). This resulted from a numerical increase in production and a trend for a decrease in feed intake. During the final 6 weeks of the trial the feed intake and both FCR values were significantly lower ($P<0.05$) while the differences in egg mass were still insignificant. There was also a trend for phytase to increase the weight gained by birds across the 12 weeks of the trial.

4.4.3 The Effect of Glucanase and Phytase Inclusion on Egg Quality

The inclusion of phytase had no significant effect on any of the egg quality criteria measured during this study (Table 4.4). The inclusion of glucanase in the diet did improve all three egg shell quality traits. Glucanase inclusion significantly increased both the shell thickness and shell strength in a quadratic manner. Further inclusion beyond 10,000 GU kg^{-1} gave no greater benefit in terms of egg shell quality and numerically decreased at 40,000GU kg^{-1}. This decrease was significant in shell strength but not shell thickness. There was also a trend for an quadratic increase in shell weight with glucanase dose. Increasing dietary glucanase inclusion sequentially increased the shell weight from 5.6 to 5.8g.

The inclusion of glucanase had no other significant effects, although the inclusion of 10,000 GU kg^{-1} did appear to improve egg quality in a number of criteria.

A higher than standard phytase inclusion increased the concentration of cholesterol in egg yolks (Table 4.6); while there was also a trend for 10,000GU kg^{-1} glucanase to reduce yolk cholesterol concentration relative to no glucanase supplementation. There was a tendency for an interaction

between phytase and glucanase. The glucanase effect described above was only visible in the diet with phytase. In the diet without phytase, glucanase had no effect on cholesterol content.

The inclusion of phytase did not significantly affect the proportion of any fatty acids (Table 4.5), but there was a trend for phytase to increase the proportion of docosahexaenoic acid (22:6) and the monounsaturated fatty acids (Table 4.6). Glucanase supplementation had a quadratic effect on the proportion of oleic acid (18:1) in egg yolks, increasing at first before falling again with the highest inclusion level. There was also a trend for glucanase to reduce the proportion of arachidonic acid (20:4) in egg yolks in a linear manner. In addition to this, a quadratic effect tended towards significance with glucanase dose up to 20,000 GU kg^{-1} increasing the proportion of docosahexaenoic acid (22:6) before falling with 40,000GU kg^{-1}. Glucanase had a significant quadratic effect on the proportion of monounsaturated fatty acids, with an increase up to 20,000 GU kg^{-1} before a reduction. A number of interactions were also found between phytase and glucanase in relation to the proportions of fatty acids. Increasing glucanase initially increased the proportion of palmitoleic acid (16:1) and then reduced it at higher dose when phytase was included in the diet. The opposite pattern was seen when phytase was not included in the diet. The same interaction was found with the proportion of polyunsaturated fatty acid; in the diet without phytase glucanase had no effect on docosapentaenoic acid (22:5) proportion. However, in the diet with phytase, 10,000 GU kg^{-1} glucanase increased the proportion of this fatty acid relative to the control as well as the higher glucanase doses. Glucanase increased the proportion of monounsaturated fatty acids up to 20,000 GU kg^{-1}. Phytase inclusion increased the proportion of monosaturated fatty acids, but as the glucanase dose increased the phytase effect was reduced.

Table 4.3 The effect of glucanase and phytase on laying hen performance

Glucanase GU kg^{-1}	Phytase FTU kg^{-1}	Production (%)	Egg Weight (g)	FI g	Egg Mass g	Adj Egg Mass g	FCR	Adj FCR	Weight Gain (g)
0	0	96.3	57.6	115.0	55.0	55.5	2.11	2.09	125.6
10000	0	96.1	60.4	118.6	57.7	58.1	2.07	2.05	178.3
20000	0	97.2	58.9	117.1	56.9	57.3	2.07	2.06	181.6
40000	0	95.0	59.1	114.3	55.9	56.2	2.06	2.05	112.6
0	2500	97.3	58.7	114.1	56.8	57.2	2.02	2.00	144.1
10000	2500	95.5	59.1	114.2	55.7	56.4	2.07	2.04	216.8
20000	2500	97.4	58.8	115.1	57.0	57.3	2.03	2.02	178.8
40000	2500	97.2	59.5	113.4	57.6	57.9	1.98	1.97	179.1
0		96.8	58.2	114.6	55.9	56.3	2.06	2.05	134.9^{c}
10000		95.8	59.7	116.4	56.7	57.3	2.07	2.05	197.5^{a}
20000		97.3	58.9	116.1	56.9	57.3	2.05	2.04	180.2^{ab}
40000		96.1	59.3	113.8	56.8	57.0	2.02	2.01	145.9^{bc}
	0	96.1	59.0	116.2	56.4	56.7	2.08	2.06	149.5
	2500	96.8	59.0	114.2	56.8	57.2	2.02	2.01	179.7
SEM		1.2	0.7	1.6	1.0	0.9	0.04	0.04	24.1
p Value									
Glucanase	Linear	NS	NS	NS	NS	NS	NS	NS	NS
	Quadratic	NS	NS	0.110	NS	NS	NS	NS	<0.005
	Cubic	NS	0.086	NS	NS	NS	NS	NS	NS
Phytase		NS	NS	0.083	NS	NS	0.056	<0.05	0.053
Glucanse × Phytase		NS	NS	NS	NS	NS	NS	NS	NS

$^{a-c}$ Means within columns with no common superscript differ significantly ($P<0.05$)

Table 4.4 The effect of glucanase and phytase on egg quality

Glucanase ($GU\ kg^{-1}$)	Phytase ($FTU\ kg^{-1}$)	Egg Weight (g)	HU	Yolk Colour	Yolk Wt (g)	BMS Score	Shell Thickness (µm)	Shell Weight (g)	Strength (kgf)
0	0	58.4	92.4	3.9	13.8	2.3	355.7	5.53	4.52
10000	0	61.3	93.2	4.1	14.1	2.0	370.2	5.88	4.83
20000	0	59.8	95.0	4.2	13.8	2.6	373.9	5.84	4.99
40000	0	60.1	93.1	4.0	13.7	2.3	367.2	5.74	4.63
0	2500	59.7	92.1	4.0	13.8	2.0	360.5	5.68	4.69
10000	2500	60.2	92.7	4.0	14.1	1.8	364.9	5.74	4.87
20000	2500	59.6	92.5	4.0	13.9	2.3	372.4	5.82	4.73
40000	2500	60.5	93.6	4.1	13.7	2.8	369.1	5.83	4.64
0		59.1	92.3	4.0	13.8	2.2	358.1[b]	5.60	4.61[b]
10000		60.7	93.0	4.1	14.1	1.9	367.6[a]	5.81	4.85[a]
20000		59.7	93.8	4.1	13.9	2.5	373.2[a]	5.83	4.86[a]
40000		60.3	93.3	4.1	13.7	2.5	368.1[a]	5.79	4.63[b]
	0	59.9	93.4	4.1	13.8	2.3	366.7	5.75	4.74
	2500	60.0	92.7	4.0	13.9	2.2	366.7	5.77	4.73
SEM		0.855	1.087	0.109	0.187	0.363	4.804	0.100	0.166
P-Value									
Glucanase	Linear	NS	NS	NS	NS	NS	NS	NS	NS
	Quadratic	NS	NS	NS	NS	NS	0.014	0.054	0.042
	Cubic	0.102	NS	NS	NS	0.153	NS	NS	NS
Phytase		NS	NS	NS	NS	NS	NS	NS	NS
Glucanse × Phytase		NS	NS	NS	NS	NS	NS	NS	NS

Table 4.5 The effect of glucanase and phytase on egg yolk fatty acid concentration as a proportion of total fatty acid content

Glucanase	Phytase	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:2	20:4	22:4	22:5	22:6	24:0
0	0	0.38	25.14	1.74^{ab}	8.30	31.74	23.37	1.29	0.20	2.47	0.61	0.17^{b}	1.29	0.16
10000	0	0.36	24.56	1.45^{c}	7.96	31.27	25.90	1.32	0.26	2.18	0.20	0.15^{b}	1.33	0.17
20000	0	0.33	24.13	1.69^{ab}	8.74	32.49	24.08	1.24	0.26	2.07	0.24	0.18^{b}	1.46	0.20
40000	0	0.39	25.06	1.72^{ab}	8.59	32.18	23.42	1.31	0.19	2.01	0.25	0.22^{ab}	1.38	0.20
0	2500	0.33	24.99	1.61^{bc}	8.52	31.39	24.69	1.37	0.24	2.26	0.18	0.19^{ab}	1.35	0.15
10000	2500	0.36	25.55	1.85^{a}	8.32	33.46	22.07	1.42	0.25	2.26	0.18	0.26^{a}	1.45	0.20
20000	2500	0.34	24.73	1.84^{a}	8.33	33.52	23.18	1.19	0.28	1.82	0.23	0.18^{b}	1.54	0.18
40000	2500	0.34	24.41	1.57^{bc}	8.84	31.91	24.45	1.21	0.19	1.85	0.20	0.19^{ab}	1.48	0.11
0		0.35	25.07	1.67	8.41	31.57^{b}	24.03	1.33	0.22	2.37	0.40	0.18	1.32	0.16
10000		0.36	25.05	1.65	8.14	32.37^{ab}	23.98	1.37	0.25	2.22	0.19	0.20	1.39	0.19
20000		0.33	24.43	1.77	8.53	33.01^{a}	23.63	1.22	0.27	1.94	0.24	0.18	1.50	0.19
40000		0.36	24.73	1.65	8.72	32.05^{ab}	23.93	1.26	0.19	1.93	0.23	0.20	1.43	0.15
	0	0.37	24.72	1.65	8.40	31.92	24.19	1.29	0.23	2.18	0.32	0.18	1.37	0.18
	2500	0.34	24.92	1.72	8.50	32.57	23.60	1.30	0.24	2.05	0.20	0.20	1.45	0.16
	SEM	0.008	0.157	0.028	0.109	0.228	0.375	0.032	0.013	0.086	0.041	0.011	0.026	0.011
Glucanase	Linear	NS	NS	NS	NS	NS	NS	NS	NS	0.066	NS	NS	NS	NS
	Quadratic	NS	NS	NS	NS	<0.05	NS	NS	NS	NS	NS	NS	0.06	NS
	Cubic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Phytase		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.09	NS
Glucanse × Phytase		NS	NS	<0.001	NS	NS	0.066	NS	NS	NS	NS	<0.05	NS	NS

Table 4.6 The effects of glucanase and phytase on egg yolk cholesterol content and fatty acid proportions

Glucanase	Phytase	Cholesterol	SFA	MUFA	PUFA
0	0	7.1	34.05	28.06[b]	28.97[ab]
10,000	0	7.7	32.96	32.72[ab]	31.31[a]
20,000	0	7.4	33.39	34.21[a]	29.36[ab]
40,000	0	7.5	34.20	33.92[a]	28.34[b]
0	2500	10.3	33.97	33.04[b]	30.10[ab]
10,000	2500	6.9	34.27	35.35[a]	27.30[b]
20,000	2500	8.3	33.44	35.40[a]	28.03[b]
40,000	2500	12.0	33.60	33.48[ab]	29.29[ab]
0		8.7	34.01	30.55[b]	29.54
10,000		7.3	33.62	34.04[ab]	29.31
20,000		7.9	33.42	34.80[a]	28.70
40,000		9.8	33.90	33.7[ab]	28.81
	0	7.4	33.65	32.23	29.50
	2500	8.2	33.82	34.32	28.68
	SEM	0.437	0.195	0.235	0.365
Glucanase	Linear	NS	NS	NS	NS
	Quadratic	0.079	NS	<0.05	NS
	Cubic	NS	NS	NS	NS
Phytase		<0.05	NS	0.09	NS
Glucanase × Phytase		0.099	NS	<0.05	<0.05

[a-b] Means within columns with no common superscript differ significantly (P<0.05)

4.4.4 The Effect of Glucanase and Phytase Inclusion on the Concentration of Phytate-P in Laying Hen Excreta

There was a significant interaction between phytase and glucanase in relation to total phosphorus concentration in the excreta of laying hens. Within the diet without phytase, 40,000GU kg^{-1} decreased total phosphorus concentration relative to other glucanase doses. However, in the phytase diet glucanase did not significantly affect total phosphorus concentration.

Glucanase had a significant quadratic effect on the non-phytate phosphorous concentration in the excreta of the laying hens. The inclusion levels 10,000 and 20,000 GU kg^{-1} resulted in a significantly higher concentration of non-phytate phosphorus relative to the 0 and 40,000 GU kg^{-1} concentrations.

Phytase significantly decreased the concentration of phytate-phosphorus from 0.75 to 0.50 g kg^{-1} (P<0.01). There was a trend for a linear glucanase effect, with the concentration of phytate phosphorus decreasing as glucanase concentration increased.

Table 4.7 The effect of glucanase and phytase in laying hen diets on the concentration of total, non-phytate and phytate phosphorous in excreta (g kg^{-1})

Glucanase	Phytase	Total-P	Non-Phytate-P	Phytate-P
0	0	1.87ab	1.00bc	0.87
10000	0	2.39ab	1.52a	0.87
20000	0	2.34ab	1.52a	0.83
40000	0	1.1c	0.64c	0.46
0	2500	1.76abc	1.21ab	0.55
10000	2500	1.54bc	1.09ab	0.45
20000	2500	1.58bc	1.12ab	0.46
40000	2500	1.97ab	1.44a	0.53
0		1.81	1.10b	0.71
10000		1.96	1.31a	0.66
20000		1.96	1.32a	0.64
40000		1.53	1.04b	0.49
	0	1.92	1.17	0.75
	2500	1.71	1.21	0.50
SEM		0.101	0.066	0.049
P Value				
	Linear	NS	NS	0.086
Glucanase	Quadratic	0.159	<0.05	NS
	Cubic	NS	NS	NS
Phytase		NS	NS	<0.01
Glucanse × Phytase		<0.005	<0.001	NS

4.5 Discussion

4.5.1 Comparison to Breeders Expected Performance

The hens used in this study conformed well with the breeder expectations (Joice and Hill, 2014). The birds were 50 g heavier than expected at the start of the trial, but by the end of the trial were at their target weight. The egg weights were on target while the feed intake was slightly higher in the birds on this trial. The egg production was 5% higher than the breeder expected values.

4.5.2 The Effect of Glucanase and Phytase on Laying Hen Performance

In this study, the inclusion of glucanase was found to benefit bird weight gain and egg weight. Previous studies have found that glucanase can benefit performance in laying hens, broilers (Hesselman, 1983) and piglets (Owusu-Asiedu et al., 2012). However, increasing glucanase concentration started to reverse the benefits seen at 10,000GU kg^{-1} on egg weight and weight gain. Increasing glucanase to 40,000GU kg^{-1} returned the weight gain back to the level of the control. Similar results have been seen in broilers fed xylanase (Mendes et al., 2013). Where xylanase was included at 0, 75, 100 and 200% of the recommended inclusion level; 75% inclusion gave the best results, with decreases in performance at 100 and 200%. The authors suggested that the higher inclusion of xylanase enzyme may result in a different end product from the hydrolysis of NSP. They hypothesised that greater carbohydrase activity would affect the oligosaccharides produced, which in turn would affect their prebiotic activity and microflora fermentation. This is possible, but many within the industry have independently suggested another mode of action. To begin with, the addition of glucanase benefits performance through the hydrolysis of the soluble β-glucans that are naturally present during the digestion of ingredients such as barley. The soluble β-glucans are relatively readily available for hydrolysis. Their breakdown will improve protein and diet digestion in addition to the AMEn of the diet by decreasing digesta viscosity (Burnett, 1966, Choct and Annison, 1990, Choct and Annison, 1992, Danicke et al., 1999). As the dose of glucanase increases, the enzyme breaks through the cell wall and releases the nutrients encapsulated (Bedford, 2002). This increases the nutrient digestibility and AMEn of diets, even if the ingredients of the diet contain relatively little amounts of soluble β-glucans. So far this mechanism is well

documented with multiple studies providing evidence (Bedford, 2000). From now on the hypothesis becomes speculative with little evidence. The theory suggests that greater glucanase inclusion allows a further break down of the cell walls. This gives no more benefit as the encapsulated nutrients have already been released from the cell. However, the cell wall destruction will start to increase the amount of soluble β-glucans present in the lumen of the tract. This will start to increase digesta viscosity, impede digestion and decrease the AMEn of the diet. As stated above, a number of colleagues in the industry have seen similar effects in unpublished in-house trials (Broom, 2014). It is possible that even greater glucanase inclusion may start to benefit the animal again as they break down the newly solubilised β-glucans. With the factors described above, the prebiotic effects of glucanase and the potential release of sugars from β-glucans it is clear that glucanase affects the digestion of the diet in a complicated manner. This may explain the trend for a cubic response in egg weight when supplemented with glucanase observed here.

The addition of 2500 FTU kg^{-1} phytase gave a decrease in FCR which resulted from a small increase in egg numbers and a decrease in feed intake. In addition to this, phytase gave a trend for an increase in weight gained. Previous studies in laying hens have failed to show the benefits of higher than standard inclusion of phytase (Augspurger et al., 2007). In Chapter 3, the inclusion of 2,500FTU kg^{-1} did not benefit performance, however, as stated within that Chapter the trial was too short to asses performance differences. Augspurger et al. (2007) found that 10,000 FTU kg^{-1} improved egg performance, but no more than standard inclusions. Meyer and Parsons (2011) similarly found that 15,000FTU kg^{-1} did not benefit performance compared to standard inclusions. Both of these trials were long enough to find a performance effect, so the difference in results must be due to difference in trial design. In this trial, an improvement in FCR was seen in laying hens at the very start of their lay cycle. At this age, the birds have a large nutritional requirement to enable continued growth, an increase in egg production and an increase in egg weight. Augpurger and colleagues (2007) performed their trial on birds in their second lay cycle after a moult with average egg weights of 65g and production around 84%.

