The effects of super-dosing phytase in the growing pig

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Declaration of Authorship

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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SL helped design the experiment, conducted the research, analysed the data and wrote the manuscript. Co-authors assisted in designing the experiment and revising the submitted manuscript.

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Abstract

Microbial phytase enzymes are commonly added to monogastric diets at 500 FTU/kg feed (as fed) to improve phytate phosphorus (P) bioavailability and reduce P excretion. The use of super-doses of phytase (>1,500 FTU/kg as fed) is currently generating much interest, as recent studies have demonstrated that such high doses can improve the growth performance of monogastrics. However, the underlying mechanism for this response remains unclear. At present, there is a dearth of information available on the effects of super-dosing in the pig. Therefore, this research set out to determine the effects of super-dosing phytase on the growing pig, with a view to shedding light on underlying mechanism, particularly in relation to the possible involvement of *myo*-inositol (MYO). Through three separate feeding trials, this work found that super-dosing phytase improved the growth performance of weaner pigs, but had no influence on the growth performance of grower or finisher pigs. Ileal digesta were collected and analysed for inositol phosphate content by HPIC. Standard doses of phytase resulted in a small accumulation of $InsP_4$ and $InsP_3$ phytate esters in the ileal digesta of weaner and finisher pigs. This build-up was effectively diminished when supplemented with a phytase super-dose, resulting in more complete phytate degradation. High doses of phytase consistently increased circulating levels of MYO in both the portal and peripheral blood in weaner, grower and finisher pigs. Super-doses of phytase were consistently associated with increases in Ca, P and Mg bioavailability across all stages of production. Moreover, there were also indications of improved trace mineral (Zn, Cu, Fe, Mn) digestibility; however, this effect was less consistent. From this thesis it can be concluded that super-doses of phytase are associated with an increase in phytate hydrolysis and a concomitant increase MYO absorption. However, these effects do not always translate into improved pig performance. Further research is needed to elucidate the factors responsible for the inconsistencies observed.

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Abbreviations

-RT	Minus reverse transcriptase
A. niger	*Aspergillus niger*
AA	Amino acid
ACTB	Beta-actin
ADFI	Average daily feed intake
ADG	Average daily gain
AHDB	Agriculture and Horticulture Development Board
AIA	Acid insoluble ash
AID	Apparent ileal digestibility
ANOVA	Analysis of variance
ATTD	Apparent total tract digestibility
BW	Body weight
C. braakii	*Citrobacter braakii*
CD36	Cluster of differentiation 36
cDNA	Complimentary DNA
CP	Crude protein
d	Day
DCP	Dicalcium phosphate
DE	Digestible energy
dEB	Dietary electrolyte balance
DEFRA	Department for Environment, Food and Rural Affairs
DI	Deionised
DM	Dry matter
DMI	Dry matter intake
DMT1	Divalent-metal transporter
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ESC	Enzyme Services & Consultancy
FCR	Feed conversion ratio
FFDW	Fat-free dry weight
FTU	Phytase unit
GIT	Gastrointestinal tract
GLM	General linear model

GOI	Gene of interest
h	Hours
Hb	Haemoglobin
Hct	Haematocrit
HMBS	Hydroxymethylbilane synthase
HPIC	High-performance ion-chromatography
HPLC	High-performance liquid-chromatography
HPRT	Hypoxanthine phosphoribosyltransferase 1
ICP-OES	Inductively coupled plasma optical-emission spectroscopy
InsP	Inositol phosphate
K_m	Michaelis constant
M3	Third metatarsal
MCHC	Mean corpuscular haemoglobin concentration
ME	Metabolisable energy
min	Minutes
mRNA	Messenger RNA
MYO	*Myo*-inositol
NaPi-IIb	Na-dependent phosphate transporter-2b
NC	Negative control
NTC	No template control
P. lycii	*Peniophora lycii*
PC	Positive control
PCR	Polymerase chain reaction
PepT1	Peptide transporter 1
Pi	Inorganic phosphate
PP	Phytate phosphorus
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RNase	Ribobnuclease
SBM	Soybean meal
SD	Standard deviation
SE	Standard error
SEM	Standard error of mean
SGLT1	Sodium/glucose transporter 1
SMIT2	Sodium/*myo*-inositol transporter 2

TFRC	Transferrin receptor
UV	Ultraviolet
ZIP14	Zrt- and Irt-like protein 14

Chapter 1
General Introduction

1.1 British pig industry

The British pig industry currently comprises 4.7 million pigs and produces approximately 900,000 tonnes of pork each year (DEFRA, 2015). Whilst the majority of this produce remains in the UK, just under one third is exported, with most of this going to Germany, Ireland and China (DEFRA, 2015). Pig production systems in the UK are varied and include straw-based housing, slatted or partial slatted housing and outdoor units. The sustainability of the British pig industry is currently under threat, as for much of the last 5 years the average cost of pig production has exceeded the average sum farmers receive for their produce (AHDB, 2016). One of the main drivers of pig farming profitability in the UK is the cost of feed. At present, feed accounts for up to 60% of the total cost of pig production (AHDB, 2016). Thus, there is a real need to find cheaper, more sustainable ways of feeding livestock in order to improve the viability of pig production in the UK.

1.2 Phosphorus

Phosphorus (P) is an essential macro-mineral required by all organisms for normal growth, development and metabolism. As the second most prevalent mineral in the pig, P accounts for approximately 1% of its body weight (NRC, 2005). More biological functions have been described for P than for any other mineral in the body (McDonald et al., 2011). Between 60 and 80% of the total body P is present in teeth and bone tissue in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), which serves as one of the primary structural components of tooth enamel and bone, and provides these tissues with tensile strength (Crenshaw, 2001). The remaining 20 to 40% of the total body P is present in both soft tissues and bodily fluids. Phosphorus serves important acid-base buffering functions in bodily fluids as a component of bicarbonate (HCO_3^-). Moreover, in soft tissue, P forms an integral component of phospholipids, phosphorylated proteins, mono-, di- and tri-phosphates and nucleotides, and therefore has key functions in intermediary metabolism, cellular

signalling, protein synthesis and cell membrane integrity (Crenshaw, 2001). Phosphorus deficiency can result in bone mineral resorption which can lead to rickets in young pigs or osteomalacia in adult pigs. Other P deficiency symptoms include poor fertility, depressed appetite and low growth rate (McDonald et al., 2011).

Depending on the stage of growth, a growing pig may require up to 4.89 g of available P per day for optimal growth and nutrient use efficiency (NRC, 1998). Pig farmers in the UK raise their livestock on predominantly grain based diets which are often limiting in available P. This is because the majority of the P in grains is tied up in a largely unavailable complex called phytic acid (Eeckhout and de Paepe, 1994; Ravindran et al., 1994). To ensure the P requirements of the animal are met, inorganic rock phosphates such as monocalcium phosphate (MCP) or dicalcium phosphate (DCP) are commonly added to the diets. However, these inorganic phosphate supplements are not a sustainable source of P, as they are both costly and a finite resource. Furthermore, the UK and the majority of Europe hold no rock phosphate deposits of their own and thus are heavily reliant on imports from Africa and Asia. With some experts predicting the depletion of global phosphate reserves within the century, there is a real need to find an alternative and more sustainable source of phosphorus (Cordell et al., 2009).