Meyer and Parsons (2011) used laying hens in mid-lay from 32 weeks of age. Although the birds used in this trial are closer in age, their production was still far from our averages with production at 85% and average egg

weights of 60g. Both these trials also used smaller White Leghorn birds compared to the larger Bovan Brown used in this experiment.

The addition of higher than standard inclusion level of phytase has, however, been shown to benefit the performance of other monogastrics (Cowieson et al., 2010). In broilers, Walk and colleagues, (2014) found that increasing phytase to just 2,000FTU kg^{-1} gave benefits to FCR that could not be explained by increasing phosphorus and calcium in the diet. It is more likely that the benefits from high phytase inclusion are related to the extra-phosphoric effects: improving the digestion of protein, carbohydrates and minerals, reducing the endogenous secretions and losses and so increasing the AMEn of the diet. Phytase may also benefit performance through the release of *myo*-inositol. Little is known about the requirements for *myo*-inositol, but it has been shown that *myo*-inositol can increase the growth rate of fish (Waagbø et al., 1998), rats (Katayama, 1997), chicks (Hegsted et al., 1941b) and broilers (Cowieson et al., 2013). It is possible that growing animals have a requirement for *myo*-inositol that mature animals do not. The concentration of serum inositol is known to be higher in neonates and foetuses compared to their adult counterparts (Quirk and Bleasdale, 1983). *Myo*-inositol is also highly concentrated in breast milk, which suggests young children have a large requirement (Bromberger and Hallman, 1986, Pereira et al., 1990). *Myo*-inositol did not benefit laying hens at 50 weeks of age, having a numerically negative effect on egg numbers, egg mass and FCR (Żyła et al., 2012). This could explain the difference seen between this trial, with start of lay chickens that are still growing, and the experiments with fully grown older birds.

No significant interactions were seen between phytase and glucanase, however, both enzymes did affect the weight gained by birds. Including 10,000GU kg^{-1} in the diet increased weight gained by 42% while phytase inclusion resulted in a 15% increase. The inclusion of 10,000 GU kg^{-1} and 2,500 FTU kg^{-1} meanwhile gave a 73% increase. This implies they had an additive effect and can both be used within laying hen diets to give nutritional benefits. If a significant interaction had been found, this effect could have been classified as synergistic which is discussed in more depth in Section 4.5.4. Previously, fibrolytic enzymes and phytase have been shown to have

additive effects in relation to AMEn and amino acid digestibility (Wu et al., 2004, Selle et al., 2009a).

4.5.3 The Effect of Glucanase and Phytase on Egg Quality

Phytase had no significant effect on any of the egg quality characteristic measured during this trial. In the previous trial, it was shown that the inclusion of 2,500FTU kg^{-1} increases mineral solubility, which gave an increase in shell quality. This may be because the calcium levels in this diet were slightly higher than the previous trial. This would have made it harder for phytase to improve shell quality. It could be that the difference between these trials is due to the bird age. As birds get older their calcium requirement increases, and so increasing calcium availability is likely to have a greater effect in older birds. That said, the inclusion of glucanase did increase shell quality in this trial. Elmenawey et al. (2010) also found that an amylase, xylanase enzyme mix improved both shell weight and thickness. This is contrary to the findings of others, who found carbohydrases had no effect on shell quality (Pirgozliev et al., 2010a) or in fact a negative effect (Yaghobfar et al., 2007). Given the low concentration of calcium encapsulated within cereal grains, it is probable that any improvement in shell quality is due to glucanase reducing digesta viscosity and so improving calcium availability. A detailed look at the data shows that in diets without glucanase, phytase does give small numerically benefits to shell weight, thickness and strength. However, once glucanase is included phytase had no consistent numerical benefit. The negative effects seen by Yaghobfar and colleagues (2007) could be due to egg size. Although insignificant they show a large increase in egg weight with enzyme supplementation. As eggs increase in size the quality of egg shells decreases as the shell is spread over a larger area it is thinner. The lack of a phytase effect and presence of a glucanase effect was unexpected but of interest. It may be that the presence of phytic acid is not restricting calcium availability as much as β-glucans within the gastrointestinal tract, or that glucanase is benefiting shell quality in another way unrelated to calcium availability.

This trial showed that 2,500 FTU kg^{-1} phytase significantly increased the cholesterol concentration in egg yolks. Zyla et al. (2012) found that the

inclusion of 300 FTU kg^{-1} gave decreased cholesterol concentration. The difference in results is likely to be due to the dose of phytase used. Zyla and colleagues found that increasing phytase activity beyond 300 FTU kg^{-1} in the diet started to increase the cholesterol concentration. In addition to this they found that adding *myo*-inositol directly to the diet gave a large increase in cholesterol concentration. It is likely that the *myo*-inositol released from the hydrolysis of phytic acid is the cause of the effects seen in this study. *Myo*-inositol is known to affect fat metabolism, reducing incidences of fatty liver syndrome in rats (Katayama, 1997) and increasing the HDL cholesterol in women with polycystic ovaries (Gerli et al., 2007). Some earlier work showed that increasing dietary *myo*-inositol concentration increased the mobilisation of cholesterol, which eventually decreased the levels of cholesterol in the blood, heart and liver in laying hens (Herrmann, 1946). Lee et al. (2005) showed that adding 15 g kg^{-1} phytic acid to a mouse diet reduced the hepatic total lipids and total cholesterol.

Glucanase inclusion affected the fatty acid profile of the egg yolks, increasing the proportion of 18:1 and 22:6 while decreasing 20:4. There were also a number of interactions between phytase and glucanase. Many of the glucanase dose responses seen follow this same rise and fall seen with weight gained and egg weight. It may be that glucanase is affecting the fatty acid profile through its actions on the energy and fat in the diet. As the dose is increased, the glucanase decreases the digesta viscosity with additional inclusion possibly reversing these effects, as already descibed; the diet AMEn would then follow this same pattern affecting energy intake and altering fat metabolism within the liver, which in turn would change the accumulation of certain fatty acids within the growing ova. It may also be that glucanase itself is affecting the digestion of fat. Mathlouthi et al. (2002) showed that supplementation of a diet with carbohydrases increases the intestinal concentration of bile acids, which aid the digestion and transportation of fat to the brush border membrane of the small intestines. In addition to this, Engberg and colleagues (2004) found that xylanase supplementation in broiler diets increased pancreatic lipase activity by 23%. Hanczakowska et al. (2012) showed that including fibrolytic enzymes in the diet can improve fat digestibility. Phytase has also been shown to improve

the AMEn of diets (Ravindran et al., 2006), as well as the ileal digestibility of fats (Camden et al., 2001). Zyla et al. (2012) also found that phytase inclusion could affect the proportion of fatty acids which could not be explained by the greater absorption of phosphorus or *myo*-inositol. Whether through changes in fat metabolism or improvement in digestion it is clear that the inclusion of both glucanase and phytase can affect the fatty acid profile in egg yolk. More in depth research is needed to understand these complicated relationships before theories can be conclusively accepted.

4.5.4 The Effect of Glucanase and Phytase on Phytate-P Concentration in the Excreta of Laying Hen

The concentration of phytate-P in the excreta can be used as an indicator of phytic acid hydrolysis. As hydrolysis increases, the concentration of phytate-P that survives the gastrointestinal tract and is excreted will decrease. The use of a higher than standard phytase inclusion increased the phytic acid hydrolysis in comparison to a positive control diet without phytase supplementation. This shows that the phytase in the diet has had the desired effect of breaking down the dietary phytic acid. However, the concentration of phytate-P in the excreta was still relatively high and shows that although the feed industry considers 2,500FTU kg^{-1} to be a "superdose", there is still room for greater phytate destruction and a prevention of the anti-nutritional effects. Shirley and Edwards (2003) found that phytase continued to increase phytate hydrolysis up to 12,000FTU kg^{-1} in broilers. This data suggests that 2,500 FTU kg^{-1} would release approximately 70% of the phytate-P. At standard inclusion levels, laying hens are normally fed less phytase than broilers to gain the same level of phytic acid hydrolysis, as the tract transit time is longer in layers giving the phytase longer to act. That said, it may be that to gain the higher than standard phytase benefits a greater dose is needed to overcome the greater concentration of calcium ions in the digesta. In the diet with phytase, glucanase gave no benefit to phytic acid hydrolysis. For glucanase to benefit the breakdown of phytic acid a number of requisites must be met (Woyengo and Nyachoti, 2011). Firstly, both phytic acid and phytase must be present for any hydrolysis to occur. Secondly, soluble non-starch polysaccharides must be present to reduce digesta viscosity or phytic acid must be encapsulated within β-glucan cell walls. Phytic acid hydrolysis must be restricted by either poor phytase activity due to the high digesta viscosity or the unavailability of phytic acid

due to its encapsulation. Next, glucanase inclusion must be able to reverse the β-glucan effect on digesta viscosity or break open cell wall. This would alleviate the β-glucan effects, improving digesta viscosity and releasing phytic acid from the cell (Woyengo and Nyachoti, 2011). Finally, for an observable increase in animal performance, the phytic acid must be limiting digestion and animal production.

In the phytase diet, glucanase did not benefit phytic acid hydrolysis and so at least one of the requirements for the synergism stated above was not met. It could be that glucanase did not increase the availability of phytic acid for hydrolysis; alternatively the phytase included may have already been hydrolysing phytic acid at its maximum capacity and greater substrate availability would not speed up the reaction. Previous studies have found a synergistic phytic acid effect with the inclusion of fibrolytic enzymes and phytase in broiler diets (Juanpere et al., 2005; Nortey et al., 2007), whereas other studies have similarly found no synergistic effect (Woyengo et al., 2008; Tiwari et al., 2010). Given the complicated nature of fibrolytic enzyme activities, it is likely that a large number of variables can affect the synergistic relationship the enzymes can have with phytase.

Woyengo and Nyachoti (2011) reviewed the interactions of glucanase and phytase and proposed that synergism is more likely if a mixture of carbohydrases are used rather than just one. This is because the cell walls of oilseeds are more complicated than those of cereals such as barley (Bedford and Partridge, 2001), and it is the oilseeds that have the greatest concentration of phytic acid (Selle and Ravindran, 2007). The destruction of their cell walls and the release of phytic acid would be greater with a cocktail of enzymes rather than just one. Synergisms are also more likely to occur if the concentrations of NSPs in the diet are high as they are more likely to restrict phytic acid hydrolysis (Adeola and Bedford, 2004). There are a larger number of other factors that can impede phytic acid hydrolysis, discussed in Section 1.4.4, such as high dietary non-phytate phosphorus or calcium. All of these are likely to have an influence on the relationship between enzymes.

In the diet without phytase, glucanase increased phytic acid hydrolysis with small increases followed by a large jump with the inclusion of 40,000GU kg^{-1}. This may be due to a greater concentration of glucanase decreasing digesta viscosity, which allowed a greater amount of phytic acid to reach the brush border membrane where there are phytase enzymes produced by the bird. It may also be the glucanase breaking open cell walls which disturbs the cell. This would free not just the phytic acid but also some of the plant’s own

phytase; allowing the two to mix would increase phytic acid hydrolysis. It is also possible that the differences seen in excreta phytate-phosphorus concentration are not due to the hydrolysis of phytic acid in the proximal gastrointesinal tract and small intestines. They could be a result of post-ileal microbial hydrolysis of phytic acid. Glucanase activity can break down β-glucans into oligosaccharides which can act as prebiotics, increasing microbial fermentation and biomass within the large intestines (Choct et al., 2001). The microflora in the intestines are known to affect total tract phytate-phosphorus hydrolysis (Wise and Gilburt, 1982), and for this reason future research should investigate the concentration of phytate-phosphorus in the ileum.

4.5.5 Conclusions

The addition of glucanase can benefit laying hens, increasing weight gained and egg weights presumably through aiding digestion, with 10,000GU kg^{-1} giving the greatest benefits. This may allow a dilution of dietary nutrients or the inclusion of lower quality, cheaper ingredients into the laying hen diet without harming production. Doses greater than 10,000 GU kg^{-1} seem to reverse the benefit of lower glucanase inclusion, which may be due to the breakdown of the cell wall creating more soluble β-glucans and increasing digesta viscosity once more.

The addition of an above standard inclusion dose of phytase has been shown to benefit laying hens with a decrease in FCR from 2.06 to 2.01 and an increase in weight gained from 150g to 180g. The FCR decrease was a result of a lower feed intake, slightly higher egg production and stable egg weights. The weight gained by laying hens is a sign of nutritional status, and an increase indicates the birds were fed a more nutritious diet.

It is important to note that neither glucanase or phytase appeared to hinder each other as a near additive effect was seen in weight gain. This shows that they can be used in combination within a laying hen diet without impeding each other.

In contrast to the previous trial the inclusion of phytase did not increase the shell quality which could be due to the age difference of the birds and the calcium content of the diet. However, glucanase has also been shown to improve egg shell quality, although the literature provides somewhat contrasting views which may be due to differences in egg sizes. In this trial

10,000 GU kg^{-1} increased shell thickness from 358 to 368μm and shell strength from 4.61 to 4.85kgf.

Phytase addition increased total tract phytic acid hydrolysis. Glucanase did not benefit phytic acid hydrolysis in the diet with phytase, but appears to increase hydrolysis when included in a diet without phytase.

Phytase has been shown to significantly increased the cholesterol content of the egg yolk, while both enzymes affected the fatty acid profile. This suggests both enzymes can affect the metabolism and digestion of fats.

Chapter 5
The Effect of Graded Levels of Phytase Beyond Industry Standards in Laying Hens Diets

5.1 Abstract

This experiment was conducted to assess the effects of increasing phytase inclusion above the standard doses within the laying hen diets. The trial had 6 dietary treatments: positive control (PC) formulated to meet bird requirements (Digestible Ca 4%, Available P 0.320%, Digestible Na 0.175%), negative control (NC) with slightly reduced levels of calcium, phosphorus and sodium equivalent to that released by standard phytase inclusion (Digestible Ca 3.857%, Available P 0.189%, Digestible Na 0.145%); NC plus 250 (NC+250 – conventional recommended inclusion rate), 1,000 (NC+1,000), 4,000 (NC+4,000) or 16,000 (NC+16,000) FTU kg^{-1} of microbial phytase. Mid-lay birds were group housed and fed their respective diets for 12 weeks during which their performance was recorded. In addition, egg quality measurements were analysed every three weeks. At the end of the trial, the birds were killed, dissected and the digesta from the gizzard and ileum were taken for phytate-phosphorus analysis. A peripheral blood sample was also taken and analysed for mineral content. Livers were also dissected, weighed and analysed for *myo*-inositol and mineral content.

The inclusion of phytase above the standard level was shown to increase egg weight from 62.7 to 63.7g. Together with a trend for an increase in egg numbers this gave a sustained increase in egg mass production from 58.9g/d to 60.7g/d in comparison to a positive control without an increase in feed intake. This shows that laying hens can benefit from the above standard inclusion of phytase. It was shown that the higher doses increased phytic acid hydrolysis, with reduced concentrations of ileum phytate phosphorus (1.18 g kg^{-1}) in comparison to the PC, NC and standard inclusions (1.38, 1.44 and 1.29g kg^{-1} respectively). The higher doses were

also shown to increase the bioavailability of *myo*-inositol, phosphorus, manganese and zinc.

5.2 Introduction

The process of phytase releasing phosphorus from phytic acid limits phytase inclusion to approximately 500 FTU kg^{-1} for economic reasons. Increasing phytase doses will increase the cost of the diet in a linear manner, but gives diminishing returns of phosphorus released form phytate (Simons et al., 1990a, Schöner et al., 1991, Huyghebaert et al., 1992). Therefore, the benefit of replacing inorganic phosphorus with phytase has a limit. Shirley and Edwards (2003) disagree with this and believe there have been underestimations in the amount of phosphorus released by higher doses. Following this, they showed that more phosphorus can be economically released using higher doses.

The standard inclusion of exogenous phytase is approximately 250 – 500 FTU kg^{-1}. However, inclusion levels have been increasing towards 1000 FTU kg^{-1} as nutritionists strive for more economic diets and lower levels of inorganic phosphates. The inclusion of above standard doses is attracting interest from nutritionists and academics alike. A phytase dose is considered to be above the standard inclusion level when the phytase activity is greater than or equal to 2500 FTU kg^{-1}, although more potent phytases have been shown to produce the benefits of high doses at just 1,500FTU kg^{-1} (dos Santos et al., 2013). Numerous studies have shown benefits of including high doses, including improvements in weight gain, FCR, AMEn, mineral digestibility and amino acid digestibility (Cowieson et al., 2011).

The majority of this work has been conducted in broilers and has shown improvements in performance and efficacy (Harper et al., 1999, Shirley and Edwards, 2003, Augspurger and Baker, 2004, Cowieson et al., 2006, Karadas et al., 2010, Walk et al., 2014). Karadas et al. (2010) showed that a phytase dose of 12,500FTU kg^{-1} improved the FCR in comparison to a standard inclusion of 500 FTU kg^{-1} (1.28 vs 1.43). In addition, 12,500 FTU kg^{-1} gave birds a higher concentration of antioxidants, alpha-tocophenol and co-enzyme Q_{10} in the liver in comparison with the negative control and

standard phytase inclusion. These were equal to, but not significantly higher than, the levels of antioxidants in the liver of birds fed the positive control diet.

The benefits of higher phytase doses have also been seen in turkeys (Esteve-Garcia et al., 2005, Pirgozliev et al., 2007), weaner piglets (Kies et al., 2006, Brana et al., 2006) and grower pig (Brana et al., 2006). Pirgozliev et al. (2007) showed that in both turkeys and broilers an increase from a standard dose to 2500 FTU kg^{-1} gave significant increase, in weight gain, feed intake and the AMEn of the diet. There was also a non-significant improvement in FCR. Brana and colleagues (2006) found that grower pigs grew 7% faster and with 6% better efficiency when fed 10,000FTU kg^{-1} in comparison to a positive control diet. In weaner piglets improvements were also seen, however, these were only significantly different from the negative control and the standard inclusion rates, and not the positive control diet. On the other hand, Kies et al. (2006) found that increasing phytase dose up to 15,000FTU kg^{-1} in a weaner diet continued to deliver improvements in feed intake, weight gain, FCR, diet digestibility and mineral digestibility. In laying hens, both Augspurger et al. (2007) and Meyer and Parsons (2011) found that high phytase doses had no greater effect than standard phytase inclusion. In contrast, the previous study (Chapter 4), in this thesis found that 2,500 FTU kg^{-1} improved the FCR of laying hens in comparison with a positive control.

With an increase in phytic acid digestibility, it would be logical to assume the benefits seen in performance could arise from greater calcium and phosphorus availability. Santos et al. (2013) showed this was not the case with 1,500 FTU kg^{-1} improving bird FCR in an adequate phosphorus diet. The lack of effect of toe ash, calcium and phosphorus is seen as a validation that these minerals were not important in delivering the improved efficiency. Walk et al. (2014) reiterated this, showing diets that included 1,500 FTU kg^{-1} improved broiler FCR in comparison with a positive control, negative control, negative control with 500FTU kg^{-1} and a positive control diet with additional dicalcium phosphate. This shows that extra calcium and phosphorus does

not benefit FCR, and so the benefits of the high dose must be extra-phosphoric. In addition to this, they found there was no benefit to toe ash with the higher dose. Finally, an analysis of the phytic acid esters revealed a reduction in the concentration of IP6 and IP5 and an increase in the concentration in the *myo*-inositol in the gizzard with the inclusion of phytase. The concentration of IP6, IP5 and *myo*-inositol were correlated to weight gain and FCR, but not toe ash.

Inclusion of these higher levels is believed to benefit the animal through almost complete phytic acid hydrolysis (Cowieson et al., 2011). Once in the small intestines, phytic acid quickly binds to cation nutrients to form insoluble phytate complexes that are refractory to endogenous phytase activity. The inclusion of high levels of phytase increases hydrolysis within the foregut and prevents the higher esters of phytic acid reaching the small intestines from the gizzard (Cowieson et al., 2011). The lower esters can then flow into the duodenum as they have weaker bonds, are more soluble and so are more readily hydrolysed by the endogenous phytase (Persson et al., 1998). Greater phytase inclusion will increase the amount of phytate-phosphorus released from phytic acid and so allow further reductions in inorganic phosphorus use (Shirley and Edwards, 2003). Although phosphorus availability is increased, it is the extra-phosphoric benefits of high phytase inclusion that are seen as potential tools to improve performance, increase efficiency and possibly allow dietary dilutions (Walk et al., 2014).

It is probable that one of the main benefits of increasing phytase dose is the reversal of the anti-nutritional effects of phytic acid described in Section 1.4.5. This causes increases in the availability of dietary nutrients such as proteins, minerals and carbohydrates while reducing endogenous secretions and losses. It is also possible that the increase in *myo*-inositol availability is responsible for some of the benefits seen. *Myo*-inositol is involved with the transport of fats (Katayama, 1997) and has been shown to improve growth performance in a number of animals (Hegsted,1941; Katayama, 1997; Waagbo et al., 1998; Cowieson et al., 2013).

Myo-inositol based compounds are known to have several important physiological functions. It has been shown to play an important role in the

control of cell volume and osmolality through calcium related chloride secretions (Kane et al., 1992; Shears, 1998). In addition to this, *myo*-inositol has been shown to mimic the effects of insulin (Dang et al., 2010, Yamashita et al., 2013).

The *myo*-inositol phospholipid glycosylphosphatidylinositol is a vital molecule that anchors proteins to the extracellular side of the cell membrane (Brown and Waneck, 1992). Approximately 150 different proteins have been shown to use *myo*-inositol in this way with a wide variety of functions, including enzymes, antigens and adhesive molecules (Hooper, 1997; Low, 1989; Brown and Waneck, 1992).

The *myo*-inositol phosphate esters are also of importance; IP3 and IP2 are water soluble and so can act as secondary messengers. They act by controlling the cellular calcium concentration and by stimulating specific protein phosphorylation process via protein kinase C (Berridge, 1993). This pathway is known to be involved in cell fertilisation, growth, secretions, sensory perception, signalling and proliferation (Shears, 1998).

IP6 and IP5 are the most abundant *myo*-inositol esters present in the body, but little is known about any metabolic function they may have. As with plant seeds, it is possible that they are used to store *myo*-inositol and various minerals. They could also have a role in providing protection from oxidative stress, through chelating minerals and averting the synthesis of reactive oxidative compounds (Doria et al., 2009). Finally, because of their capacity to bind to various molecules such as proteins, an as of yet unknown biological function cannot be dismissed.

The previous studies within this thesis have shown that the inclusion of high phytase doses in laying hen diets can improve mineral solubility and availability (Chapter 3), in addition to improving the FCR of laying hens and the hydrolysis of phytic acid (Chapter 5). However, it was found that the hydrolysis of phytic acid was incomplete as substantial phytate-phosphorus was still found within the excreta. Shirley and Edwards (2003) showed in broilers that supplemental phytase continued to increase phytic acid hydrolysis up to 12,000FTU kg^{-1}. Their data suggested that the 2,500 FTU kg^{-1} used in the previous chapter would only release approximately 70% of

the phytate-P. It may be that due to the higher calcium content of the laying hen diet this value is lower in laying hens. The glucanase data also raised questions over post-ileal phytic acid hydrolysis. The concentration of phytic acid in the ileum would be a better indicator of hydrolysis that the bird can take advantage of, as microbial fermentation will undoubtedly affect the concentration of phytate-P in the excreta. Additionally, the concentration of phytate-P in the gizzard may give a better indicator still, as Walk et al. (2014) found the concentration of IP6, IP5 and *myo*-inositol in the gizzard was correlated to weight gain and FCR.

5.2.1 Study Objectives

The objective of this study was to investigate the effect of increasing phytase dose in comparison to a positive control diet, a negative control diet and standard phytase inclusions on the performance of the laying hen, quality of eggs and the hydrolysis of phytic acid within the gastrointestinal tract up to the ileum.

5.2.2 Hypotheses

Birds on the negative control diet will have reduced performance in comparison with those on the positive control diet

The addition of a standard phytase inclusion on top of the negative control diet will restore laying hen performance to the level of those fed the positive control diet.

The supplementation of phytase above standard inclusions will improve laying hen performance in comparison with birds fed the positive control, negative control and standard inclusion diets.

Supplementing diets with phytase will increase the hydrolysis of phytic acid within the gastrointestinal tract; reducing the concentration of phytate-P within the gizzard and ileum.

Supplementing diets with phytase will increase the concentration of *myo*-inositol and minerals in the liver of laying hens.

Supplementing diets with phytase will increase the concentration of minerals in the blood plasma of laying hens.

5.3 Materials and Methods

An experiment was conducted to assess the effect of increasing phytase above the standard inclusion on laying hen performance, egg quality and the hydrolysis of phytic acid.

5.3.1 Experimental Design and Treatments

The experiment consisted of six dietary treatments including a positive control diet (PC) that met the birds' requirements in every regard and a negative control diet (NC). The negative control diet had a reduced level of phosphorus, calcium and sodium. These reductions were equivalent to the formulation matrix values normally applied to the standard Quantum Blue phytase dose of 250FTU kg^{-1} (0.143% digestible calcium, 0.13% available phosphorus and 0.03% digestible sodium). The NC diet also had four different inclusions of phytase added on top of the formulation to give the remaining treatments; NC + 250FTU kg^{-1}, NC + 1000 FTU kg^{-1}, NC + 4000 FTU kg^{-1} and NC + 16,000 FTU kg^{-1}. The NC + 250FTU kg^{-1} and NC + 1000 FTU kg^{-1} are considered to be standard inclusions (SI) while the NC + 4000 FTU kg^{-1} and NC + 16,000 FTU kg^{-1} are considered to be above standard inclusions (ASI).

5.3.2 Animal Housing

At 35 weeks of age a total of 108 Bovan Brown laying hens were allocated to 36 cages balancing for bird weight. After one week of recording performance, the cages were allocated to treatments balancing bird weight, egg production, egg weight and feed intake giving each treatment 6 replicates of three birds within one animal house.

5.3.3 Feeding Regime and Performance Recording

The birds were fed their experimental diets for 12 weeks during which their performance criteria were recorded. Every three weeks one day's eggs were collected and analysed for the egg quality and component weights as described in Section 2.3.5.

5.3.4 Dissections

After 12 weeks the birds were killed by cervical dislocation over three consecutive days. To begin, a peripheral blood plasma sample was taken for mineral analysis. The gut was then liberated from the bird and the proventriculus, gizzard and ileum were collected. Each section of the gastrointestinal tract was clamped and then weighed while full of digesta. The digesta was then removed from the tract and mixed before the pH was recorded from three points. The empty weight of the tract segment was then recorded. The digesta was then frozen at -20°C until it was analysed for phytate-phosphorus concentration as described in Section 2.4.4. The whole liver was also taken and frozen at -20°C until it was analysed for mineral and *myo*-inositol concentration. Approximatly 1g of liver was weighed and added to a sealed 15ml falcon tube with 4ml of 2% nitric acid and 1 g of acid cleaned glass beads. This tube was then vortexed for 30 seconds 3 times and then shaken for 30 minutes. This homogenised the liver sample into a solution. A 1 ml sample of this solution was then centrifuged at 13,000 x g for 10 minutes within an eppendorf and was analysed in line with the blood methodologies as described in Sections 2.4.3.2 and 2.4.3.4.

5.3.5 Statistical Analysis

Univariate general linear model (GLM) analysis of variance (ANOVA) tests within the statistical programme IBM SPSS Statistic 21 were used to analyse the data. All data was tested for normality using the Kolmogorov-Smirnov test. The homogenity of variance of data was also analysed within the model. Polynomial contrasts were used within the model to assess the negative control treatments for linear, quadratic and cubic phytase affects. Specific orthogonal contrasts were also applied to assess the difference between specific treatments: the PC vs NC, the PC vs NC+ 250 FTU kg^{-1}, NC vs NC+ 250 FTU kg^{-1} diets, PC vs ASI, NC vs ASI and finally SI vs ASI. Significance was assigned at $P < 0.05$.

Table 5.1 Ingredients and calculated nutrient composition of experimental diets

Ingredient (g kg^{-1})	Positive Control	Negative Control
Barley	75.0	75.0
Wheat	477.9	492.4
Hipro Soya Ext	119.6	117.4
Rapeseed Ext	175.0	175.0
ABN Pullet Grower Plain	2.50	2.50
L-Lysine HCl	0.79	0.83
DL-Methionine	1.17	1.16
Novafill 20	0.125	0.125
ABN 50/20 Citran Pigment	1.50	1.50
Econase XT	0.15	0.15
Limestone Coarse	89.2	88.1
Monocalphos 22.7	8.75	2.97
Salt PDV [25kg]	2.87	2.85
Sodium Bicarb	1.64	0.56
Soya Oil GM	43.70	39.50
Nutrient Composition (g kg^{-1})		
Dry Matter	884.1	882.7
Crude Oil	61.0	56.9
Crude Protein	173.0	173.5
Crude Fiber	41.7	41.9
Crude Ash	129.1	122.7
Crude Starch	330.8	339.1
Crude Sugar	40.4	40.5
Calcium (Total)	40.0	38.6
Calcium (Digestible)	40.0	38.6
Phosphorus (Total)	5.9	4.6
Phosphorus (Available)	3.1	1.8
Sodium (Total)	1.75	1.45
AMEn (MJ kg^{-1})	11.4	11.4

Quantum Blue Phytase was added on top of the negative control to give the diets: NC + 250FTU kg^{-1}, NC + 1000 FTU kg^{-1}, NC + 4000 FTU kg^{-1} and NC + 16,000 FTU kg^{-1}.

5.4 Results

5.4.1 Diet Analysis

The diet analysis in Table 5.2 shows that the diets conformed to the formulation in Table 5.1. The crude oil was slightly high but equal across treatments with a slight drop in the 16000FTU kg^{-1} diet. Crude protein, fibre and phytate phosphorus were all on target and consistent across the treatments. Ash and calcium were higher than the formulation required, but they were lower in the NC diet as designed. The available phosphorus was at the required level within all the diets with half the concentration in the NC diets compared to the PC. The sodium levels also fall from the PC to NC diets, however, the difference is not quite as large as the formulation required.

Table 5.2. Analysed nutrient composition of diets

Nutrient Composition (g kg^{-1})	PC	NC	NC +250	NC +1000	NC +4000	NC +16000
Dry Matter	896	899	907	900	903	895
Ether Extract	65.1	65.6	64.7	63.8	65.3	61.3
Crude Protein	172	174	173	174	174	172
Crude Fibre	41	46	46	44	48	42
Ash	154	145	148	148	145	146
Calcium	56.8	43.6	40.6	42.0	42.6	41.8
Phosphorus	5.9	4.3	4.4	4.5	4.4	4.2
Av. Phosphorus	3.1	1.6	1.7	2.0	1.7	1.5
Phytate-P	2.8	2.7	2.7	2.5	2.7	2.7
Sodium	1.9	1.6	1.7	1.7	1.7	1.7

5.4.2 The Effect of Increasing Phytase Inclusion on Laying Hen Performance

As shown in Table 5.3. The treatments had no significant effect on the feed intake of laying hens. The NC+1000 and NC+16000 diets have the numerically highest feed intakes, however, this is because both treatments included one cage of chickens that appeared to consume over 130 g a day per bird. This is a lot higher than the expected feed intake and was presumably due to feed wastage. There was a trend (P=0.089) for a linear increase in egg production with an increase in phytase inclusion, and a

significant difference between the egg production of the NC birds and those of the above standard inclusion diets.

The addition of phytase gave a significant rise in the weight of eggs with 16,000FTU kg^{-1} giving the heaviest eggs. The ASI diets gave eggs that were heavier than those from the NC and the SI diets. Egg mass increased in a linear manner with phytase inclusion from 58.1 to 60.75g d^{-1}. The egg mass of the ASI diets was significantly higher than the NC, PC and SI diets. Phytase had no effect on bird FCR, but there was a trend (P=0.072) for a quadratic increase in the adjusted FCR with a trend for a difference between the NC and ASI diets (2.01 and 1.94 respectively). There was also a trend for a quadratic increase in weight gain with a significant difference between birds on the NC and ASI diets.

No significant differences were seen between the PC, NC and the 250 FTU kg^{-1} diets.

Table 5.3 The effect of increasing phytase inclusion on laying hen performance

Dietary treatment	Feed Intake	Production	Egg Weight	Egg Mass	Adj Egg Mass	FCR	Agj FCR	Weight Gain
	(g)	(%)	(g)	(g/d)	(g/d)			(g)
PC	116	92.5	63.15	58.52	58.89	2.01	1.98	158.7
NC	117	93.1	62.26	58.1	58.30	2.03	2.01	134.3
NC+250	114	93.6	62.32	58.62	59.57	1.94	1.92	169.1
NC+1,000	121	95.6	63.09	60.12	61.61	1.99	1.97	193.3
NC+4,000	116	96.0	63.63	60.49	61.07	1.92	1.90	203.6
NC+16,000	119	95.4	63.92	60.75	60.44	1.98	1.97	191.9
SEM	2.550	1.420	0.450	1.010	0.520	0.042	0.016	20.37
Contrast P Values								
Linear	NS	0.089	<0.05	<0.001	<0.001	NS	NS	NS
Quadratic	NS	NS	0.084	NS	NS	NS	0.096	0.071
Cubic	NS	NS	NS	NS	NS	NS	NS	NS
PC vs NC	NS	NS	NS	NS	NS	NS	NS	NS
PC vs 250	NS	NS	NS	NS	NS	NS	NS	NS
NC vs 250	NS	NS	NS	NS	NS	NS	NS	NS
PC vs ASI	NS	NS	NS	<0.05	<0.01	NS	NS	NS
NC vs ASI	NS	<0.05	<0.05	<0.05	<0.01	NS	0.072	<0.05
SI vs ASI	NS	NS	<0.05	<0.05	<0.005	NS	NS	NS

PC = Positive Control; NC= Negative Control, SI= Standard Inclusions (NC+250 and NC+1,000); ASI=Above Standard Inclusions (NC+4,000 and NC+16,000)

5.4.3 The Effect of Increasing Phytase Inclusion on Laying Hen Egg Quality

The addition of phytase had no significant effect on egg shell quality; no significant differences were found in any of the contrasts of shell strength, weight or thickness, as shown in Table 5.4.