1.3 Phytate

Phytate, the salt form of phytic acid (*myo*-inositol hexakisphopshate, $InsP_6$) is invariably present in plant based feedstuffs where it serves as the primary form of inositol and phosphate storage (Lott et al., 2000). Phytate P (PP) can account for between 60 to 80% of the total phosphorus content of many common plant based ingredients, including: cereals, cereal by-products and oilseeds (Table 1.1). Although phytate phosphorus (PP) accounts for a significant proportion of the total dietary-P intake of the pig, phytate is generally insoluble at intestinal pH, and thus this source of P is largely unavailable to the animal. As discussed below, this poses a problem from both a nutritional and an environmental standpoint (Section 1.3.1).

Table 1.1. Mean concentration of total and phytate phosphorus and phytase activity of some commonly used feedstuffs

Feedstuff	No. of studies	Total P (%)[a]	Phytate P (%)[a]	Phytase activity (FTU/kg)[b]
Corn	6	0.27 (0.23-0.32)	0.19 (0.17-0.22)	36 (15-70)
Wheat	5	0.34 (0.29-0.42)	0.23 (0.18-0.26)	1465 (1193-1637)
Barley	3	0.34 (0.31-0.37)	0.20 (0.19-0.22)	799 (582-1016)[c]
Sorghum	3	0.30 (0.26-0.36)	0.20 (0.17-0.23)	24 (24-24)[c]
Soybean meal	5	0.67 (0.57-0.84)	0.37 (0.35-0.40)	62 (na)[d]
Rapeseed meal	3	1.16 (1.05-1.35)	0.74 (0.70-0.76)	41 (na)[d]
Wheat bran	4	1.10 (0.92-1.16)	0.76 (0.57-0.97)	2836 (928-4624)

[a] Results are means of data sourced from Eeckhout and de Paepe (1994), Ravindran et al. (1994), Viveros et al. (2000), Godoy et al. (2005), Pontoppidan et al. (2007), Tahir et al. (2012). Data in parentheses are the range. Expressed as % of DM.
[b] Means of data from Eeckhout and de Paepe (1994), Viveros et al. (2000) and Godoy et al. (2005) studies. Data in parentheses are the range. Expressed as % of DM.
[c] Mean from two studies
[d] Data from one study.

The structure of phytic acid, as originally described by Johnson and Tate (1969) over 4 decades ago, is a *myo*- configured inositol ring with a phosphate group attached to each of the 6 carbon atoms (Figure 1.1). The phosphate group attached to the second carbon 2 (C2) is axially orientated whereas the remaining 5 phosphate groups have an equatorial orientation. Each phosphate group contains two ionisable protons with pKa values ranging from 1.5 to 10. Six of these protons have pKa values between 1.5 and 2 and are thus strongly acidic; 3 of the protons have pKa values of between 5.7 and 7.6 and are thus slightly acidic; and the remaining 3 protons have pKa values above 10 and are thus basic (Costello et al., 1976). As a result, the phytate molecule possesses a negative charge across a wide range of pH values, and the strength of this charge increases as the ambient pH rises.

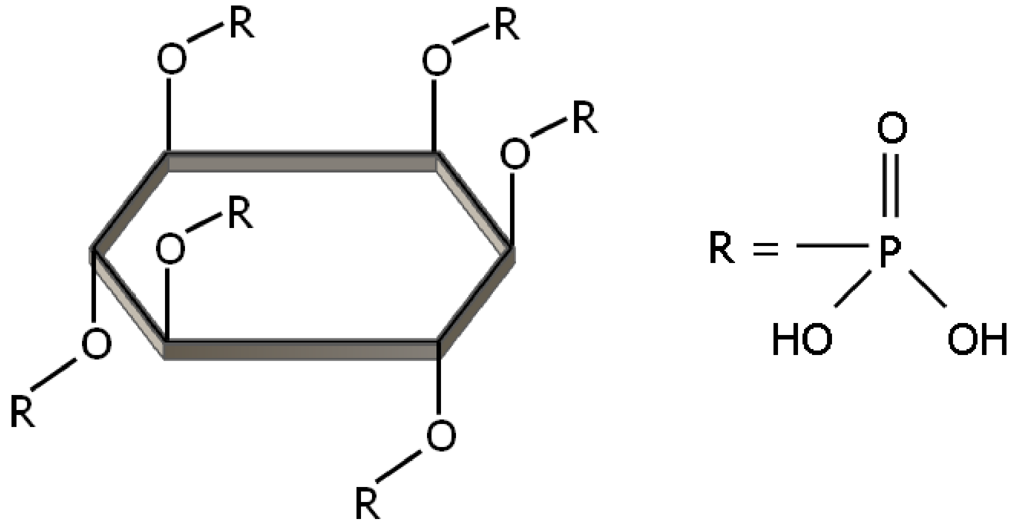

Figure 1.1. Schematic of phytic acid (myo-inositol hexakisphosphate), molecular mass 660.03 g/mol.

In the plant phytate exists primarily as a K and Mg salt and is usually found within storage vacuoles of aleurone cells as phytate globoid particles (Joyce et al., 2005). Lott et al. (1985) demonstrated that the size of these globoids is influenced by the ratio of cations (Mg and Ca) to K, with a high cationic to K ratio resulting in the formation of larger more insoluble globoids, and a low cationic to K ratio resulting in smaller more soluble globoids. Throughout maturation, seed protein storage vacuoles begin to accumulate large quantities of these globoids (Jiang et al., 2001), which at germination are degraded by intrinsic phytase enzymes, providing the developing embryo with a source of minerals and inositol. The location of phytate varies among different ingredients, for example, the majority of phytate in wheat, barley and rice is situated in the aleurone cells, whereas in corn the majority of phytate is located in the germ cells (O'Dell et al., 1972). In oilseeds such as soybean the phytate tends to be dispersed throughout the seed (Erdman, 1979). Less phosphorylated esters of phytate (*myo*-inositol penta-, tetra-, tri-, di- and mono-phosphate) are also found in feedstuffs of plant origin, but at much smaller concentrations (Pontoppidan et al., 2007b).

1.3.1. The phytate problem

The low availability of PP in monogastrics is mainly a function of solubility and not a lack of adequate phytase activity which many suggest (Cowieson et al., 2011). One of the main determinants of phytate solubility within the gastro-intestinal tract (GIT) is pH (Cheryan et al., 1983). Phytate is more soluble at low pH values (i.e. in the stomach) than it is at higher pH values (i.e. in the intestines; Campbell and Bedford, 1992; Schlemmer et al., 2001), and this is largely due to mineral-phytate interactions occurring in the GIT. As phytate passes through the GIT the pH becomes progressively higher, more protons begin to dissociate from the phosphate groups, and thus the net negative charge of the phytate molecule increases. Consequently, the affinity of phytate to chelate with cations increases as the molecule travels along the GIT. Upon chelating with cationic minerals a stable, insoluble mineral-phytate complex is formed rendering both the mineral and the phytate molecule unavailable for digestion (Maenz, 2001).