Increasing phytase did have an effect on the blood and meat spot score of eggs. There was a significant quadratic response to the addition of phytase with the score increasing from the NC to the 4000 FTU kg^{-1} diet before falling slightly in the 16000 FTU kg^{-1} diet. Both the NC and the 250 FTU kg^{-1} diets gave eggs that had fewer meat and blood spots than the PC diet. The ASI diets had significantly higher blood and meat spot scores than the NC and SI diets. The PC and ASI diets were not significantly different.

The statistical model indicates that the presences of phytase in laying hen diets decreased the Haugh unit score of the egg in a linear manner ($P<0.05$) The contrasts showed no significant differences, however, individual treatment comparisons using post-hoc bonferroni tests, show that the Haugh unit score of 86.3 from eggs laid by birds receiving the 16,000 FTU kg^{-1} diet was significantly lower than all other diets except the NC diet.

No significant effects were seen on the weight of egg yolks, but the inclusion of phytase did increase the colour of egg yolks in a linear manner ($P<0.001$). The colour of the eggs from the NC diet were lighter than those on the PC diet (12.3 vs 12.6, $P<0.05$). There was also a trend for the NC eggs to be lighter than the eggs from the 250FTU kg^{-1} diets. The ASI eggs had significantly deeper coloured egg yolks than the eggs from the NC and SI diets.

Table 5.4. Effect of increasing phytase inclusion on egg quality

Dietary treatment	Egg Weight (g)	HU	Yolk Colour	Yolk Weight (g)	BMS	Shell Thickness (µm)	Shell Weight (g)	Shell Strength (kgf)
PC	63.4	89.2	12.6	16.0	0.89	369.2	6.0	4.5
NC	62.6	86.3	12.3	16.0	0.35	377.7	6.1	4.8
NC+250	63.4	88.1	12.5	15.9	0.42	374.7	6.1	4.8
NC+1,000	64.1	89.3	12.2	16.1	0.53	379.5	6.3	4.9
NC+4,000	63.0	88.0	12.6	15.8	0.93	368.6	6.0	4.6
NC+16,000	62.9	83.6	12.8	16.4	0.55	363.2	6.0	4.9
SEM	0.362	0.760	0.049	0.115	0.058	0.003	0.049	0.064
Contrast P Values								
Linear	NS	<0.05	<0.001	NS	NS	NS	NS	NS
Quadratic	NS	NS	NS	NS	<0.001	NS	NS	NS
Cubic	NS	NS	NS	NS	NS	NS	NS	NS
PC vs NC	NS	NS	<0.05	NS	<0.01	NS	NS	NS
PC vs 250	NS	NS	NS	NS	<0.01	NS	NS	NS
NC vs 250	NS	NS	0.061	NS	NS	NS	NS	NS
PC vs ASI	NS	NS	NS	NS	NS	NS	NS	NS
NC vs ASI	NS	NS	<0.05	NS	<0.01	NS	NS	NS
SI vs ASI	NS	0.117	<0.01	NS	<0.05	NS	NS	NS

PC = Positive Control; NC= Negative Control, SI= Standard Inclusions (NC+250 and NC+1,000); ASI=Above Standard Inclusions (NC+4,000 and NC+16,000);BMS =Blood Spot Score; HU=Haugh Unit

5.4.4 The Effect of Increasing Phytase Inclusion on the Phytate-P Concentration in the Gastrointestinal Tract

The treatments had no significant effect on the concentration of total or phytate phosphorus in the gizzard of laying hens as shown in Table 5.5.

Table 5.5. The effect of increasing phytase inclusion on the concentration of total and phytate-P in the gastrointestinal tract (g kg^{-1})

Dietary treatment	Gizzard and Proventriculus		Ileum	
	Total-P	Phytate-P	Total-P	Phytate-P
PC	1.03	0.40	2.68	1.38
NC	0.99	0.37	2.56	1.44
NC+250	0.95	0.33	2.81	1.32
NC+1,000	1.02	0.39	2.57	1.26
NC+4,000	0.98	0.37	2.46	1.22
NC+16,000	0.96	0.33	2.50	1.14
SEM	0.017	0.012	0.042	0.023
Contrasts P Values				
Linear	NS	NS	NS	<0.001
Quadratic	NS	NS	NS	.084
Cubic	NS	NS	NS	NS
PC vs NC	NS	NS	NS	NS
PC vs 250	NS	NS	NS	NS
NC vs 250	NS	NS	0.087	NS
PC vs ASI	NS	NS	NS	<0.01
NC vs ASI	NS	NS	NS	<0.001
Standard vs ASI	NS	NS	<0.05	<0.05

PC = Positive Control; NC= Negative Control, SI= Standard Inclusions (NC+250 and NC+1,000); ASI=Above Standard Inclusions (NC+4,000 and NC+16,000)

The inclusion of phytase did however, affect the concentration of phosphorus in the ileum. There was a trend for 250FTU kg^{-1} to increase the total phosphorus concentration in the ileum in comparison to the NC diet. The concentration of phytate phosphorus in the ileum was lower when birds were fed the ASI diets in comparison to the NC diets ($P<0.001$). The inclusion of phytase significantly decreased the concentration of phytate-phosphorus in the ileum in a linear manner. Contrast analysis revealed that the concentration of phytate-phosphorus in the ileum digesta of the ASI fed

birds was significantly lower in comparison to that of birds fed the PC, NC and SI diets.

5.4.5 Effect of Increasing Phytase Inclusion on the Liver *Myo*-inositol and Mineral Concentration

There was a trend for phytase to increase liver weights in a linear and quadratic manner, with significantly heavier livers in the NC and ASI birds, in comparison with the PC birds. The concentration of *myo*-inositol in the liver of laying hens increased in a linear manner with phytase dose, as shown in Table 5.6. The *myo*-inositol liver concentration was higher in birds fed the ASI diets in comparison to the NC and SI diets($P<0.05$).

The dietary treatments also significantly affected the concentration of minerals in the liver. There was a quadratic decrease in sodium levels with phytase inclusion up to 4000 FTU kg^{-1} before an increase with 16000FTU kg^{-1}. The liver sodium concentration was also higher in birds fed to NC diet in comparison with those fed the PC diet ($P<0.05$). Liver magnesium concentrations decreased as phytase inclusion increased ($P<0.01$), with livers form birds fed the NC diet being significantly lower than those from birds fed the PC and 250 FTU kg^{-1} diets. In addition to this, birds fed the ASI diets had significantly lower liver magnesium concentrations in comparison to those on the PC and SI diets. Liver phosphorus decreased as phytase inclusion increased ($P<0.05$), with a trend for the birds on the ASI diets to be lower than those on the PC diets. There was a significant linear and quadratic decrease in liver potassium. The concentration of potassium remained quite consistent in livers throughout the treatments before dropping suddenly in those birds fed the 16,000 FTU kg^{-1} diet. This gave a difference between the ASI and PC liver potassium concentration ($P<0.05$). There was a trend for liver calcium concentration to increase and a significant increase in manganese concentration with increasing phytase inclusion ($P<0.05$). The ASI diets resulted in elevated liver manganese levels that were higher than in those from birds fed the NC diet ($P<0.05$). Finally, liver zinc concentration increased in a linear manner with phytase inclusion.

Table 5.6 The effect of increasing phytase inclusion on the concentration of *myo*-inositol (mg g^{-1}) and minerals (mg kg^{-1}) in the liver

Dietary treatment	Liver weight (g)	*Myo*-inositol	Na	Mg	P	K	Ca	Mn	Fe	Cu	Zn
PC	35.7	2.61	638	362	2403	3229	195	1.08	71.7	2.59	13.8
NC	46.85	1.45	790	318	2335	3092	196	1.03	71.4	2.58	13.2
NC+250	42.04	1.79	705	377	2211	3051	224	1.14	73.6	3.24	13.3
NC+1,000	39.34	1.84	712	320	2403	3166	191	1.09	64.6	2.66	15.9
NC+4,000	38.88	2.95	610	345	2409	3261	195	1.22	63.4	2.98	19.2
NC+16,000	51.98	4.1	823	290	2032	2666	237	1.12	65.3	2.65	20.9
SEM	1.63	0.34	22.33	7.147	41.28	52.077	7.14	0.02	1.36	0.1	1.115
Contrast P Values											
Linear	0.064	<0.05	NS	<0.01	<0.05	<0.001	0.1	NS	NS	NS	<0.05
Quadratic	0.069	NS	<0.01	NS	0.088	<0.05	NS	<0.05	0.06	NS	NS
Cubic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
PC vs NC	<0.05	NS	<0.05	<0.05	NS	NS	NS	NS	NS	NS	NS
PC vs 250	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.06	NS
NC vs 250	NS	NS	NS	<0.05	NS	NS	NS	NS	NS	0.06	NS
PC vs ASI	<0.05	NS	NS	<0.05	0.098	<0.05	NS	0.101	0.06	NS	<0.05
NC vs ASI	NS	<0.05	NS	NS	NS	NS	NS	<0.05	0.08	NS	<0.05
SI vs ASI	NS	<0.05	NS	<0.05	NS	NS	NS	NS	NS	NS	<0.05

PC = Positive Control; NC= Negative Control, SI= Standard Inclusions (NC+250 and NC+1,000); ASI=Above Standard Inclusions (NC+4,000 and NC+16,000)

5.4.6 The Effect of Increasing Phytase Inclusion on Blood Mineral Concentration

The dietary treatments had a significant effect on the concentration of minerals within blood plasma. Feeding birds diets with increasing phytase inclusion levels increased their blood plasma phosphorus ($P<0.05$) and manganese concentrations in a linear manner ($P<0.01$). Birds fed the ASI and PC diet had a blood phosphorus concentration that was higher than that of birds fed the NC diet ($P<0.05$). In addition to this, birds fed the NC diets also had a significantly reduced concentration of sodium ($P<0.05$), iron ($P<0.05$) and copper ($P<0.05$) in comparison with those fed the PC and ASI diets. The birds fed the ASI diets had a blood plasma manganese concentration that was significantly higher than that of birds fed the SI ($P<0.01$) and NC diets ($P<0.01$).

Table 5.7 The effect of increasing phytase inclusion on blood plasma mineral concentration (µg ml^{-1})

Dietary treatment	Na	Mg	P	K	Ca	Mn	Fe	Cu	Zn
PC	2591	26	69.8	193	209	0.04	1.05	0.23	2.18
NC	2010	21.5	44.3	230	162	0.03	0.56	0.17	1.67
NC+250	2433	23.9	50.5	248	183	0.03	0.9	0.22	1.82
NC+1,000	2397	25.4	59.3	180	199	0.03	0.86	0.21	2.04
NC+4,000	2467	25.8	62.3	241	192	0.04	1.17	0.21	2.19
NC+16,000	2525	27.5	71	219	197	0.04	1	0.23	2.2
SEM	80.1	1	3.3	15.7	7.7	0	0.07	0.01	0.09
Contrast P Values									
Linear	NS	NS	<0.05	NS	NS	<0.01	NS	NS	NS
Quadratic	NS	NS	NS	NS	NS	0.08	<0.05	NS	NS
Cubic	NS	NS	NS	NS	NS	NS	NS	NS	NS
PC vs NC	<0.05	NS	<0.05	NS	0.1	0.13	<0.05	<0.05	0.12
PC vs 250	NS	NS	0.08	NS	NS	NS	NS	NS	NS
NC vs 250	NS	NS	NS	NS	NS	NS	NS	0.1	NS
PC vs ASI	NS	NS	NS	NS	NS	NS	NS	NS	NS
NC vs ASI	<0.05	0.09	<0.05	NS	NS	<0.01	<0.05	<0.05	0.06
SI vs ASI	NS	NS	NS	NS	NS	<0.01	NS	NS	NS

PC =Positive Control; NC=Negative Control, SI=Standard Inclusions (NC+250 and NC+1,000); ASI=Above Standard Inclusions (NC+4,000 and NC+16,000)

5.5 Discussion

5.5.1 Comparison to Breeders Expected Performance

The birds used in this trial met the breeder expectations with slightly higher production numbers (94% vs 92%) and slightly reduced egg weights (63 vs 64 g respectively) giving an egg mass on target at 59g d^{-1}(Joice and Hill, 2014). The daily feed intake was at 117 g rather than 115 g which also increased the FCR marginally but this was probably due to the feed wastage discussed in Section 5.4.1. The bird weight at the start of the trial was on target, however, during the trial the birds gained more weight than required, making birds 100g over their expected weight by the end of the experimental period.

5.5.2 The Effect of Increasing Phytase Inclusion on Laying Hen Performance

The increase in egg weight seen in this diet indicates that laying hen performance can be improved by inclusions of phytase greater than standard. This effect together with the trend for an increase in egg production resulted in a higher daily egg mass. This further supports the claim that using high phytase doses can benefit laying hen performance, as the egg mass production by these birds was significantly higher than birds fed the PC ($P<0.01$), NC ($P<0.01$) and standard inclusion diets ($P<0.005$) without significantly increasing feed intake.

Previous trials in broilers and pigs have also seen improvements in performance with above standard inclusion levels of phytase in comparison with standard levels (Cowieson et al., 2011). However, the studies using laying hens have failed to find significant benefits when including higher phytase doses relative to standard inclusions and positive control diets (Augspurger et al., 2003; Meyer and Parsons, 2011).

In the previous experiment described in Chapter 4, above standard inclusion of phytase improved FCR relative to a positive control diet, but did not significantly improve egg mass or decrease feed intake. In this trial, an increase in egg mass did not translate into an improved FCR.

As phytase breaks down the phytic acid, preventing its anti-nutritional effects, the AMEn (Miles and Nelson, 1974) and digestibility of protein (Liao et al., 2005) in the diet can be increased.

The improvement in FCR of the birds in Chapter 4 is through a small, insignificant decrease in feed intake and increase in egg numbers.Both egg numbers and feed intake are affected by adjusting energy levels, whereas egg weight is considered to be controlled by protein (Leeson, 2008). Therefore it is likely that the birds in Chapter 4 benefited from phytase increasing the AMEn of their diets.

In this trial, the egg numbers increased slightly indicating a possibly improved AMEn, but the increase in egg mass was due more to the production of heavier eggs. The weight of eggs is controlled more by protein intake rather than energy (Keshavarz and Nakajima, 1995). No effect on feed intake was visible in this trial either, which indicates the birds in this trial may have benefited more from improvements in protein digestibility rather than AMEn of the diet from phytic acid hydrolysis.

This difference between the trials may be due to difference in the birds age. It may be that younger birds adapt to higher AMEn diets more easily as they are still growing and their feed intake is increasing, albeit only slightly.

The lack of effect on egg weight in the Chapter 4, in comparison to this trial, may also be explained by age differences. The weight of eggs is influenced by the concentration of protein and amino acids in the diet with an increase in dietary protein causing an increase in egg size. However, this effect is influenced by age, Waldroup et al. (1995) showed that increasing methionine from 0.23% to 0.38% increased egg weight and that this effect was greatest in older birds. Any benefit of greater protein and amino acid availability from the addition of phytase is therefore likely to have a greater effect on egg weight in older birds. Age could also affect the utilisation of *myo*-inositol released from phytic acid. As previously mentioned, younger animals appear to have a greater need for *myo*-inositol than their mature equivalents.

5.5.3 The Effect of Increasing Phytase Inclusion on Egg Quality

In this trial phytase had no significant effect on egg shell quality. This is in agreement with the Chapter 4 trial, but disagrees with the results from Chapter 3. The reason for the absence of a phytase effect in this trial may be because of the unexpectedly high level of calcium found in the diet as seen

in Table 5.2. This provided the bird with ample calcium for egg shell formation and so it is unlikely that improving calcium solubility in the digesta, as shown in the first trial, would benefit shell formation. It is notable that in Chapter 3 an increase in egg shell strength was seen in combination with an increase in blood calcium concentration whereas in this experiment neither blood calcium or egg shell quality were increased by phytase supplementation.

Within this trial the addition of phytase had an effect on the average blood and meat spot score of eggs. These blood spots are caused by haemorrhage of blood that is supplying the follicle during its formation. Meat spots are blood spots that have changed colour within the egg whites due to a change in pH (Nalbandov and Card, 1944) . The incidence of blood spots is thought to be related to bird stress, but was found to have a genetic element (Jeffrey and Pino, 1943). When birds had seasonal laying cycles, eggs earlier in the season had a greater number of spots (Lerner and Taylor, 1947). Nutrition has been shown to affect incidences of blood spots with the inclusion of alfalfa in the ration decreasing their incidence rate (Sauter et al., 1965). An increase in vitamin A has also been shown to reduce blood spot occurrences (Bearse et al., 1960) as has supplementation with vitamin K (Day and Woody, 1964). Sumiati and Piliang (2005) showed that supplementing laying hen diets with 400 FTU kg^{-1} increased the concentration of vitamin A in eggs by 24%. It is possible therefore that phytase may have altered the balance of vitamin availability which effected the incidences of blood spots.