Undigested PP results in elevated P levels in the excreta. High levels of P in livestock manure is the main source of agricultural P pollution (Jongbloed and Lenis, 1998), therefore, the inability of the animal to degrade phytate is of serious environmental concern. When applied to agricultural land as fertiliser, P accumulates in the soil and upon repeated application the capacity for soil to store P is exceeded. This increases the likelihood of P entering surface waters as runoff or leachate and polluting these waters due to eutrophication. Pretty et al. (2003) estimated that the combined social, environmental and response cost of freshwater eutrophication in England and Wales amounts to between £75 million and £114.3 million each year. Therefore, it is important to find alternative ways of disposing of high P manure, or rather, lowering the P content of the excreta. One way in which the P content of the excreta can be effectively reduced is through the application of exogenous phytase supplementation.

1.4 Phytase

Phytase (*myo*-inositol hexakisphopshate phosphohydrolase) enzymes are a group of hydrolases with the capacity to catalyse the systematic cleavage of phosphates from phytic acid. Phytase was first identified in 1907 in rice bran by Suzuki et al. (1907) and has since been found in numerous microorganisms and a variety of plant and animal tissues (Kornegay, 2001). These enzymes are commonly classified according to their initial site of phytate hydrolysis, as either a 3-phytase or a 6-phytase. A 3-phytase initiates phytate hydrolysis at the C3 position, whereas a 6-phytase starts with the phosphate at the C6 position.

There are currently four known subfamilies of phytase, each with distinct structural and catalytic properties, these include: histidine acid phosphatases (HAPs), ß-propeller phosphatases (BPPs), protein tyrosine phosphatases (PTPs) and purple acid phosphatases (PAPs). HAPs are the most well characterised group of phytases due to their widespread commercial use as a feed additive for livestock. Furthermore, most of the currently identified phytases are HAPs (Mullaney and Ullah, 2003). The remaining three classes of phytase enzymes were discovered comparatively recently and are less well characterised (Mullaney and Ullah, 2003; Tonks, 2006).

HAPs (EC. 3.1.3.8) optimally catalyse dephosphorylation at an acidic pH using phosphohistidine intermediates, and in general have specificity for a broad range of substrates (Van Etten, 1982). Members of this group are found in both prokaryotes and eukaryotes, and possess a conserved active site RHGxRxP motif (Van Etten et al., 1991). BPP phytases (EC. 3.1.3.8), also known as alkaline phytases, specifically target Ca-phytate complexes and are highly dependent on the binding of Ca^{2+} ions for catalysis (Fu et al., 2008). BPPs are unable to use $InsP_2$ or $InsP_1$ as substrate, thus their final breakdown product is $InsP_3$ (Greiner et al., 2007). The PAP (EC. 3.1.3.2) phytases are a group of metallophosphatases most commonly found in plant tissue cereals (Brinch-Pedersen et al., 2014). These enzymes possess a bi-nuclear Fe^{3+}- x catalytic core, where x denotes an Fe^{3+}, Mg^{2+}, Mn^{2+} or Zn^{2+} ion (Lei et al., 2013). The remaining class of phytase, the PTPs (EC. 3.1.3.48), are a group of recently discovered phytases that have been detected in ruminal Gram positive bacteria (Nakashima et al., 2007). These PTP enzymes are characterised by a common active site HCx_5R sequence motif (Tonks, 2006).

1.4.1 Phytase use in animal nutrition

Exogenous microbial phytase enzymes are commonly supplemented to pig and poultry diets to improve PP availability and concurrently minimise P excretion. At the time of writing, all currently available commercial phytase enzymes are HAPs and this is largely owing to their broad substrate specificity, favourable pH activity profile and strong catalytic activity towards phytate (Lei et al., 2013).

These phytase enzymes catalyse phytate hydrolysis in the gastric regions of the tract (Jongbloed et al., 1992; Kemme et al., 2006), where the phytate is most soluble, yielding orthophoshates that are available to the animal for absorption. Theoretically, phytase cleaves phosphate moieties from the phytate molecule one at a time, yielding a series of lower phytate esters (inositol penta- to mono-phosphate; $InsP_5$ to $InsP_1$) and finally *myo*-inositol (MYO). Yet, *in vivo*, complete degradation of phytate to MYO is rarely achieved due to the axial orientation of the phosphate group at C2, which is recalcitrant to the action of most phytase enzymes (Wyss et al., 1999). It is, however, thought that endogenous mucosal phosphatases have the capacity to cleave this phosphate from the inositol ring (Hu et al., 1996).

Nelson et al. (1971) were the first of many to show that dietary microbial phytase supplementation can effectively improve PP availability in the target animal. The authors found that supplementation of an *Aspergillus ficuum* derived phytase to a corn-soybean meal (SBM) diet significantly improved broiler chick growth performance, bone ash and PP digestibility. Nevertheless, it was not until 1991 that the first commercial phytase product, an *Aspergillus niger* (*A. niger*) derived enzyme, Natuphos®, was marketed. Even then, the adoption of this technology was largely confined to the Netherlands and parts of the east coast of the USA, regions where there was legislative enforcement to minimise P output from livestock production facilities.

Since the turn of the century, there has been a rapid rise in the use of microbial phytases in monogastric nutrition which has seen the global inclusion rate of phytase to pig diets increase from approximately 8% in 1999/2000 (Kornegay, 2001) to an estimated 70% at present (Lei et al., 2013). This incredible growth rate can largely be attributed to a reduction in phytase costs and the development of more efficacious second and third generation bacterial phytases. In addition, the EU-wide ban on the inclusion of meat-and-bone meal (a P rich ingredient) to monogastric diets, the

growing recognition of the need to minimise agriculture's contribution to global nutrient pollution, and the improved understanding of the anti-nutritional effects of phytate, have all contributed to the significant increase in phytase use (Selle and Ravindran, 2008).

1.4.2 Sources of phytase within the GIT of the pig

Phytase enzymes are ubiquitously present throughout nature. Common sources include plants and microorganisms, particularly bacteria, fungi and yeast. Microbial phytases are generally 3-phytases, whereas plant phytases are usually 6-phytases (Dvorakova, 1998). Although less common, phytase has also been detected in some animal tissue such as the intestinal mucosa and liver (McCollum and Hart, 1908; Maenz and Classen, 1998). Sources of phytase that may hydrolyse dietary phytate within the GIT of pig include: (i) plant phytases, (ii) intestinal mucosa, (iii) GIT microbial phytases or (iv) supplementary microbial phytases.

1.4.2.1 Plant phytases

Phytase has been detected in numerous species of commonly used feed ingredients, although its activity varies greatly between different feedstuffs (Table 1.1). Grains such as wheat, triticale and barley contain significantly more phytase activity than ingredients such as maize or SBM. Studies in both pigs (Pointillart et al., 1987; Steiner et al., 2006) and poultry (Paik, 2003) have shown that plant phytases can indeed improve PP bioavailability. However, as these enzymes are largely heat sensitive their activity is significantly reduced or even eliminated during the pelleting process (Jongbloed and Kemme, 1990). Consequently, the contribution of phytase activity originating from the dietary ingredients is usually disregarded.

1.4.2.2 Mucosal phytases

As with plant phytases, the contribution of mucosal phytases to phytate hydrolysis within the GIT is frequently dismissed. Although studies have demonstrated that the intestinal mucosa of the pig is capable of producing phytase (Spitzer and Phillips, 1945; Pointillart, 1993), its activity is thought to be limited by the absence of available substrate. This is due to the insolubility of the phytate molecule at intestinal pH. Schlemmer et al. (2001) determined the solubility of $InsP_{2-6}$ in the small intestine of the pig (mean pH 6.6 $\pm$ 0.3) and found the solubility of $InsP_2$, $InsP_3$, $InsP_4$, $InsP_5$ and $InsP_6$ to be 75%, 31%, 8%, 7% and 2% respectively. Thus,

as the degree of phosphorylation decreases, the solubility of phytate at intestinal pH increases. This can be attributed to a diminishing affinity to chelate with cationic minerals.

Hu et al. (1996) performed an *in vitro* study in which pig duodenal, jejunal and ileal mucosal samples were incubated with purified $InsP_{3\text{-}6}$ isolates to determine the associated phytase activity. The authors found that mucosal phytase efficacy was highest in the jejunum and lowest in the ileum. In addition, the hydrolytic efficacy of mucosal phytase decreased as the number of phosphates on the substrate increased. The authors postulated that mucosal phytases are more important in the breakdown of the lower phytate esters ($InsP_3$ and below).

1.4.2.3 GIT microbial phytases

It is also thought that dietary phytate can be hydrolysed by phytase derived from the microbiota residing in the large intestine (Seynaeve et al., 2000; Rutherfurd et al., 2014b). Schlemmer et al. (2001) offered finisher pigs (BW 92 kg) one of two dietary treatments; a control diet with high intrinsic phytase activity, or the same diet with low intrinsic phytase activity (heat treated). The authors found that the apparent total tract degradability of phytate was similar for both treatments; however, apparent P digestibility was markedly lower in pigs fed the phytase inactivated diet than the control diet. The authors surmised that the phytate degradation taken place in the pigs fed the low phytase diet was most likely occurring in the hind-gut, beyond the site of major P absorption. As P is primarily absorbed in the proximal half of the small intestine (Low, 1980), the contribution of gut microfloral phytases to phytate degradation is of little use to the pig.

1.4.2.4 Exogenous microbial phytases

Phytases produced by microbes have been shown to possess greater catalytic activity for phytate within the GIT than plant phytases (Rapp et al., 2001). This was demonstrated in a study by Zimmermann et al. (2002), as they compared the efficacy of two cereal phytases (wheat and rye) with a microbial *A. niger* derived phytase (Natuphos®) in grower pigs offered a P marginal diet. The authors reported a 2.5-fold improvement in PP availability in pigs receiving the microbial phytase treatment compared to those receiving the cereal phytase treatments. This difference in efficacy can be attributed to differences in pH optima, as microbial phytases possess greater catalytic activity at lower pH values (such as those experienced in

the stomach) than plant phytases (Greiner and Konietzny, 2006) . In addition, microbial phytases are less susceptible to pepsin induced proteolysis (Phillippy, 1999).

The advent of genetic engineering technology in the 1980s was pivotal in the successful development of large-scale microbial phytase production. The first generation of commercial phytase feed enzymes were mainly of fungal origin (most notably *A. niger*) owing to their extracellular secretory properties and their tolerance to low pH values (Kim et al., 1998). However, a major drawback of these fungal phytases is that they tend to be most active at a near neutral pH (Lei et al., 2013). For example, *A. niger* is most active at pH 5-5.5 (100%) and although 60% of its activity is retained at pH 2, there is a large drop in activity at pH 3.5 (Tran et al., 2011). This drop in phytase activity is significant given that the pH of the pig's stomach is typically around 3.5 (Yi and Kornegay, 1996). Therefore, large amounts of this enzyme must be added to pig diets in order to liberate sufficient PP (Lei and Stahl, 2001).

The isolation of an *Escherichia coli* (*E. coli*) derived phytase in 1999 led to the development of a second generation of bacterial phytases (Rodriguez et al., 1999). These bacterial phytases possess a number of superior biochemical properties over the traditional fungal phytases within the GIT (Augspurger et al., 2003). For instance, several comparative *in vitro* studies have demonstrated that *E. coli* derived phytases have greater specific activity for phytate across a wide range of physiologically relevant pH values (2.5 to 4.5) than fungal phytases (Morales et al., 2011; Tran et al., 2011). In addition, bacterial-derived enzymes are more resistant to proteolysis from pepsin, trypsin and chymotrypsin and hence remain stable and active in the GIT for longer (Kumar et al., 2003; Igbasan et al., 2000; Morales et al., 2011).

An 'ideal' phytase has been described as one that is highly effective at hydrolysing phytate in the GIT of the target animal, resistant to proteolysis, thermostable and cheap to manufacture (Lei and Stahl, 2001). As no known wild type phytase meets all of these criteria, the search for an ideal phytase has shifted towards improving the characteristics of existing ones through protein engineering. The two techniques commonly employed to induce changes to the protein molecule include rational design and directed evolution (Yao et al., 2012). Rational design involves making

select changes to the protein structure based on similar proteins with desired characteristics, whereas directed evolution involves making random changes to the protein structure, and then screening for mutants exhibiting superior biochemical properties.

In the study of Garrett et al. (2004), directed evolution was successfully employed to improve the thermostability and gastric performance of an *E. coli* (*AppA*) phytase. Using gene site saturation mutagenesis technology, the authors generated a library of cloned constructs incorporating all possible amino acid substitutions at 431 sites of the gene. Following a heated incubation high-throughput screening assay, a construct (Phy9X) comprising 8 single-site amino acid substitutions displayed a 12 °C higher melting temperature and was found to be 3.5-times more stable in simulated gastric fluid. Other studies have successfully optimised phytase thermostability (Jermutus et al., 2001; Zhang et al., 2007), specific activity (Tian et al., 2011) and the pH optima profile (Kim et al., 2006) from a variety of microbial sources. At present, the phytase market is dominated by this third generation of modified microbial phytase feed enzymes.

1.4.3 Measuring phytase activity

Phytase activity is commonly measured in phytase units (FTU) and expressed per unit of feed. One FTU denotes the amount of enzyme necessary to release 1 µmol of Pi/minute from an excess of sodium phytate (0.0051 mol/L) at 37 °C and pH 5.5 *in vitro* (Engelen et al., 1994). As different phytases have different pH activity profiles and reaction kinetics, the *in vivo* P release value associated with 1 FTU/kg for a particular product may differ to that of another (Goncalves et al., 2016). These enzymatic differences make it difficult to compare different phytase products. This is further compounded by the number of alternative units of measurement for phytase activity that exist, each with their own definition. Such complications can make drawing comparisons between studies that have used different phytase products somewhat challenging. A standardised method for measuring phytase activity is needed to simplify phytase application for end users. For consistency, phytase activity will be expressed in FTU throughout this thesis.

Questions have been raised over the suitability of the FTU measurement assay for estimating *in vivo* phytase efficacy, with many suggesting that the assay should be performed under more physiologically relevant conditions, for example at a lower

pH and with a more appropriate substrate. Logically, a more physiologically relevant assay would give a better indication of the expected enzyme efficacy *in vivo*.