Williams (1992) reviewed the factors that affect egg Haugh unit score and concluded that nutrition was not of great importance in comparison to storage and genetics. However, in this trial, phytase significantly affected albumen height. Although the analysis suggested this was a linear relationship, numerically it appears to be quadratic, increasing with the standard doses before falling in the above standard inclusions. This is probably due to the increased absorption of minerals as the formation of phytate complexes is reduced. Trace minerals were also shown to be important with selenium improving albumen height (Arnold et al., 1974). Since then, supplemental magnesium was shown to reduce the thinning of the albumen during time in storage (Monsey et al., 1977) while vanadium contamination of phosphorus was exposed to be a factor reducing albumen

height (Sell et al., 1982). Jenson and colleagues (1978) found that including brewers grains in laying hen diets increased the quality of the albumen, which they believed to be due to the presence of trace minerals. Subsequently, they found that iron, copper, zinc, manganese and selenium all improved albumen quality while vanadium, tin, nickel and molybdate had no effect. It seems likely that in this trial, the release of minerals initially benefited albumen quality restoring the Haugh units to the level of the PC diet, but as the dose rose trace elements such as vanadium become more available and had a negative effect. If the inclusion of high phytase is used commercially, the levels of some trace minerals may need to be reduced as the concentration at which they are detrimental to the bird and egg is lowered.

In this study, phytase inclusion also increased the yolk colour score indicating a deepening of colour. Kozlowski and Jeroch (2011) showed that in calcium and phosphorus deficient diets the colour score was reduced, the addition of phytase, however, reversed this effect. Similarly, Englmaierová et al. (2012) found phytase deepened the colour of egg yolks when supplemented to a diet deficient in phosphorus, but not a diet that meets the birds requirements. This experiment also showed a lightening of yolk colour in the deficient diets. It may be the phosphorus is needed for the absorption and metabolism of carotenoids which go on to be transported to the developing yolk and intensify the colour. Pirgozliev et al. (2010b) showed that 3000 FTU kg^{-1} phytase increased the bioavailability of carotenoids which increased their concentration in the liver of broilers.

5.5.4 The Effect of Increasing Phytase Inclusion on Phytate phosphorus hydrolysis

As described in Section 4.5.4 the concentration of phytate phosphorus in the excreta or ileum is related to the amount of phytic acid hydrolysis within the tract. In the previous experiment it was seen that the inclusion of 2,500FTu kg^{-1} increased total tract hydrolysis of phytic acid in comparison to a positive control. In this trial the addition of phytase had no significant effect on the hydrolysis of phytic acid in digesta reaching the gizzard. Contrary to this, Walk et al. (2014) found that 1,500 FTU kg^{-1} significantly reduced the concentration of the higher ester of phytic acid and increased the concentration of *myo*-inositol, indicating greater phytic acid hydrolysis. There

were also good correlations between the ester concentrations in the gizzard and FCR.

It is likely that the phytase is hydrolysing phytic acid within the foregut, as it is the site of greatest phytase activity (Table 1.5). However, there was a great deal of variation between gizzard measurements from different birds which may have hidden these effects.

It was found however, that phytase did significantly affect the concentration of phytate-P in the ileum (P<0.001), indicating that higher dietary inclusion of phytase produced greater amounts of phytic acid hydrolysis within the gastrointestinal tract. Phytase continued to hydrolyse phytic acid with diminishing returns up to 16,000FTU kg^{-1} with the above standard inclusions giving significantly greater hydrolysis than the PC (P<0.01), NC (PC<0.001) or standard inclusion diets (P<0.05). This is in line with the work of Shirley and Edwards (2003) who showed hydrolysis of phytic acid continued to increase up to the higher dose of 12,000 FTU kg^{-1} with diminishing returns in broiler diets. It is clear that the continued breakdown of phytic acid seen here is responsible for the improvement seen in laying hen performance, whether through the reduction in the anti-nutritional effects of phytic acid or the release of *myo*-inositol.

5.5.5 The Effect of Increasing Phytase Inclusion on Liver *Myo*-inositol and Mineral Concentration

This experiment found that greater hydrolysis of phytic acid in ileal digesta through the inclusion of increasing dietary phytase doses resulted in a higher concentration of *myo*-inositol in the liver of laying hens. Cowieson and colleagues (2014) found similar effects in broilers, where increasing phytase from 0 or 1,000FTU kg^{-1} to 3000FTU kg^{-1} increased the concentration of *myo*-inositol in blood plasma. *Myo*-inositol is known to affect the transport of fats (Katayama, 1997) from the liver (Anderson, 1914) and intestines (Chu and Geyer, 1983). *Myo*-inositol has been shown to mimic insulin by stimulating the translocation of GLUT4 to the plasma membrane (Dang et al., 2010, Yamashita et al., 2013). Although chickens do not possess GLUT4 (Tokushima et al., 2005), chickens have been shown to be sensitive to insulin (Sweazea and Braun, 2006). Cowieson et al. (2014) suggested that through the mimicry of insulin, *myo*-inositol benefits birds by affecting glucose transport, gluconeogenesis and protein deposition. *Myo*-inositol can

also control cell osmolality and is known to act as an osmoprotectant in plants (Klages et al., 1999). Osmoprotectants are important for maintaining cell water volumes and so the conformation of proteins (Biggers et al., 1993). Intestinal cells have to survive with various osmotic environments as the blood plasma is hypo-osmotic in comparison to the intestinal lumen (Mongin, 1976). Osmoprotectants have been shown to benefit intestinal health and digestion of nutrients (Remus et al., 1995; Eklund et al., 2006a). *Myo*-inositol derived compounds also play pivotal roles in cell metabolism. For example, glycosylphosphatidylinositol anchors a large variety of proteins to the extracellular side of the cell membrane (Brown and Waneck, 1992). The supplementation of diets with *myo*-inositol has been shown to benefit growth in fish (Waagbø et al., 1998), rats (Katayama, 1997) and chicken (Hegsted et al., 1941a). Two studies in broilers have shown *myo*-inositol can benefit growth and efficiency (Cowieson et al., 2013, Żyła et al., 2013), whereas one study in laying hens found *myo*-inositol had small non-significant negative effects on performance of birds fed a slightly deficient diet (Żyła et al., 2012). It is possible that supplementation of *myo*-inositol can only benefit animals when they are supplied with adequate phosphorus because it is required to synthesise a number of the *myo*-inositol derivatives utilised by the animal, for example IP_3. Using higher phytase inclusions to provide the bird with *myo*-inositol from phytic acid would overcome this as the phosphorus released from phytic acid can be utilised to regenerate *myo*-inositol based compounds within the bird once absorbed.

Despite a large number of significant results, the effect of phytase on the concentration of minerals in the liver was quite inconsistent. The clearest effect seen was on the concentration of zinc in the liver, through a distinct rise with phytase inclusion. The addition of 1000FTU kg^{-1} to a weaner diet was also shown to increase liver zinc levels, but had no effect on the concentration of calcium, phosphorus, magnesium, sulphur, copper, iron and selenium (Jolliff and Mahan, 2012). The same study also found, there was a near significant trend for an increase in liver manganese concentration. Martinez et al. (2004) showed that 500 FTU kg^{-1} increased phosphorus levels in liver and blood plasma but had no effect on the levels of copper. Other studies have also found that phytase increased the bioavailability of trace minerals like zinc, iron, manganese and magnesium (Czech and Grela, 2008). Zinc is an essential trace mineral that is needed for normal cell growth, development and differentiation. Zinc is integral for DNA synthesis, RNA transcription in addition being an important co-factor or component of many enzymes. Zinc has also been shown to be important in preventing liver

cirrhosis (Vallee et al., 1956). Given the importance of zinc as a nutrient, it is possible that the increased availability of zinc in the diet may have benefited the birds on this trial, increasing egg weight and egg mass.

5.5.6 The Effect of Increasing Phytase Inclusion on Blood Mineral Concentration

The blood plasma concentration of minerals was also affected by the phytase treatment, with a trend for an increase in the levels of manganese and phosphorus. The increase in blood phosphorus indicates that the higher levels of phytase improved the availability of phosphorus which was utilised by the bird. This suggests that some of the benefit of increasing phytase inclusion may be from greater phosphorus absorption and therefore contradicts the experiment described in Chapter 3 where an increase in phosphorus solubility did not translate into higher levels of circulating phosphorus. In addition to this, dos Santos et al. (2013) found that increasing phytase within a broiler diet to 1,500 FTU kg^{-1} improved FCR but not toe ash, signifying the benefits were not phosphoric in nature. Walk et al. (2014) supported this, showing that supplementing a slightly deficient broiler diet with 1,500 FTU kg^{-1} improved FCR in comparison to a positive control diet with additional dicalcium phosphate. This shows that the extra calcium and phosphorus does not deliver the same benefit of higher phytase inclusion. The increase in blood phosphorus seen here may be a result of the high dietary calcium content in all diets. The requirements for and digestion of calcium and phosphorus are inexorably interlinked. High levels of dietary calcium have been shown to reduce the retention of phosphorus with numerical decreases in circulating phosphorus (Keshavarz, 1986). In addition to this, increasing the ratio of calcium to phosphours has also been shown to reduce the retention of phosphorus in laying hens (Pastore et al., 2012). Tamin et al. (2004) showed that increasing dietary calcium not only reduced the utilisation of phytate phosphorus but also decreased the digestion of non-phytate phosphorus. This was a result of the formation of calcium phosphates within the lumen. While more soluble than phytate, calcium phosphates are not very soluble above pH 5 (Holt et al., 1925; Selle et al., 2009b). It is therefore possible that in this trial the high calcium concentration restricted phosphorus availability on the NC and SI diets. As a result, the birds increased their phosphorus absorption as phytase continued to release phytate-phosphorus.

5.5.7 Conclusions

The similarity in performance between the PC, NC and NC+250 FTU kg^{-1} diets indicate that the standard equivalence values used in the formulation of diets seem to be accurate.

The supplementation of laying hen diets with phytase doses greater that the standard inclusion levels have been shown to increase the egg weight and egg mass without increasing feed intake. It is possible that this is due to increased phosphorus or zinc availability, however, it is more likely that this is due to a reduction in the anti-nutritional effects of phytic acid and the release of *myo*-inositol.

Greater dietary phytase inclusion increased the hydrolysis of phytic acid in ileal digesta and increased the bioavailability of *myo*-inositol with increased liver concentrations.

Chapter 6
The Effect of Including Betaine and Phytase in Laying Hens Diets

6.1 Abstract

This experiment was conducted to assess the effect of supplementing a laying hen diet with a phytase dose above the standard level, and betaine alone and in combination with phytase. The trial had a 2×2 factorial design with and without phytase and betaine inclusion at 2,500 FTU kg^{-1} and 2 g kg^{-1} respectively on top of a basal diet that met the bird's requirements. Twenty one week old laying hens were group housed and fed their respective diets for 12 weeks during which their performance was recorded. In addition, egg quality measurements were analysed every three weeks. At the end of the trial one week's eggs were collected and analysed for egg quality over the next 4 weeks to measure how the egg quality changed over time when birds had been fed the different diets. Finally, the birds were killed, and a peripheral blood sample was taken and analysed for mineral content, plasma urea nitrogen (PUN) and *myo*-inositol concentrations. When supplemented alone both betaine and phytase improved FCR; from 2.11 to 1.99 and 2.03 respectively. However, when fed in combination the FCR of laying hens was not significantly different from that of the control. Phytase significantly increased the circulating concentration of *myo*-inositol, however, betaine reversed this effect. Feeding phytase was shown to increase egg shell quality while dietary betaine supplementation reduced the loss of water from eggs through evaporation. Both betaine and phytase reduced the intensity of yolk colours when fed alone, however, when fed in combination the yolk colour was restored to the level of the control. Both betaine and phytase supplementation decreased the concentration of PUN. Finally, birds which had been fed phytase had decreased circulating concentrations of magnesium, copper and zinc.

6.2 Introduction

The use of betaine as a supplement in animal diets has increased in recent years (Ratriyanto et al., 2009). Betaine is a stable, nontoxic, naturally

occurring derivative of the amino acid glycine (Yu et al., 2004). Sugar beets and molasses are common sources of betaine but purified forms are also available; these include anhydrous betaine, betaine monohydrate and betaine hydrochloride (Kidd et al., 1997, Eklund et al., 2005). Betaine has been shown to improve animal performance and nutrient digestibility, but these effects are inconsistent (Overland et al., 1999; Attia et al., 2005; Eklund et al., 2006a; Eklund et al., 2006b).

Betaine inclusion at 1g kg^{-1} has been shown to improve growth rate by 13% and FCR by 8% in grower finisher pigs (Zou et al., 2002). Betaine supplementation at 1.5 g kg^{-1} increased weight gain and decreased FCR in broilers (Virtanen and Rosi, 1995) and similar effects have been seen in ducks(Wang et al., 2004) and turkeys (Noll et al., 2002). In laying hens, betaine has been shown to benefit performance (Zou and Lou, 2002, Park et al., 2006). Zou et al. (2002) showed that the addition of 0.6g kg^{-1} betaine significantly improved egg production by 8.7% and FCR by 9.0%, but found no difference in egg weight. Alternatively, Park et al. (2006) found that 1.2g kg^{-1} betaine supplementation increased egg weight but not production. In contrast to these studies, Harms and Russell (2002) found that supplemental betaine gave no advantage in a choline adequate diet. Betaine is also known as a carcass modifier as it can increase the percentage of lean meat in animal products (Wang and Xu et al., 1999). For example, Zhan and colleagues (2006) found that feeding betaine in the diet increased the breast meat yield in broilers, while Cadogan et al. (1993) showed that betaine supplementation could reduce backfat thickness in gilts.

In addition to these benefits, betaine supplementation is known to be particularly advantageous when animals are under some form of stress. Ryu and colleagues (2003) showed that betaine improved egg production and shell quality of laying hens when experiencing heat stress. Betaine has also been shown to benefit animals undergoing microbial immune challenges (Augustine et al., 1997). Finally, during water salinity stress betaine supplementation improved the growth and FCR of broilers (Honarbakhsh et al., 2007).

Betaine has a number of metabolic roles through which it can benefit animal nutrition and performance; for example, as a trimethylated glycine compound betaine can act as a methyl donor (Kidd et al., 1997). Methionine, choline and betaine are the most important methyl carriers in livestock, and so increasing dietary betaine may allow a reduction in an animal's requirement of methionine and choline (Siljander-Rasi et al., 2003). Methylation is vital for

normal cell functions including protein and energy metabolism, and through these actions betaine can benefit lean growth. As a methyl donor, betaine allows increased utilisation of amino acids for protein synthesis; as a result fewer amino acids go for deamination and the synthesis of adipose tissue (Wallis, 1999). Betaine has also been shown to decrease the activity of lipogenic enzymes (Huang et al., 2006) and increase lipase activity (Zhan et al., 2006, Zou et al., 1998). Methyl groups can also be donated to form lecithin which is involved with the transport of fats (Saunderson and Mackinlay, 1990). Betaine sparing choline will increase choline availability for other metabolic functions (Dilger et al., 2007), for example the formation of very low density lipoproteins (VLDL) and phosphatidylcholine. Zou et al. (1998) showed an increase in the concentration of VLDL when betaine was supplemented in a layer diet. VLDL prevent the deposition of fats in the liver and increase the rate of their removal (Yao and Vance, 1989). Phosphatidylcholine is required for the formation of cell membranes and so it is essential for cell secretions and therefore normal cellular function. Choline sparing also allows greater formation of acetylcholine, which is a neurotransmitter involved in the nervous system (Dilger et al., 2007). Betaine has also been shown to affect fat metabolism through increasing the concentration of methylated compounds , such as carnitine and creatine, within the liver (Xu and Zhan, 1998, Zhan et al., 2006). Carnitine is involved with the transport of long chain fatty acids for oxidation.

Betaine also has an important role as an osmoprotectant in bacteria (Pichereau et al., 1999), plants (Xing and Rajashekar, 2001), fish (Clarke et al., 1994) , mammals (Law and Burg, 1991), and birds (Lien et al., 1993). Osmoregulation is defined as the cell's capacity to maintain its structure and metabolic functions though controlling the flow of water in and out of itself (Kidd et al., 1997). Changes in cell water volumes will adjust the intracellular ionic environment which can lead to changes in protein conformation (Biggers et al., 1993). Changes in water volume can therefore alter cell function and lead cells into more anabolic or catabolic states (Häussinger, 1998). Water homeostasis is vital for cells that experience differing osmotic conditions (Klasing et al., 2002), such as those in the kidney (Bagnasco et al., 1987), liver (Weik et al., 1998) and small intestines (Mongin, 1976) Osmoprotectants are a class of organic molecules that are highly soluble (Chambers and Kunin, 1985). These can reach high concentrations within cells or organelles that are exposed to an osmotic or ionic stress and thereby prevent the accumulations of inorganic ions (Petronini et al., 1992). High concentrations of ions will be detrimental to enzyme activities and cell

membranes, whereas osmoprotectants can become concentrated without disturbing cellular functions. They can also help cells maximise water retention against osmotic gradients in times of cellular dehydration (Klasing et al., 2002).

Cells involved in nutrient digestion and absorption require osmolytic protection mechanisms as they transport water, ions and nutrients from the lumen of the small intestines to the blood plasma. The luminal digesta of the intestines is hyperosmotic in comparison to blood plasma (Mongin, 1976). It is through the protection of the intestinal cells that betaine can benefit nutrient digestibility (Eklund et al., 2006). Increases in dietary betaine have been shown to increase the accumulation of betaine in the small intestines and in particular the duodenum (Klasing et al., 2002). Betaine supplementation has been shown to improve the integrity of the gastrointestinal tract (Kettunen et al., 2001).