1.4.4 Effect of phytase on P bioavailability

The recent success of feed phytase technology in livestock nutrition is largely down to its consistently tangible effect on improving dietary P availability. Phytase's capacity to effectively hydrolyse phytate in the proximal regions of the digestive tract and free up otherwise unavailable PP is well established and was first demonstrated in pigs by Simons et al. (1990). The authors reported a 26% increase in P digestibility in grower pigs fed a low-P maize-SBM diet supplemented with 1,000 FTU/kg of an *A. niger* phytase. This was met with a concomitant 35% reduction in P excretion.

A plethora of studies have since confirmed this phytase effect in weaner, grower and finisher pigs (Dungelhoef et al., 1994; Harper et al., 1997; Augspurger et al., 2003). At the commonly recommended inclusion rates of 500 to 1,000 FTU/kg, phytase generally improves P digestibility by anywhere between 8 and 34% (Table 1.2). Such improvements in P availability are associated with reductions in P excretion, thus highlighting the major role these enzymes have in mitigating agricultural P pollution. As with many other enzyme catalysed reactions, P release from phytate due to phytase follows a law of diminishing returns, therefore, the amount of P liberated gradually diminishes with each incremental increase in phytase activity.

The amount of supplementary Pi that may be displaced by phytase is known as the phosphorus equivalency value. These values are normally calculated in feeding trials whereby pigs are fed a P deficient diet supplemented with increasing amounts of Pi or phytase, and a P sensitive response, such as growth performance, P digestibility or bone ash is measured and the two are compared statistically using linear or non-linear equations.

Many studies have determined P equivalency values in pigs for a range of different phytase enzymes (Harper et al., 1997; Augspurger et al., 2003; Adeola et al., 2006). Prior to the advent of third generational phytases, feed industries commonly assigned a P matrix value of 1.0 to 1.2 g/kg to their phytase products at the recommended dose of 500 FTU/kg (Selle and Ravindran, 2008). At the same dose, the new generation of modified phytases are typically assigned a P matrix value of

between 1.3 and 1.4 g/kg (Adeola and Cowieson, 2011). However, from the literature, it is clear that there is considerable variation in the P equivalency values generated between studies. Phytase efficacy in the gut is influenced by a variety of factors including diet and nutrient composition (particularly total P and Ca levels), therefore, these factors as well as the differences in choice of P sensitive response variable, Pi source, type and level of phytase activity in the studies may have contributed to the variable P equivalency estimates seen in the literature. Factors influencing phytase efficacy within the GIT will be discussed further in Section 1.7.

In order to maximise the economic and ecological use of phytase it is important for accurate P equivalency values to be determined. Underestimation of the P equivalency value of a phytase product will likely result in excessive P wastage, whereas overestimation may lead to compromised growth and weaker bones. Poulsen et al. (2007) suggested that all phytase enzymes should be tested under the appropriate dietary conditions, thus a product may have different P equivalency values for different diets.

Table 1.2. Summary of results on the effect of phytase on apparent total tract P digestibility (%) in pigs from selected studies

Diet	Phytase source[b]	Percent increase in P digestibility over control[c]	Reference
Wheat-barley	*P. lycii* (375)	11	Poulsen et al. (2007)
Wheat-barley	*A. niger* (500)	18	Poulsen et al. (2007)
Wheat-barley	*A. niger* (500)	20	Poulsen et al. (2010)
Wheat-barley	*A. niger* (750)	23	Poulsen et al. (2007)
Wheat-barley	*A. niger* (830)	8	Blaabjerg et al. (2012)
Wheat-barley	*P. lycii* (750)	17	Poulsen et al. (2007)
Wheat-barley	*P. lycii* (1,000)	34	Vigors et al. (2014)
Wheat-SBM[a]	*E. coli* (500)	13	Sands and Kay (2007)
Wheat-SBM	*E. coli* (1,000)	19	Sands and Kay (2007)
Corn-Barley	*A. niger* (1,000)	9	Emiola et al. (2009)
Corn-SBM	*A. niger* (500)	13	Johnston et al. (2004)
Corn-SBM	*P. lycii* (500)	9	Veum et al. (2006)
Corn-SBM	*E. coli* (500)	10	Veum et al. (2006)
Corn-SBM	*E. coli* (500)	8	Jendza et al. (2005)
Corn-SBM	*E. coli* (500)	11	Adeola et al. (2004)
Corn-SBM	*E. coli* (750)	15	Adeola et al. (2014)
Corn-SBM	*E. coli* (1,000)	25	Jendza et al. (2005)
Corn-SBM	*C. braakii* (500)	21	Almeida et al. (2013)
Corn-SBM	*C. braakii* (1,000)	25	Almeida et al. (2013)
Corn-SBM	*C. braakii* (1,000)	21	Guggenbuhl et al. (2012a)
Corn-SBM	*C. braakii* (1,105)	34	Rutherford et al. (2014)

[a]SBM = soyabean meal
[b]Number in parenthesis represents phytase activity (FTU/kg)
[p]Difference in apparent phosphorus digestibility (%) between phytase treatment and no phytase control.

1.5 Phytate as an anti-nutrient

Extensive research in the area of phytase in monogastric nutrition over the last two decades has revealed that phytate is a highly reactive compound with anti-nutritional effects that encompass more than just an unavailable source of P. The negative effects of phytate on mineral availability, most notably on di- and trivalent cations, has been known for some time (Davies and Nightingale, 1975; Nwokolo and Bragg, 1977). More recently however, it has been shown that phytate can also interact with amino acids (AA), starch and lipids within the GIT and thereby reduce protein and energy digestibility (Selle et al., 2000; Lee et al., 2006; Ravindran et al., 2006; Cowieson and Ravindran, 2007). Furthermore, phytate-protein interactions in the foregut of the animal are thought to trigger an array of nutritional consequences including: increased endogenous AA and mineral secretions, reduced nutrient absorption, disruption of the luminal dietary electrolyte balance (Selle and Ravindran, 2008) and a reduction in the efficacy of digestive enzymes such as α-amylase, sucrose, maltase and pepsin (Dersjant-Li et al., 2015).

Consequently, the ingestion of phytate can have adverse effects on monogastric growth performance and nutrient use efficiency. For example, Woyengo et al. (2012) found that supplementing a casein-cornstarch diet with 2% phytate for 21 days reduced piglet ADG and increased FCR by 37% and 25% respectively. Similarly, with broilers, Onyango and Adeola (2009) demonstrated a 28% decrease in ADG and an 8% increase in FCR upon the addition of 2% phytate to a casein diet for 2 weeks. Here, the authors also noted that the adverse effects of phytate on growth performance could in part be mitigated by phytase supplementation at 1,000 FTU/kg.

1.5.1 Phytate effect on mineral availability

Following a review of the literature, Cheryan (1980) surmised that phytate can chelate almost all divalent cations and that the stability of the complexes, in order of strongest to weakest, is as follows: Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+}. Several others have also found that phytate forms stronger complexes with Zn^{2+} and Cu^{2+} than with other minerals, and there is general agreement that the strength of the phytate-mineral interaction increases as the relative atomic mass of the mineral

increases (Pontoppidan et al., 2007a). Bound cations may interact with one phosphate from the phytate molecule, or form a cationic bridge between two phosphates from either the same or different molecules (Erdman, 1979). These mineral chelates may exist as an insoluble complex that is resistant to hydrolysis, or alternatively an available soluble chelate (Cheryan, 1980).