In the chicken, betaine, *myo*-inositol and taurine are the most important osmolytes used for osmotic regulation (Lien et al., 1993). This is of interest as the previous chapter indicated that phytase supplementation increased the bioavailability of *myo*-inostiol when phytase was included at high doses. Given the trend for greater use of both betaine and high phytase doses in the poultry industry, it is of interest to see how the two supplements interact with each other. A number of other parallels exist between betaine and *myo*-inositol regarding their effects on metabolism. Phytase has been shown to affect the transport of fat soluble compounds (Pirgozliev et al., 2010b) . Similarly, betaine has been shown to affect the transport of fats (Ratriyanto et al., 2009). Betaine is the oxidised form of choline, therefore it is of interest that both *myo*-inostiol and choline have been shown to reduce fatty liver disease (Wolford and Murphy, 1972). The cellular uptake of both osmolytes has also been shown to overlap somewhat. Skate erythrocytes increased the uptake of both betaine and *myo*-inositol when subjected to hypertonic stress (Goldstein and Davis, 1994). This study continued to look at the osmolyte transporters and found that they were both activated by the water volume of the cell and were both suppressed by the same inhibitors indicating overlap in their control. Similarly, when canine MDCK kidney cells were exposed *in vitro* to a hypertonic medium and found that sodium coupled transporters increased the uptake of both betaine and *myo*-inositol fivefold (Handler and Kwon, 1996). The authors found that although they utilised different transporters the control of omsolyte uptake was similar. The hypotonic medium stimulated the transcription of the transporter genes,

while post-translational regulation of the transporters was inhibited by phosphorylation in both cases.

To date, only one other published study has looked at the effects of adding betaine and phytase to an animal's diet in combination. Debicki-Garnier and Hruby (2003) supplemented diet with and without betaine in addition to 0, 500 or 1000 FTU kg^{-1} phytase. They found that both betaine and phytase improved the feed intake, end body weight and body weight gain of broilers, but there was no significant effect on the FCR. There were no significant interactions with the exception of one: the ash excreted was lower when broilers were fed betaine and phytase together in comparison with phytase alone. This suggests that betaine increased the retention of phosphorus and calcium following phytic acid hydrolysis, presumably through an improved gastrointestinal tract integrity and greater nutrient absorption.

6.2.1 Objectives

The objective of this study was to investigate the effects of supplementing a laying hen diet with a higher than standard phytase dose and betaine, paying particular attention to the performance of laying hens in addition to egg quality. Finally, the concentration of *myo*-inositol and minerals in the blood was analysed to assess the effect of phytase and betaine on *myo*-inositol and mineral bioavailability.

6.2.2 Hypotheses

Betaine and phytase supplementation will improve laying hen performance

Phytase inclusion will increase the concentration of circulating *myo*-inositol

An interaction between betaine and phytase supplementation will be seen in laying hen performance due to the overlap in betaine and *myo*-inositol function.

6.3 Materials and Methods

An experiment was conducted to assess the effect of including betaine and phytase on laying hen performance, egg quality and the concentration of *myo*-inositol and minerals in the blood

6.3.1 Experimental Design and Treatments

The experiment was a 2×2 factorial design, with or without the inclusion of betaine at 2g kg^{-1} and the addition of 0 or 2,500 FTU kg^{-1} Quantum Blue™ phytase on top of a nutritionally adequate base diet giving four dietary treatments described in Table 6.1.

6.3.2 Animal Housing

At 21 weeks of age 240 Bovan Brown laying hens were allocated to 60 cages balancing for bird weight within each replicate. Each cage within a replicate was then randomly allocated a treatment diet.

6.3.3 Feeding Regime and Performance Recording

The birds were fed their experimental diets for 12 weeks during which their performance criteria was recorded. Every three weeks one day's eggs were collected and analysed for the egg quality as described in Section 2.3.5.

6.3.4 Egg Keeping Quality

During the last week of the experiment all eggs were collected every day from Monday to Friday, weighed and labelled. One fifth of the eggs were analysed as above on the day of collection and the remainder stored in cardboard trays at room temperature (21ºC) for 7, 14, 21 or 28 days. The relative humidity ranged from 65 to 76%. On each consecutive collection day, a different fifth of the eggs were analysed so that by the end of the week all replicates had had a collection day analysis of their eggs. Likewise in the subsequent weeks, on each particular day within the week a different fifth of the eggs were analysed, so that by the end of the experiment eggs from each replicate of each treatment had been analysed on each sampling day. The egg analysis was similar to that carried out throughout the experiment detailed in Section 2.3.5, however, the Haugh unit micrometer broke on some days and so there are some gaps within this data set.

Table 6.1. Ingredients and calculated nutrient composition of experimental diets

Ingredient (g kg^{-1})	Control	Betaine	Phytase	Betaine & Phytase
Wheat	606.3	604.3	605.8	603.8
Rapeseed Solv Ext	30	30	30	30
Soybean meal 48	205.1	205.1	205.1	205.1
Wheat Bran	4.9	4.9	4.9	4.9
Soya oil	40	40	40	40
Salt	2.9	0.29	0.29	0.29
DL Methionine	1.2	1.2	1.2	1.2
Limestone	82.7	82.7	82.7	82.7
Dicalcium Phos	16.1	16.1	16.1	16.1
Choline chloride	5.8	5.8	5.8	5.8
Vitamin premix	4.9	4.9	4.9	4.9
Phytase	0	0	0.5	0.5
Betaine	0	2	0	2
Nutrient Composition (g kg^{-1})				
Crude Oil	52.6	52.6	52.6	52.6
Crude Protein	175.0	175.0	175.0	175.0
Crude Fiber	25.7	25.7	25.7	25.7
Calcium (Total)	37.5	37.5	37.5	37.5
Calcium (Digestible)	37.5	37.5	37.5	37.5
Phosphorus (Total)	07.1	07.1	07.1	07.1
Phosphorus (Available)	04.5	04.5	04.5	04.5
Sodium (Total)	01.5	01.5	01.5	01.5
AMENn (MJ kg^{-1})	2800	2800	2800	2800

6.3.5 Feather Score

Throughout the duration of the trial it was noticed that the birds within some cages were feather pecking more than others. To investigate this further, the feather scores of all the birds were recorded in the final week of the trial using the methods of Bilcik and Keeling (1999). Briefly, the body was split into different regions: head, neck, back, rump, belly, breast, legs, tail, wing primary feathers and wing covert feathers. Each section was inspected and given a score from 0 to 5: 0=Intact feathers, 1= up to three feathers missing, some scruffy, 2= greater than three feathers missing, 3= a bald patch smaller than 50% area, 4= bald patch greater than 50% area.

6.3.6 Dissections

After 12 weeks the birds were killed by cervical dislocation. A peripheral blood plasma sample was taken for analysis. This sample was analysed for plasma urea nitrogen, *myo*-inositol and mineral analysis.

6.3.7 Statistical Analysis

Two factor univariate general linear model (GLM) analysis of variance (ANOVA) tests within the statistical programme IBM SPSS Statistic 21 were used to analyse the data; when statistically significant, replicate was included in the model. All data was tested for normality using the Kolmogorov-Smirnov test. The homogenity of variance of data was also analysed within the model. For the egg keeping quality analysis the data were analysed as a 2 x 2 x 5 factorial with betaine inclusion, phytase inclusion and age of egg (0, 7, 14, 21 or 28 days of age) used in the model and individual egg as the unit of replication. When significant post-hoc bonferroni tests were performed for multiple comparisons. Significance was assigned at $P < 0.05$.

6.4 Results

6.4.1 Diet Analysis

The diet analysis is shown in Table 6.2. The protein, fibre, calcium and phosphorus content of the diets were in line with the calculated composition in Table 6.1. The only notable deviation was a slightly elevated level of fat.

Table 6.2. Analysed nutrient composition of the diets

Nutrient Composition ($g\ kg^{-1}$)	Control	Betaine	Phytase	Betaine & Phytase
Dry Matter	897.	900	900	904.
Ether Extract (OIL A)	61.4	60.1	61.4	58.0
Crude Protein	175	175	177	178
Crude Fibre	26	27	27	27
Ash	124	117	119	120
Calcium	38.0	37.4	35.8	36.8
Phosphorus	6.4	6.4	6.2	6.1
Available Phosphorus	4.5	4.6	4.4	4.4
Phytate-P	1.9	1.8	1.8	1.7
Sodium	1.5	1.5	1.2	1.3

6.4.2 The Effect of Betaine and Phytase on Laying Hen Performance

The effect of the dietary treatments on the performance of the birds over the whole trial are shown in Table 6.3. There was a trend for phytase addition to increase feed intake, in addition to a trend for betaine supplementation to decrease feed intake. There were no significant effects on egg production. There was no significant effect on egg mass, however, a trend for an interaction between dietary treatments was seen in adjusted egg mass.

The trends seen on egg mass and feed intake resulted in a trend for an interaction in FCR and a significant interaction in the adjusted FCR. Supplementation of both betaine and phytase alone gave significant improvements in FCR to 1.99 and 2.03 respectively in comparison to the FCR of birds on the control diet (2.11; Table 6.3). However, when supplemented together there was no significant benefit with an FCR of 2.07. The FCR of birds fed both supplements was not significantly different from either the control or the supplements fed alone; however, there was a trend for a difference between the addition of betaine without phytase and betaine with phytase. The addition of 2,500 FTU kg^{-1} to the laying hen diet significantly increased the weight gained by laying hens from 159 to 201 g while betaine had no effect.

Table 6.3. The effect of betaine and phytase on laying hen performance

Betaine (g kg^{-1})	Phytase (FTU kg^{-1})	FI (g)	Egg Production (%)	Egg Weight (g)	Egg Mass (g)	Adj Egg Mass (g)	FCR	Adj FCR	Weight Gained (g)
0	0	110	87.7	59.9	52.5	52.6	2.11	2.11^{a}	181
0	2500	113	92	60.4	55.3	55.6	2.04	2.03^{b}	192
2	0	108	90	60.5	54.1	54.4	2.01	1.99^{b}	136
2	2500	110	89	60.4	53.7	53.8	2.07	2.07^{a}	209
0		112	89.85	60.2	53.9	54.1	2.08	2.07	187
2		109	89.5	60.4	53.9	54.1	2.04	2.03	173
	0	109	88.9	60.2	53.3	53.5	2.062	2.05	159
	2500	112	90.5	60.4	54.5	54.7	2.055	2.05	201
SEM		1.0	1.7	*0.43*	*1.20*	1.05	*0.04*	0.03	*20*
P-Value									
Betaine		0.081	NS	NS	NS	NS	NS	NS	NS
Phytase		0.075	NS	NS	NS	NS	NS	NS	<0.05
Betaine × Phytase		NS	NS	NS	NS	0.089	0.067	<0.05	NS

[a-b] Means within columns with no common superscript differ significantly (P<0.05)

6.4.3 The Effect of Betaine and Phytase on Egg Quality

The effect of the dietary treatments on egg quality across the whole trial are presented in Table 6.4. The treatments did not affect the shell quality, BMS score or albumen quality. There was however, a significant interaction effect on the yolk colour. The addition of phytase and betaine alone reduced the yolk colour score from 12.27 to 11.68 and 11.81 respectively but had no effect when included within the diet together. There was also a trend for phytase to increase the yolk weight.

6.4.4 The Effect of Betaine and Phytase on Egg Quality during Storage

The effects of storing eggs for up to 4 weeks at room temperature on their percent weight loss are presented in Table 6.5. There were no differences in egg weight between any of the treatments at the start of this experiment. The loss of egg weight was slowed by the inclusion of betaine in the laying hen diet. By day 14 of storage, eggs from birds fed the betaiine supplemented diets had lost only 1.6% of their start weight in comparison to the 1.8% weight loss of eggs from birds fed the diets without betaine ($P<0.05$). This significant difference remained so for the remainder of their storage.

Table 6.6. shows the effect of the diets on egg quality over the 4 weeks of storage. The egg weight, Haugh unit and yolk colour all decreased over time while the yolk weight increased from 15.1 to 16.7 g. The storage time also significantly affected shell thickness, increasing between day 0 and day 7 from 345 µm to 354 µm followed by a decrease to 346 µm by day 28. Phytase significantly decreased the Haugh units of the egg albumen from 52.8 to 50.5. In addition this, phytase increased yolk weight (16.2 vs 16.39 g, $P=0.051$), shell weight (6.05 vs 6.14 g, $P<0.01$) and shell strength (4.02 vs 4.20 kgf, $P<0.05$). There was also a trend for an increase in shell thickness and egg weight.

Table 6.4 The effects of betaine and phytase on egg quality

Betaine (g kg^{-1})	Phytase (FTU kg^{-1})	Haugh Unit	Yolk colour	Yolk weight (g)	BMS score	Shell thickness (μm)	Shell weight (g)	Shell Strength (kgf)
0	0	85.2	12.27^{a}	14.32	1.44	0.36	5.91	4.18
0	2500	84.2	11.68^{b}	14.72	1.56	0.36	5.9	4.28
2	0	86.7	11.81^{b}	14.31	1.78	0.36	5.89	4.17
2	2500	85.1	12.18^{a}	14.38	1.98	0.36	5.9	4.16
0		84.7	11.975	14.52	1.5	0.36	5.905	4.23
2		85.9	11.995	14.345	1.88	0.36	5.895	4.165
	0	85.9	12.04	14.315	1.61	0.36	5.9	4.175
	2500	84.7	11.93	14.55	1.77	0.36	5.9	4.22
SEM		1.05	0.122	0.13	0.288	0.003	0.04	0.079
P-Value								
Betaine		NS	NS	NS	NS	NS	NS	NS
Phytase		NS	NS	0.078	NS	NS	NS	NS
Betaine × Phytase		NS	<0.001	NS	NS	NS	NS	NS

$^{a-b}$ Means within columns with no common superscript differ significantly ($P<0.05$)

Table 6.5 The effect of betaine and phytase on weight loss during storage

Betaine ($g\ kg^{-1}$)	Phytase ($FTU\ kg^{-1}$)	Start Egg weight (g)	Percent weight loss relative to start weight			
			Day 7	Day 14	Day 21	Day 28
0	0	62.16	0.825	1.820	3.057	3.967
0	2500	62.68	0.854	1.755	2.835	3.730
2	0	62.84	0.820	1.569	2.596	3.399
2	2500	63.06	0.795	1.634	2.661	3.503
0		62.42	0.839	1.787	2.946	3.848
2		62.95	0.808	1.602	2.629	3.451
	0	62.5	0.822	1.695	2.827	3.683
	2500	62.87	0.825	1.694	2.748	3.616
SEM		0.55	0.028	0.08	0.146	0.182
P-Value						
Betaine		NS	NS	<0.05	<0.05	<0.05
Phytase		NS	NS	NS	NS	NS
Betaine × Phytase		NS	NS	NS	NS	NS

Table 6.6. The effect of betaine, phytase and storage time on egg quality

Day	Betaine ($g\ kg^{-1}$)	Phytase ($FTU\ kg^{-1}$)	Egg weight (g)	Haugh units	Yolk Colour	Yolk Weight (g)	Shell thickness µm	Shell weight (g)	Shell Strength kgf
0			62.57	86.08	11.7	15.13	345	6.05	4.191
7			62.52	58.26	11.9	16.58	354	6.08	4.022
14			61.86	42.47	11.2	16.38	347	6.08	4.175
21			61.27	33.07	11	16.69	347	6.16	4.136
28			60.4	38.4	11.3	16.67	346	6.1	4.022
	0	0	61.14	51.85	11.7^{a}	16.22	347	6.02	3.986
	0	2500	61.72	50.26	11.1^{b}	16.39	349	6.13	4.244
	2	0	61.88	53.79	11.1^{b}	16.17	346	6.09	4.045
	2	2500	62.16	50.73	11.8^{a}	16.38	349	6.15	4.162
	0		61.43	51.06	11.4	16.31	348	6.075	4.115
	2		62.02	52.26	11.45	16.28	348	6.12	4.104
		0	61.51	52.82	11.4	16.2	347	6.055	4.016
		2500	61.94	50.5	11.45	16.39	349	6.14	4.203
SEM			0.264	0.989	0.136	0.097	1.6	0.03	0.084
P-Value									
Time			<0.001	<0.001	<0.001	<0.001	<0.005	NS	Ns
Betaine			<0.05	NS	NS	NS	NS	NS	NS
Phytase			0.105	<0.05	NS	0.051	0.061	<0.01	<0.05
Betaine × Phytase			NS	NS	<0.001	NS	NS	NS	NS

[a-b] Means within columns with no common superscript differ significantly ($P<0.05$)

6.4.5 The Effect of Betaine and Phytase on Laying Hen Feather Score

Examination of the feather cover showed that there was a significant interaction between the betaine and phytase inclusion in relation to back feather score ($P<0.05$, Shown in Fig 6.1.) There was a tendency for birds that received dietary betaine supplementation alone to have a poorer feather score on their backs in comparison to birds fed the control diet ($P<0.094$). Bird fed phytase and betaine in combination had a lower feather score indicating better feather cover in comparison to those fed betaine alone ($P<0.05$). Similar patterns to those shown in Fig 6.1 were also seen in the feather score of the neck and head of chickens with trends for dietary interactions. The diets had no significant effects on the feather score of the flight feathers, the belly or legs of hens.

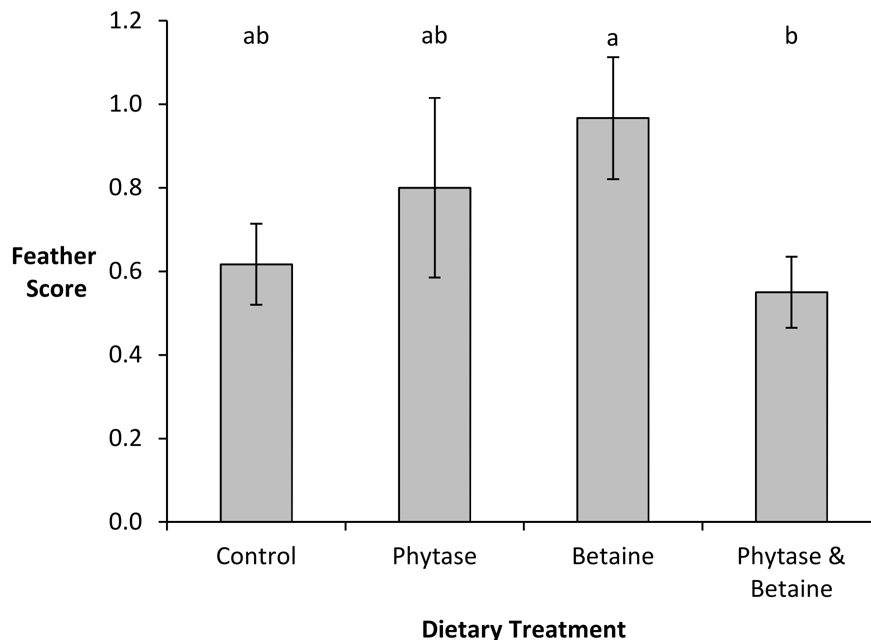

Figure 6.1 The effect of betaine and phytase on the mean feather score of the back of laying hens with standard error bars. [a-b] Means within columns with no common superscript differ significantly (P<0.05)

6.4.6 The Effects of Betaine and Phytase on Blood Plasma Parameters

The addition of phytase and betaine either alone or together had a significant effect on some of the blood measurements, as shown in Table 6.7. There was a significant interaction between the dietary treatments and the concentration of PUN in laying hens. The PUN was significantly decreased by phytase with a significant for an interaction. The addition of both phytase alone reduced PUN relative to the control from 4.81 to 3.28 mg dl^{-1}, whereas, betaine supplementation alone reduce PUN to 3.50 mg dl^{-1}. The addition of the supplements in combination also reduced PUN to 3.49 mg dl^{-1}; this effect however, was not additive and not significantly lower than the PUN level of the supplements fed alone.

The addition of phytase to the control diet significantly increased the concentration of *myo*-inositol within the blood of laying hens from 24.1 to 25 mg l^{-1}, while the addition of betaine reduced the concentration of *myo*-inositol from 25.0 to 24.0 mg l^{-1}. A significant interaction was also seen whereby phytase alone increased *myo*-inositol concentration relative to the control while betaine alone had no effect. The supplementation of the diet with phytase and betaine in combination reduced the *myo*-inositol concentration relative to the birds fed phytase alone.

The haematocrit of blood was not significantly affected by the supplements, but there was a trend for an interaction. The addition of both supplements gave a numerical increase in the haematocrit when each was fed alone but in combination they gave a small reduction in the haematocrit value of the blood.

The mineral analysis generally showed that numerically both betaine and phytase reduced the concentration of minerals. Betaine significantly reduced the concentration of phosphorus and magnesium in the blood while phytase reduced the concentration of magnesium and copper. There was a significant interaction regarding the concentration of iron. The addition of both supplements fed alone significantly increased the concentration of iron in the blood plasma, but when supplemented in combination the effect was not large numerically and insignificant.

Table 6.7 The effect of betaine and phytase on the blood plasma parameters

Betaine	Phytase	PUN	*Myo*-inositol	Hematocrit	K	Ca	P	Mg	Zn	Fe	Cu	Mn
($g\ kg^{-1}$)	($FTU\ kg^{-1}$)	($mg\ dl^{-1}$)	($mg\ l^{-1}$)		($\mu g\ ml^{-1}$)							
0	0	4.81^{a}	24.0^{b}	21.7	270	193	70.8	30.8	2.13	1.02	0.23	0.036
0	2,500	3.28^{b}	26.0^{a}	22.3	310	157	57.9	25.0	1.60	1.64	0.20	0.043
2	0	3.50^{b}	24.1^{b}	23.2	360	154	52.5	23.1	1.91	1.56	0.24	0.038
2	2,500	3.49^{b}	24.0^{b}	21.3	371	130	43.1	17.3	1.55	1.34	0.17	0.023
0		4.05	25.0	22.0	290	175	64.4	27.9	1.86	1.33	0.22	0.039
2		3.39	24.0	22.3	365	142	47.8	20.2	1.73	1.45	0.20	0.031
	0	4.16	24.1	22.4	315	174	61.7	26.9	2.02	1.29	0.23	0.037
	2,500	3.38	25.0	21.8	340	143	50.5	21.2	1.57	1.49	0.19	0.033
SEM		0.21	0.26	0.37	31	10	3.5	1.5	0.11	0.11	0.01	0.003
P-Value												
Betaine		NS	<0.05	NS	NS	NS	<0.05	<0.01	NS	NS	NS	NS
Phytase		<0.05	<0.05	NS	NS	NS	0.105	<0.05	<0.05	NS	<0.05	NS
Betaine x Phytase		<0.05	<0.05	NS	NS	NS	NS	NS	NS	<0.05	NS	NS

[a-b] Means within columns with no common superscript differ significantly ($P<0.05$)

6.5 Discussion

6.5.1 Comparison with Breeder Expectations

The birds used in this trial were 100 grams overweight at the start and end of the trial in relation to the breeders expectations (Joice and Hill, 2014). The egg production of the birds on this trial was lower than the breeder expectations (91.6 vs 89.7%), while the eggs were 2 grams heavier. This gave an egg mass production that was on target which, with a reduced daily feed intake (113 vs 110g), gave a slightly reduced FCR (2.15 vs 2.05)

6.5.2 The Effect of Betaine and Phytase on Laying Hen Performance

In this trial, the weight gained by laying hens was higher when birds were fed a phytase diet. Similar effects have been seen in all of the trials in this thesis. This suggests that including greater levels of phytase in the diet does benefit the nutrition of the laying hens, allowing it to invest more energy and protein in maintenance and growth.

Throughout this experiment, the addition of phytase without betaine to a start of lay hen gave numerical benefits in egg production, egg weight and egg mass which gave an improved FCR in spite of a trend for an increase in feed intake. Similar effects were seen in the previous trials. In Chapter 4, including phytase at above standard levels in start of lay trials has given improvements in FCR through a small increase in egg production, decrease in feed intake and a steady egg weight. It may be that younger birds utilise the extra phosphoric benefits of phytase more readily than older birds. In Chapter 5, the addition of above standard phytase inclusions to a mid-lay diet gave significant benefits to performance, but the effect on FCR was weak and variable. As discussed in Chapter 5, Meyer and Parsons (2011) did not find any benefit of including an above standard phytase dose in birds from 32 weeks of age. Similarly, Augspurger and colleagues (2007) failed to show any significant benefits of including a high phytase diet relative to standard doses to birds in their second lay cycle after a moult. It is possible that this is because the improvement in performance was related to the release of *myo*-inositol from phytic acid, and younger growing animals have a greater requirement of *myo*-inositol. The addition of higher than standard inclusion levels of phytase has, however, been shown to benefit the

performance of other growing monogastrics (Cowieson et al., 2010). Walk et al. (2014) showed that including phytase at 2,000FTU kg^{-1} gave benefits to FCR that could not be explained by increasing phosphorus and calcium in the diet. These benefits are referred to as the extra-phosphoric effects: these include improving the digestion of protein, carbohydrates and minerals, reducing endogenous secretions and losses, increasing the AMEn of the diet and finally releasing *myo*-inositol.

The supplementation of a diet with betaine numerically decreased egg production, but increased egg weight, which resulted in no change in egg mass production. This, in combination with a trend for a reduced feed intake significantly improved FCR. Zou and Lu (2002) have previously shown that 0.6 g kg^{-1} of betaine improved egg production by 8.7% and FCR by 9.0% without affecting feed intake or egg weight. Park et al. (2006) found that betaine supplementation increased egg weight but had no effect on egg production. Gudev et al. (2011) found that including betaine at 0.7 or 1.5 g kg^{-1} increased egg numbers and egg mass but decreased egg weight; this is similar to the results of Ryu et al. (2003). When hens under heat stress were fed a diet supplemented with betaine egg production and FCR were improved. Contrary to these studies, Harms and Russell (2002) found that 0.166% betaine inclusion had no benefit on egg production, egg weight, egg mass, feed intake, FCR or weight gain.

An interesting interaction was seen regarding FCR, with both phytase and betaine improving FCR significantly when supplemented alone but in combination having a smaller and insignificant effect. Contrary to this, Debicki-Garnier and Hruby (2003) found that supplementation of a broiler diet with betaine and phytase improved the feed intake, end body weight and body weight gain of broilers, but there was no significant effect on the FCR. The differences seen here are likely to be due to the differences between broilers and laying hens. The antagonism seen in this trial between phytase and betaine suggests they should not be used together in industry. It is of interest to understand the mechanisms behind this antagonistic effect so the benefits of both supplements can be utilised commercially. It is possible that this opposition is due to their actions within the gastrointestinal tract. However, it is unlikely that betaine could significantly decrease phytic acid hydrolysis from exogenous phytase or prevent the absorption of nutrients released from phytate, as betaine is known to improve nutrient digestibility and uptake. Debicki-Garnier and Hruby (2003) showed that the addition of

betaine to a diet supplemented with phytase further reduced ash excreted, suggesting greater phytic acid hydrolysis. Phytase hydrolysing phytic acid will release cations from phytate complexes. It is possible that the release of these charged ions will affect the osmotic environment across the wall of the tract and so affect betaine uptake from the lumen of the intestines, but again this is unlikely because betaine can be moved against the osmotic gradient.

It is more likely that the antagonistic effects of betaine and phytase are due to interactions between betaine and *myo*-inositol. As discussed in the introduction, *myo*-inositol is released from phytic acid hydrolysis, and although it is chemically different from betaine in structure, they have similar metabolic niches with considerable overlap. Betaine, *myo*-inositol and taurine are the main osmoprotectants within the chicken. It is plausible that this overlap in functions reduces the uptake of both *myo*-inositol and betaine from small intestines, when their availability is increased. The mechanisms of controlling uptake of different osmolytes into cells is very similar (Goldstein and Davis, 1993, Handler and Kwon, 1996). Although, Goldstein and Davis (1993) did not find that increasing the concentration of one osmolyte reduced the uptake of another in erythrocytes.

The analysis of the *myo*-inositol in blood plasma supports this apparent antagonism, showing that the addition of phytase to the control diet increases the circulating concentration of *myo*-inositol. The supplementation of betaine in combination with phytase reversed this and brought the concentration of *myo*-inositol back to the level of the control and betaine alone. Firstly, this indicates that *myo*-inositol bioavailability may be increased by high dose phytase supplementation. This is in agreement with the phytase dose response trial (Chapter 5), which showed an increase in liver *myo*-inositol concentration with higher phytase inclusion. Similarly, Cowieson et al. (2014) found that high phytase inclusion increased the concentration of blood *myo*-inositol. The effect of the treatments on the blood *myo*-inositol also supports the theory that the increase in *myo*-inositol is important in relation to the benefits delivered by high phytase dose. This is because the addition of betaine with phytase not only reversed the increase in blood *myo*-inositol concentration, but also FCR. It is thought that *myo*-inositol may be an important contributor regarding the improvement in animal performance seen with high phytase doses (Cowieson et al., 2011). *Myo*-inositol has been shown to improve the growth of fish (Waagbo et al., 1998), rats (Katayama, 1997) and the chicken (Hegsted, 1941). Two studies in broilers have shown

myo-inositol can benefit growth and efficiency (Cowieson et al., 2013, Żyła et al., 2013).

It is noteworthy that antagonistic effects have been seen between *myo*-inositol and choline. Choline is readily oxidised to betaine within animal tissues and utilised for methylation in addition to protecting cells from osmotic stresses. Interestingly, one of the benefits of *myo*-insoitol and choline/beatine is their ability to transport fat and reduce incidences of fatty liver disease. The administration of inositol and choline alone prevented the formation of fatty livers more than when given in combination (Forbes, 1943). Tylor and McKibbin (1952) showed that the growth of *myo*-inositol deficient yeast was suppressed by choline. In rats fed a choline deficient diet, renal necrosis was aggravated by *myo*-inositol supplementation (Handler, 1946). Similarly, slipped tendons (perosis) caused by choline deficiency in turkeys (Jukes, 1941) and chickens (Agranoff and Spivey- Fox, 1959) was exasperated by *myo*-inositol supplementation, increasing in appearance and severity. It seems logical that because the uptake of betaine and *myo*-inositol are controlled in the same way, an increase in the concentration of one would inhibit the uptake of the other and exacerbate deficiencies and their symptoms. It could be hypothesised that greater availability of both betaine and *myo*-inositol could reduce the uptake of both osmolytes, still provide the osmoprotection needed by cells but reduce the other metabolic benefits that these compounds bring, that have been discussed at length in the introduction.

6.5.3 The Effect of Betaine and Phytase on Egg Quality

The analysis of egg quality across the duration of the trial revealed one significant effect. An interaction between treatments, while both phytase and betaine supplemented alone made the yolks paler, together they restored the yolk colour to equal the control diet. In Chapter 5, it was found that phytase inclusion could increase the intensity of colour in egg yolks. Which is in contrast to the findings here. The difference between these two studies is the inclusion level of phytase. In the previous trial, 250 FTU kg^{-1} numerically increased the colour score of the NC diet restoring it to the level of the PC. The inclusion of 1000 FTU kg^{-1} actually gave a numerical decrease in yolk colour relative to the PC and 250 FTU kg^{-1} diet. Finally, the deepening of yolk colour relative to the PC was not seen until phytase was included at 4000 and 16000 FTU kg^{-1}. In this experiment, 2,500 FTU $^{-1}$ was used as the phytase dose. Other experiments have shown that standard

inclusion levels of phytase can restore yolk colour to phosphorus deficient diets (Kozłowski and Jeroch, 2011, Englmaierová et al., 2012). It is possible that two mechanisms are in place here; initially phytase frees phosphorus and so reverse a deficiency allowing a restoration of yolk colour but not intensifying it greater than the control. As phytase dose is increased to 1000 to 2,500 FTU kg^{-1}, some of the *myo*-inositol is released from the near complete hydrolysis of phytic acid and seems to negatively affect yolk colour. *Myo*-inositol can affect the transport of fats and fat soluble compounds such as carotenoids (Pirgozliev et al., 2010b), as can betaine (Matthews and Southern, 2000). Further supplementation of phytase increases the *myo*-inositol released from phytic acid, and this increases the level of carotenoids transported to the egg yolk. Pirgozliev et al. (2010) showed that 3000 FTU kg^{-1} phytase increased carotenoid concentration in the liver of broilers. Matthews and Southern (2000) showed that betaine could increase and decrease the concentration of circulating carotenoids in broilers depending upon an immune challenge from *Eimeria acervulina.* It is interesting that both a further increase in phytase and betaine on top of phytase appears to reverse some of the negative effects of 1000-2500 FTU kg^{-1}. Whether through changes in carotenoid metabolism or digestion, it is clear that there are complex relationships between *myo*-inositol, betaine and carotenoids which can effect egg pigmentation when fed in a commercial situation.

Weight loss that occurs during storage was found to be reduced by betaine. From the time of laying, the egg loses weight as water evaporates through the pores of the shell (Williams, 1992). Although unpublished, Dr Cadogan discussed the results from a pilot trial as part of Dansico Sub-project 2.1.1., stating that including 1g kg^{-1} dietary betaine could increase the content of betaine in eggs almost threefold (PoultryCRC, 2011). This is interesting because betaine has previously been shown to maximise water retention against osmotic gradients and evaporation in intestinal cells (Kettunen et al., 2001) and plant leaves (Lai et al., 2014). It is likely that in this trial, dietary betaine supplementation has increased the concentration of betaine within the egg and so reduced the evaporation of water from the egg. Time also significantly decreased the Haugh unit score as expected; this was due to a loss of carbon dioxide and an increase in the alkalinity of the albumen, which starts albumen liquefaction (Li-Chan and Nakai, 1989, Williams, 1992). Similarly, as anticipated, the weight of the egg yolk increased as water and amino acids moved across the vitelline membrane from the albumen (Heath, 1977).

Although the dietary treatments did not affect the changes in quality over time, differences in egg quality were seen that were not observed throughout the rest of the trial. This is probably because the differences are more pronounced towards the end of the trial, and because a greater total number of eggs were analysed in order to assess the effect of time.

Both betaine and phytase significantly increased egg weight, which supports the numerical patterns seen throughout the 12 weeks of the trial that failed to reach significance, shown in Table 6.3. Phytase also increased the egg yolk weight in line with egg weight. Phytase could benefit egg and yolk weight through the extra-phosphoric benefits, increasing nutrient digestibility or the release of *myo*-inositol. Betaine could benefit egg weight through an increase in nutrient digestibility through its affects as an osmoprotectant in the intestine, through a sparing effect of methionine or choline as a methyl donor, or through the direct effects of methyl donation(Ratriyanto et al., 2009). Finally, an increase in egg betaine content may increase the uptake of water into the egg during its formation in the oviduct which would increase egg weight. This may explain why unlike phytase, there was not an increase in yolk weight and a significant reduction in relative yolk weight was seen.