The extent of phytate-mineral complex formation that occurs within the GIT, and the solubility of these complexes, is driven by the relative proportions of mineral:phytate and the ambient pH (Weaver and Kannan, 2002). Typically at pH values of 6 - 7 or above, such as those experienced in the distal intestines, mineral complexes begin to precipitate from solution. This is particularly true when the mineral:phytate ratio is high. In general, as the mineral:phytate ratio increases, the pH necessary for complex precipitation decreases (Grynspan and Cheryan, 1983; Pontoppidan et al., 2007a). Conversely, at low pH, there is greater protonation of the phytate molecule, and thus the potential for the formation of insoluble complexes is minimised (Maenz, 2001). Using a potentiometric technique, Persson et al. (1998) found that stability of the phytate-mineral chelates decreased as the level of phosphorylation on the phytate molecule decreased. It was concluded that degradation of phytate to at least $InsP_3$ is necessary to minimise insoluble mineral complex formation at higher pH levels and thus improve both mineral and phytate bioavailability.

Oberleas et al. (1962) were among the first to demonstrate the inimical effects of phytate on mineral availability in pigs. Here, weaned pigs were fed a purified casein control diet with or without supplementary phytate (0.7%) or zinc (Zn; 100 mg/kg) for 6 weeks. Pigs fed the diet containing supplementary phytate had a depressed rate of growth and displayed signs of Zn deficiency, such as parakeratosis. These effects were not observed in pigs offered the same diet with supplementary Zn. The authors concluded that phytate was inhibiting Zn absorption.

Numerous *in vivo* studies have since confirmed an inverse relationship between phytate concentration and cationic mineral digestibility in a range of monogastric species. Woyengo et al. (2009a) fed piglets a casein-maize based diet supplemented with 0, 5, 10 or 20 g/kg sodium phytate and reported quadratic reductions in the AID of Na, K and P, and linear reductions in the AID of Ca and Mg in response to increasing phytate concentration. Interestingly, the digestibility coefficients of both

Mg and Na were negative in pigs receiving the 20 g/kg of sodium phytate treatment (-0.03% and 0.18% respectively), suggesting that phytate is stimulating the endogenous secretions of these minerals. It is not yet fully understood how phytate stimulates endogenous mineral secretions; however, it has been proposed the presence of insoluble phytate-protein complexes trigger endogenous secretions of Na (as sodium bicarbonate) into the intestines (discussed below in Section 1.5.3; Cowieson et al., 2004). Alternatively, it is possible that through chelating with enzymatic cofactors, phytate is stimulating compensatory mineral outputs via negative feedback mechanisms (Woyengo et al., 2009).

Ravindran et al. (2006) reported a similar effect of phytate on mineral absorption in broilers, as they observed reductions in the AID coefficients of Ca, P, Fe, and Na in response to a 3.2 g/kg increase in dietary phytate concentration. Similarly, in fish, Gatlin and Phillips (1989) found that a 1% increase in dietary phytate reduced Zn availability in Channel Catfish. Many have also studied this inhibitory effect in humans, most notably for Fe, Zn and Ca as deficiencies of these minerals are of global concern (Hallberg et al., 1989; Hurrell et al., 2003; Hambidge et al., 2004; Hambidge et al., 2005).

1.5.1.1 Phytate-calcium interactions

Although phytate has a stronger affinity for minerals such as Cu and Zn, the formation of *de novo* phytate-Ca complexes are considered more important (Angel et al., 2002). This is because Ca is typically present in pig diets at levels of 50 to 100-fold more than other cations. In a recent review of Ca-phytate interactions in pigs and poultry, Selle et al. (2009a) concluded that up to one third of ingested Ca may be tied up in a phytase resistant Ca-phytate complex. The general consensus is that Ca-phytate complexation occurs in the small intestine, in a 4.93:1 (Ca:phytate) molar ratio (Marini et al., 1985). Precipitation of the Ca-phytate complex begins to take place at pH 4 to 4.5, and increases with increasing pH (Wise and Gilburt, 1981; Grynspan and Cheryan, 1983; Oberleas and Chan, 1997). As the level of phosphorylation on the phytate molecule decreases, the affinity of the molecule to chelate Ca diminishes in a disproportionate fashion. For example, $InsP_3$ has 10% of the binding potential of $InsP_6$ (Luttrell, 1993).

A common misconception in phytate nutrition is that monogastric species lack the enzymes necessary to hydrolyse dietary phytate. However, this is not the case, and

several studies have in fact demonstrated that monogastric species can effectively digest phytate, so long as it remains soluble in the intestines. For example, in a study by Nahapetian and Young (1980), rats were fed a high Ca (30.6 mM/ 100 g) or a low Ca (2.9 mM/ 100 g) diet and were provided with an oral dose of C^{14} labelled phytate. The authors reported a 54% reduction in phytate excretion and a significant increase in C^{14} recovery from body tissues in rats fed the low Ca diet. Similarly, Tamim et al. (2004) reported a 63% reduction in the AID coefficient of phytate in broilers fed a corn-SBM diet following an increase in dietary Ca from 2 to 7 g/kg. These studies show that phytate can be hydrolysed by the animal provided the dietary Ca concentration is low. The negative effect of Ca on phytate availability can be attributed to the formation of insoluble Ca-phytate complexes that render both constituents unavailable for absorption (Wise, 1983). Consequently intestinal Ca levels are considered the primary determinant of phytate availability.

1.5.2 Protein-protein interactions

The situation regarding phytate-protein interactions remains somewhat unclear, but as with minerals, phytate can interact with proteins and in doing so restrict AA digestibility (Table 1.3). In contrast to the studies presented in Table 1.3, Woyengo et al. (2009) reported that the addition of 2% phytate to a casein-cornstarch diet had no effect on the AID of amino acids in weaner pigs. Likewise, Knuckles et al. (1989) found that the addition of 2% phytate to a casein-cornstarch diet had no effect on amino acid digestibility in rats. These equivocal amino acid responses to phytate may be due to differences in diet composition or phytate concentration (Woyengo and Nyachoti, 2013), however, this remains to be tested.

The precise mechanism by which phytate restricts protein availability remains to be determined. Three proposals for the direct repression of AA digestibility by phytate include: (i) binary complex formation, (ii) ternary complex formation, and (iii) kosmotropic properties of phytate. It has also been suggested that phytate may negatively affect protein digestibility indirectly by stimulating endogenous AA secretions and compromising intestinal Na-dependent AA absorption systems (discussed below in Section 1.5.4).

1.5.2.1 Binary phytate complexes

Phytate can directly interact with protein via electrostatic interactions and in doing so form binary protein-phytate complexes (Cosgrove, 1966). This type of interaction occurs when the negative charges of the phosphate moieties on the phytate molecule interact with the positive charges found on basic amino acids (Hys, Lys and Arg) at pH levels below their iso-electric (IE) point (Prattley et al., 1982; Rajendran and Prakash, 1993). Using Na-phytate and beta-Globulin derived from *Sesamum indicum* L., Rajendran and Prakash (1993) studied the kinetics of binary phytate-protein complex formation by stopped-flow spectrophotometry. The authors found the reaction was bi-phasic, with an initial rapid electrostatic attraction between phytate and the basic amino acids, which was followed by a gradual accumulation of protein molecules. This accumulation of proteins results in the generation of a high molecular weight agglomeration that precipitates from solution. Consequently, these binary protein-phytate complexes are largely refractory to gastric proteolysis (Vaintraub and Bulmaga, 1991; Kies et al., 2006a).