Phytase also decreased the Haugh unit score of eggs, indicating poorer albumen quality. Similar significant results were seen in Chapter 5, while in Chapter 4 a non-significant decrease in Haugh units was seen. As discussed in Chapter 5, phytase will increase phytic acid hydrolysis, preventing the formation of phytate. The release in trace minerals will increase their availability; highly available trace minerals have previously been shown to reduce albumen quality (Sell et al., 1982).

Finally, phytase was shown to benefit egg shell quality. This is in agreement with Chapter 3 but in contrast to Chapters 4 and 5. The differences are likely to be due to the birds' calcium store status and dietary calcium levels.

Higher phytase inclusions increase the hydrolysis of phytic acid, reducing the formation of phytate complexes, and so improve calcium availability. Greater calcium availability will replenish calcium stores within the chicken and allow more calcium to be deposited on the shell membrane during shell formation (Clunies et al., 1992). The phytase benefit had a stronger effect on shell strength and weight in comparison to shell thickness because phytase also increased the egg size, and so the calcium had to be spread more thinly (Roland, 1979).

6.5.4 The Effect of Betaine and Phytase on Laying Hen Feather Score

The supplementation of a diet with betaine and phytase gave numerical increases in the feather scores on the back of laying hens. The biggest effect seen was phytase decreased feather score, indicating less pecking when supplemented to the betaine diet, but not the control. Nutrition has been shown to play an important role in the establishment of feather pecking behaviours (Van Krimpen et al., 2005). Increasing protein in a diet has been shown to decrease feather pecking (Schaible et al., 1947), while increasing energy content of a diet increases feather pecking (Elwinger, 1981). Minerals are also important, most notably, reducing sodium levels increases feather pecking (Hughes and Whitehead, 1979) while increasing magnesium has been shown to decrease feather pecking (Schaible et al.,1947). The effect of the treatment was small, even when significant, but should be noted and may justify further investigation to understand underlying mechanisms involved which are undoubtedly complicated.

6.5.5 The Effect of Betaine and Phytase on Blood Plasma Parameters

The effect of phytase and betaine on the concentration of *myo*-inositol have already been discussed in Section 6.5.2, but other dietary effects were seen on the blood plasma. There was a significant interaction with both phytase and betaine decreasing PUN by 32 and 27% respectively, but together they had no greater effect. An elevated PUN concentration can result from increased protein intake, protein catabolism or impaired kidney function; one possible caused of impaired kidney function kidney dehydration. Both *myo*-inositol and betaine are osmoprotectants that benefit kidney function and so reduce protein catabolism and PUN levels. Similarly, decreases in blood urea nitrogen (BUN) with phytase addition have been seen in pigs (Liu et al., 2010) and broilers (Jiang et al., 2013). In contrast to this, Baidoo and Walker (2003) failed to find any effect while Johnston and colleagues (2004) found that phytase increased BUN in pigs. Hagar and Al Malki (2014) showed that betaine could reduce and restore BUN levels after rats had renal injuries induced by cadmium intoxication.

The effect of the treatments on blood mineral concentration is interesting. In previous experiments significant increases have been seen in multiple minerals with phytase addition, whereas in this trial phytase significantly

decreased the concentrations of magnesium, copper and zinc. The reason of this contrast may be due to the age of the birds and the duration of the trial. Increasing the availability of minerals at an early age may increase the minerals stored within the body and reduce their requirements, and therefore the absorption and circulation of minerals after 12 weeks. This however, is just one possibility and further research is needed to investigate the dichotomy seen between the trials.

6.5.6 Conclusions

The inclusion of a high phytase dose or betaine alone benefited laying hen efficiency. In birds which received phytase this was achieved by increasing egg production and weight, while for birds which received betaine there were smaller increases in egg production and weight; but the decrease in feed intake also gave an improved FCR.

When supplemented within a diet together the benefits to FCR were reduced. This may be due to an antagonist relationship between *myo*-inositol and betaine which has been documented previously. Indeed, phytase supplementation increased the concentration of circulating *myo*-inositol while betaine supplementation on top of phytase reversed this effect. Phytase was once again shown to benefit shell quality, presumably by increasing calcium availability, while betaine reduced the weight lost by eggs over time in storage. This may be due to increased betaine within the egg reducing the loss of water through evaporation. The supplements had an interesting effect on yolk colour, reducing the intensity of colours when fed alone however, in combination the colour was restored to the level of the control. PUN was reduced by both betaine and phytase supplementation, which could be due to the osmolytes *myo*-inositol and betaine improving kidney function and reducing protein catabolism. Finally, in contrast to previous experiment phytase supplementation did not increase the concentration of circulating minerals but actually decreased the levels of magnesium, copper and zinc. This may be due to excessive mineral availability earlier in the trial but needs further investigation.

Chapter 7
General Discussion

7.1 The Effect of High Phytase Inclusion on Laying Hen Performance

The aim of this thesis was to discover if using higher than standard phytase inclusion levels could benefit laying hens. In Chapter 3, the above standard inclusion of phytase failed to significantly benefit laying hen performance; however, this is likely due to the short length of the trial. The three other experiments in this thesis, all indicated improvements in performance with high phytase doses. The experiments described in Chapters 4 and 6 showed improvements in FCR.

In Chapter 4, the FCR of laying hens was improved from 2.06 to 2.02 by 2,500 FTU kg^{-1} in comparison to a positive control diet. This was caused by small non-significant increases in egg production (96.1 vs 96.8%) and a trend for a reduced daily feed intake (116.2 vs 114.2g). Chapter 4, also showed that phytase and glucanase did not inhibit each other, and were additive in terms of the benefits in bird weight gain.

In Chapter 6, FCR was improved by 2,500 FTU kg^{-1} phytase from 2.11 to 2.03 in comparison to a control diet. This was a result of an insiginicant increase in egg production and egg weight from 88 to 92%, and 59.9 to 60.4g respectively. Betaine was shown to have an antagonistic relationship with phytase. When fed alone, betaine also reduced the FCR of laying hens from 2.11 to 1.99. However, the FCR of laying hens fed both supplements in comination (2.07), was not reduced in comparison to those fed the control diet and was significantly different from that of the hens fed the supplements alone. This may be due to the overlap in metabolic function of *myo*-inositol and betaine.

In Chapter 5, high phytase inclusions clearly demonstrated benefits in comparison with a positive control, negative control and standard inclusion levels. In particular an increase in egg mass production was quite pronounced. Birds fed the above standard inclusion levels of phytase produced 60.8g d^{-1} egg mass. This was signifcantly higher than birds fed the positive control, negative control and standard inclusion diets (58.9, 58.3 and

60.5g d^{-1} of egg mass respectively). This is in contrast with the studies of Augspurger et al. (2007) and Meyer et al. (2011); neither study found benefits from including higher phytase doses. As discussed in Chapter 4, both of these trials used white egg breeds and birds with very low egg production, whereas the production of the chickens on all the trials in this thesis were high. Augspurger et al. (2007) also used older birds that had been moulted and were in their second cycle. Trials by other authors have shown that higher phytase doses can benefit piglet and broiler performance (Pirgozlier et al., 2007, Walk et al., 2014). It is thought that these benefits are due to improvements in protein digestion, diet AMEn, reductions in endogenous losses and the greater availability of *myo*-inositol (Cowieson et al., 2011).

It can be concluded from this thesis that the inclusion of high levels of phytase, sometimes denoted as “superdoses,” can benefit laying hen performance. However, research from other authors has indicated that these benefits are not always reliable. In addition to this, the benefits seen throughout this thesis have varied between improvements in egg weight, egg mass and efficiency. Other dietary factors, such as betaine inclusion, can also inhibit these improvements. Future experiments need to investigate how the performance improvements seen in this thesis can be manipulated to provide an economic benefit to nutritionists and farmers, improve laying hen efficiencies and reduce the environmental impact of poultry farming.

7.2 The Effect of High Phytase Inclusion on *Myo*-Inositol

The inclusion of phytase in laying hen diets increased the concentration of *myo*-inositol in the liver and blood in Chapters 5 and 6 respectively. In Chapter 5, the hepatic *myo*-inositol concentration continued to rise with each increase in phytase inclusion from 1.45mg g^{-1} in birds fed the negative control diet to 4.1mg g^{-1} in birds fed the 16,000 FTU^{-1} diet. In Chapter 6, 2,500 FTU kg^{-1} increased the concentration of blood plasma from 24.0 to 26.0mg l^{-1}. However, when supplemented to a diet with 2g kg^{-1} betaine, phytase had no effect on the blood plasma concentration of myo-inositol. Cowieson et al. (2014) has similarly shown that blood plasma *myo*-inositol concentration continued to increase with phytase inclusion in broilers diets. This suggests the highest inclusion levels of supplemental phytase continue to increase the absorption of *myo*-inositol. Future work needs to asses if improvements in performance and increases in *myo*-inositol absorbtion are

due to a previously unappreciated requirement for *myo*-inositol. The link between an increased blood plasma *myo*-inositol concentration and an improved FCR, as seen in Chapter 6, suggest this may be the case.

As discussed previously, *myo*-inositol and its derivatives have a large array of metabolic functions including: fat transport, (Katayama, 1997), insulin mimicry (Dang et al., 2010, Yamashita, 2013), protection form osmotic stresses (Lien et al., 1993), anchoring proteins to cell membranes (Brown and Waneck, 1992), secondary messengers (Berridge, 1993), and possibly protecting cells form oxidative stress (Doria et al., 2009). The supplementation of diets with *myo*-inositol has been shown to benefit growth in fish (Waagbo et al., 1998), rats (Katayama, 1997) and chickens (Hegsted, 1941). Two studies in broilers have shown *myo*-inositol can benefit growth and efficiency (Cowieson et al., 2013 and Zyla et al., 2004), whereas one study in laying hens found *myo*-inositol had small non-significant negative effects on performance of birds fed a slightly deficient diet (Zyla et al., 2012). *Myo*-inositol may not be able to deliver benefits to the animal if phosphorus is deficient, as many of the metabolic functions of *myo*-inositol require it to be phosphorylated once within the animal. Future experiments should be conducted to investigate the laying hen's requirement of *myo*-inositol when fed a phosphorus adequate diet. Experiments should also be conducted to explore what other factors influence *myo*-inositol digestibility and retention. The antagonistic relationship between *myo*-inositol and betaine is particularly interesting given the increase in use of both phytase and betaine in commercial diets. An experiment should be conducted to assess if betaine inhibits the uptake of *myo*-inositol from the gastrointestinal tract or impeds its metabolic functions once within the animal.

7.3 The Effect of High Phytase Inclusion on Minerals

The effect of high phytase inclusion on mineral bioavailability varied between the different experiments. Chapter 3 showed the clearest mineral effects, with 2,500FTU kg^{-1} phytase increasing the solubility of minerals within the gastrointestinal tract and the concentration of multiple minerals within the blood plasma. For example, the solubility of calcium was increased in the from 5.1 to 7.9% soluble in the gizzard, 4.0 to 12.3% soluble in the duodenum and 0.5 to 1.3% soluble in the ileum. The results from the other studies were less consistent in relation to minerals. In Chapter 5, the dose response of phytase only gave one clear mineral result; increasing phytase

up to 16000 FTU kg^{-1} continued to increase the blood plasma zinc concentration from 1.67 to 2.2 μg ml^{-1}. In Chapter 6, 2,500 FTU kg^{-1} phytase supplementation decreased some blood plasma mineral concentrations, including magnesium (from 26.9 to 21.2 μg ml^{-1}) , zinc (from 2.02 to 1.57 μg ml^{-1}) and copper (from 0.23 to 0.19 μg ml^{-1}). It is possible that the benefits are clearer in Chapter 3 because this trial did not last as long and so the birds had less time to respond to higher availabilities of minerals. Differences in dietary mineral concentrations may also have had an effect. It is known that an excess of one mineral can reduce the availability of another (Pontoppidan et al.,2007a). The greater availability of multiple minerals may be responsible for some of the benefits seen from increased phytase inclusion. However, for this to be accepted as true, the mineral requirements of birds fed diets with and without a high phytase inclusion needs to be assessed in future trials.

7.4 The Effect of High Phytase Inclusion on Egg Quality

The inclusion of higher phytase doses has been shown to affect egg quality. Firstly, in Chapters 3 and 6 phytase was shown to benefit shell quality, whereas in Chapter 4 and 5 phytase failed to have an effect. In chapter 3, egg shell strength was increased from 3.49 to 3.93kgf. In Chapter 6, 2,500 FTU kg^{-1} phytase increased shell weight and shell strength from 6.07 to 6.12g and from 4.01 to 4.20kgf respectively. The lack of effect seen in Chapters 4 and 5 could be explained by the elevated calcium levels in the diets. In Chapters 4 and 5 the dietary calcium concentrations of the control diets were greater than 50g kg^{-1} , whereas in Chapters 3 and 6 the concentrations were less than 50 and 40 g kg^{-1} respectively. From these trials therefore, it can be concluded that the use of high phytase inclusion levels may allow a further reduction in dietary calcium concentrations while maintaining shell strength. This should be tested in future experiments to determine the calcium requirement of laying hens fed a high phytase diet.

Across all of the trials, the Haugh unit score was depressed by phytase inclusion. Although only a small effect was recorded, and this decrease was only significant in Chapters 5 and 6, it is noteworthy as this may reflect a greater risk. In Chapter 6, the Haugh unit score was reduced to 50.5 from 52.82 with phytase supplementation. The reduction in Haugh units is likely due to the release of trace minerals from phytate; some minerals such as vanadium have previously been shown to inhibit albumen quality. Chapter 3

has shown that 2,500 FTU kg^{-1} phytase inclusion increases the solubility of minerals and their bioavailability. In addition to the benefits this may bring, it may also cause some problems as birds may overdose on some minerals that are only required in small quantities. If the high doses of phytase used in these experiments become the commercial standard, the laying hen requirements for all minerals will need to be investigated further. In addition to this, the toxicity levels of some minerals will also need to be analysed; for example, the acceptable levels of fluorine and vanadium contamination in phosphorus sources may need to be reduced. However, this may not be a problem as greater phytase inclusion is likely to result in a reduced inclusion of inorganic phosphorus.

Phytase has also been shown to affect egg yolk colour in an interesting, but inconsistent manner, which is probably due to *myo*-inositol affecting the transport of fat soluble carotenoids. Although this effect is probably not large enough to affect consumer preferences, it is interesting that *myo*-inositol may affect the transport of many fat soluble molecules and vitamins. Future research should focus on how the products of phytic acid hydrolysis, such as *myo*-inositol, can affect the digestion and metabolism of essential nutrients like the fat soluble vitamins.

7.5 Final Conclusions

The inclusion of dietary phytase at levels considered to be above standard has shown benefits to laying hens fed these diets in relation to birds fed positive controls, negative controls and standard phytase inclusions. Notably, the phytase has given increases in bird weight, egg weight and egg mass. While these results are encouraging and suggest greater nutrient availability from phytase inclusion, they are not in themselves always desirable. Laying hen nutritionists and farmers want to control both bird weight and egg weight rather than increase them indefinitely. This is because an increase in egg weight will result in a fall in egg numbers towards the end of the lay. Instead of using this data to predict the commercial benefits of high phytase inclusion, it should be used to show that phytase can benefit the nutritional status of the laying hen. The next step in this area of research is to investigate if the inclusion of a high phytase dose can allow a ration dilution; reducing the protein, energy and minerals levels of a laying hen diet without detrimentally affecting performance. This work should ideally be confirmed with longer trials that investigate the effects of phytase throughout the whole life cycle of the laying hen, from hatch to end

of lay. This research may allow a cheaper diet to be formulated utilising low-priced, less nutrient dense ingredients. This research would enable cost benefit analysis to be done and recommendations to be made to laying hen nutritionists and farmers. Given that the cost of phytase is reducing, while the cost of protein, energy and phosphorus are increasing, it is likely that higher phytase inclusion levels will be more economical.

Increasingly, improved *myo*-inositol uptake is seen as a potential benefit from higher phytic acid hydrolysis and greater phytase inclusion levels. A lot more research is needed in this area as there is very little and much of it is outdated. In particular, it would be interesting to assess the impact of *myo*-inositol inclusion in a nutritionally adequate laying hen diet. It would be fascinating to explore the *myo*-inositol requirement of hens at a variety of ages to see if the hypothesis about younger birds having a greater need is correct.

The use of higher phytase inclusion levels in this thesis has been shown to benefit laying hens. Future research should investigate the mechanisms behind these improvements, paying particular attention to the increased *myo*-inositol availability.

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List of Abbreviations

Adj Egg Mass	-	Adjusted Egg Mass
Adj FCR	-	Adjusted Feed Conversion Ratio
AID	-	Apparent Ileal Digestibility
AMEn	-	Apparent metabolisable energy
ASI	-	Above Standard Inclusion
BMS	-	Blood and Meat Spots
BSE	-	Bovine Spongiform Encephalopathy
BWG	-	Body Weight Gain
BUN	-	Blood Urea Nitrogen
Duo	-	Duodenum
FCR	-	Feed Conversion Ratio
FI	-	Feed Intake
FTU	-	Phytase Units
Giz	-	Gizzard and Proventriculus
GU	-	Glucanase Units
HDEP	-	Hen Day Egg Production
HU	-	Haugh Units
Jej	-	Jejunum
MBM	-	Meat and Bone Meal
NC	-	Negative Control
NSP	-	Non-Starch Polysaccharides
PC	-	Positive Control
Phytate-P	-	Phytate Phosphorus
PUN	-	Plasma Urea Nitrogen
SI	-	Standard Inclusion
Total-P	-	Total Phosphorus
XU	-	Xylanase Units