Binary phytate-protein complex formation is determined by phytate and protein concentration, protein source and type, cation concentration (particularly Ca) and ambient pH (Kemme et al., 1999). A low pH is necessary for binary phytate-protein complex formation, therefore, it is generally agreed that the stomach is the main site of binary complex formation in the pig (Yunusova and Moiseeva, 1987; Vaintraub and Bulmaga, 1991). In the aforementioned study, Rajendran and Prakash (1993) found that phytate-beta-Globulin complexing was greatest at pH 2.3. More recently, Kies et al. (2006) tested the effect of phytate addition on protein solubility across of range of different ingredients *in vitro*. Marked reductions in protein solubility were recorded at pH 2, with minimal effects on solubility at pH 3 and above. Thus, it was concluded that a low pH (pH $\leq$2) is conducive to binary complex formation.

1.5.2.2 Ternary phytate complexes

Ternary protein-phytate complexes form when proteins, at pH values above their IE point (carrying a negative charge), interact with phytate via a cationic bridge (Prattley et al., 1982). These complexes tend to form in regions of high pH, such as the distal small intestine, using Ca as the link between the phytate and protein molecules (Reddy and Salunkhe, 1981). Ternary complexes are thought to bind primarily with low molecular weight peptides or amino acids, owing to their site of

formation, and are thus frequently dismissed as having little influence on protein digestibility (Champagne et al., 1990).

1.5.2.3 Kosmotropic properties of phytate

More recently, it has been proposed that phytate may influence protein solubility via the exertion of kosmotropic-like effects (Cowieson and Cowieson, 2011). The Hofmeister series classifies various salts as either a kosmotrope (salting-out) or a chaotrope (salting-in) depending on their propensity to precipitate proteins (Hofmeister, 1888, cited in Baldwin, 1996). A salt that improves protein stability and reduces solubility is classed as a kosmotrope, and conversely, a salt that decreases protein stability and increases solubility is classed as a chaotrope. The mode of action of the Hofmeister series remains uncertain, but is believed to stem from alterations to the surrounding water structure (Baldwin, 1996). A kosmotrope denotes an ion that can improve water structure through the formation of hydrogen bonds which, in turn, increases the stability of the water network. This structure making effect essentially depletes solubilised proteins of their hydration, resulting in protein precipitation. Chaotropes, however, have the opposite effect and can bring disorder to the hydrogen bonding network of water, thereby improving protein solubility. According to the Hofmeister series phosphate (HPO_4^{2-}) is a kosmotropic anion (Zhang et al., 2005), hence the suggestion that phytate may be altering protein solubility indirectly via interactions with the surrounding water medium.

Table 1.3. Impact of dietary phytate on apparent digestibility (%) of essential amino acids[a]

Reference	Species	Increase in dietary phytate (%)	Amino acid								
			Arg	His	Iso	Leu	Lys	Met	Phe	Thr	Val
Bohlke et al. (2005)	Pig	NR[b]	- 4.5	NS[c]	- 3.3	NS	- 8.1	NS	- 3.0	- 4.8	- 5.8
Liao et al. (2005a)	Pig	0.78	- 0.7	- 4.0	- 3.7	- 2.1	- 1.4	NR	- 4.2	- 4.6	- 4.6
Ravindran et al. (2000)	Broiler	0.53	- 0.7	- 1.6	- 1.9	- 1.6	- 1.4	NR	- 1.9	- 2.5	- 1.8
Ravindran et al. (2006)	Broiler	0.32	- 2.3	- 4.8	- 2.5	- 2.2	- 2.8	NS	- 2.8	- 4.0	- 3.6

[a]Table adapted from Woyengo and Nyachoti (2013)
[b]NR = not recorded
[c]NS = non-significant.

Bye et al. (2013) challenged this theory with an *in vitro* study involving Na-phytate and lysozyme (IE = 11.4). The authors tested the effect of phytate on lysozyme stability and solubility using direct scanning calorimetry (DSC) and precipitation methods respectively. DSC data showed that increasing the phytate concentrations from 5 mM to 100 mM did indeed increase lysozyme stability, with a 3.2 °C increase in T_m value (temperature of maximum protein unfolding), which is consistent with the kosmotrope tenet. However, at phytate concentrations of 0.4 to 5 mM, phytate had the opposite effect and reduced protein stability. This was attributed to binary phytate-complex formation. Furthermore, the solubility data were in fact contradictory to the DSC data, and showed that as the phytate concentration increased from 4 to 30 mM, the solubility of lysozyme increased. This suggests phytate was acting as a chaotrope rather than a kosmotrope. The authors concluded that phytate-protein interactions are more complex than originally thought and that phytate may indeed act as a kosmotropic anion, but at lower phytate concentrations electrostatic interactions between phytate and protein are dominant. Clearly, the ability of phytate to indirectly impede protein availability through kosmotropic effects is a complex issue that merits further investigation.

1.5.3 Consequences of phytate-protein interactions

The formation of insoluble phytate-protein complexes is thought to trigger a cascade of anti-nutritional effects in monogastric animals, including: increased endogenous AA secretions and losses, increased luminal dietary electrolyte balance (dEB = $Na^+ + K^+ - Cl^-$ meq kg^{-1}), and impaired enterocyte nutrient absorption. It is thought that the presence of pepsin resistant phytate-protein complexes in the stomach stimulates additional outputs of pepsin and hydrochloric acid (HCl) through negative feedback mechanisms. Similar responses have been demonstrated in rats with condensed tannins (Mitjavila et al., 1973), which like phytate are anti-nutrients with the capacity to bind proteins and impede protein digestion. Mitjavila et al. (1973) reported a ~60% increase in gastric pepsin and HCl output in response to frequent tannic acid administration. Further evidence to support the notion that phytate increases pepsin and HCl secretions was provided by Woyengo et al. (2010), as they found that supplementation of 2% Na-phytate to a casein-cornstarch diet reduced stomach digesta pepsin activity by 46% and reduced jejunal pH from 7.13 to 6.61 in weaners. Similar results have been observed in broiler chicks (Liu et al., 2009; Morgan et al., 2016).

Both pepsin and HCl are capable of damaging the epithelial lining of the intestine and are therefore known as endogenous aggressors (Allen et al., 1986). An increase in the secretion of these aggressors would be met with increased gastroduodenal secretions of mucin and sodium bicarbonate ($NaHCO_3$), to protect the epithelial lining of the GIT and neutralise the acid respectively (Allen et al., 1986). Cowieson et al. (2004) were among the first to show that phytate does indeed stimulate mucin (as sialic acid) and Na secretions in broiler chickens. Onyango et al. (2009) have since confirmed this, as they too reported an increase in mucin loss from the GIT in response to phytate supplementation in broilers. Interestingly, in an earlier study by Onyango et al. (2008), it was shown that phytate increased *Muc1* and *Muc2* (genes involved in mucin synthesis) gene expression in the jejunal tissue of mice. Thus, it is plausible that phytate would have similar effects on mucin and Na secretion in the pig.

That phytate may stimulate secretion of mucin into the intestine is important as it has recently been established that phytate increases endogenous AA losses (Cowieson et al., 2008; Onyango et al., 2009). Endogenous AA losses are dictated by the relative amounts of AA that are secreted and not re-absorbed in the intestine (Nyachoti et al., 1997). Thus, increased endogenous losses may be associated with increased AA secretions, reduced AA absorption, or a combination of both. It is noteworthy that pig mucin is 34.3% protein and is largely resistant to enzymatic hydrolysis. Therefore, an increase in mucin secretion in response to phytate-protein complexes will undoubtedly result in an increase in endogenous AA losses (Lien et al., 1997). The study of Cowieson and Ravindran (2007) supported the view that phytate increases endogenous AA losses, primarily in the form of mucin. Using the peptide alimentation method, the authors investigated the effect of phytate (supplemented as Na-phytate) and phytase on the composition and amount of ileal endogenous AA losses in broilers. Interestingly, they reported a significant correlation ($r = 0.762$) between the AA composition of mucin and the reductions in endogenous AA losses due to phytase. Furthermore, the authors also found that the ileal amino acid profiles were also consistent with phytate increasing endogenous AA losses through an increase in pepsin secretion.

Phytate increases Na levels in the small intestine, presumably through increased $NaHCO_3$ secretion (Woyengo et al., 2009; Woyengo et al. 2010) and in doing so increases the luminal dEB. In the pig small intestine, the active transport of sugars,

amino acids and minerals is largely driven by a transmembrane Na gradient which is maintained by Na-K-adenosine triphosphotase (ATPase) in enterocytes. Studies have demonstrated that low Na diets can impair Na-K-ATPase activity, resulting in compromised nutrient uptake (Gal-Garber et al., 2003). Therefore, it may be that phytate is impeding nutrient absorption in the small intestine (particularly endogenous or dietary amino acids and glucose) by compromising Na dependant transport systems. The work of Dilworth et al. (2005) in rats and that of Liu et al. (2008) in broilers attests this proposition, as both studies found that phytate supplementation reduced enterocyte Na-K-ATPase activity. Furthermore, Woyengo et al. (2012) investigated the effect of phytate on jejunal electrophysiological properties in the weaner pig using Ussing chambers. They reported that supplementation of casein-cornstarch diet with 2% phytate reduced jejunal short-circuit current by 7.97 $\mu A/cm^2$, which was attributed to reduced Na dependant nutrient transport.

It would appear that phytate is capable of stimulating endogenous AA secretions, chiefly as mucin and pepsin, and impairing re-absorption; however, much of the work of that has led to this hypothesis has been carried out in poultry or rats. Studies exploring the effect of phytate on endogenous AA losses in the pig are limited but future work in this area would prove instructive and improve our understanding of the anti-nutritional effects of phytate, and reciprocally, the potential for phytase application.

1.5.4 Phytate effect on energy availability

There is evidence to suggest that phytate has inimical effects on energy availability in monogastric animals (Liao et al., 2005a; Ravindran et al., 2006). The negative effects of phytate on energy digestibility were first highlighted almost half a century ago by Rojas and Scott (1969), who found that feeding chickens de-phytinised cottonseed meal improved ME digestibility. In addition, Yoon et al. (1983) found a negative correlation between dietary phytate intake and the blood glycaemic response in humans, which suggests that phytate is impairing glucose uptake. In grower pigs, Liao et al. (2005a) reported a 4.7 and a 5.2 % decrease in AID and apparent total tract digestibility (ATTD) of digestible energy (DE) respectively, in response to a 0.92 % increase in dietary phytate concentration.

Indeed, an increase in endogenous AA secretions and losses will be nutritionally and energetically expensive and most likely increase the energy and AA maintenance requirement of the animal (Nyachoti et al., 1997). Increased endogenous AA secretions are associated with an increased rate of protein turnover in the gut, increased blood flow to the gut and greater movement of digestion products along the GIT, which are all energetically expensive processes (Nyachoti et al., 1997). Therefore, increased AA gastric secretions may be one of the ways in which phytate impedes energy digestibility.

It is also possible that phytate directly impedes energy availability through interactions with carbohydrates or lipids (Thompson et al., 1987), or indirectly through interactions with digestive enzymes and their metallic cofactors (Selle and Ravindran, 2007). Both *in vivo* (Thompson et al., 1987; Lee et al., 2006) and *in vitro* (Thompson and Yoon, 1984) studies have demonstrated that phytate can reduce starch digestibility. The mechanism for this response is unclear, however, Rickard and Thompson (1997) suggested that phytate may directly depress starch digestion by (i) binding to starch directly via phosphate links, (ii) binding to starch indirectly via starch granule-associated proteins (SGAP), (iii) binding directly to amylase enzymes (iv) or by chelating with Ca, a cofactor required for normal amylase activity. There is little evidence to support the notion of direct phytate-starch complexing, however, it has been demonstrated that phytate can effectively diminish α-amylase activity (Deshpande and Cheryan, 1984). More recently, it has been shown that phytate can also suppress duodenal maltase and sucrase activity (Liu et al., 2008). Thus, the inimical effects of phytate on energy digestibility may in part stem from compromised carbohydrate digestion.

In respect of lipids, it is possible that phytate complexes with lipids, peptides and divalent cations (mainly Ca and Mg) in the intestines to form ‘lipophytins’ (Ravindran et al., 2000). The constituents of lipophytins are largely resistant to digestion and thus are excreted as metallic soaps (Atteh and Leeson, 1983). Alternatively, phytate may be indirectly impairing lipid digestion by reducing lipase efficacy (Knuckles et al., 1989; Liu et al. 2009).

1.6 Extra-phosphoric effects of phytase

Historically, exogenous phytases were supplemented to pig diets to improve dietary P bioavailability and reduce P levels in the excreta. However, over the last decade the extra-phosphoric effects of phytase have become increasingly apparent, and now it is not uncommon for phytase enzymes to be assigned matrix values for other nutrients such as Ca, Na, AA and sometimes even energy. It has been suggested that use of exogenous phytase to partially ameliorate the anti-nutritional effects of phytate may be of primary importance to liberation of PP (Adeola and Cowieson, 2011). Recent research has demonstrated that phytase can improve mineral, AA and energy digestibility and reduce endogenous secretions in the animal. However, these extra-phosphoric effects of phytase remain contentious, especially in regard to AA and energy availability.

1.6.1 Effect of phytase on mineral digestibility

Microbial phytases are able to improve the bioavailability of cations by hydrolysing ingested phytate prior to the site of mineral-complex formation. The minerals that are most influenced by phytase supplementation, other than P, are Ca, Mg, Fe and Na (Cowieson and Ravindran, 2007). Using a 2 x 2 factorial design of treatments, Shelton et al. (2005) assessed the effect of phytase (0 or 500 FTU/kg) and trace mineral premix (TMP) supplementation (0 or 1 g/kg) to a corn-SBM diet on pig growth performance. The TMP provided 127 mg Zn, 127 mg Fe; 20 mg Mn, 12.7 mg Cu, 0.80 mg I and 0.30 mg Se per kg of feed. The diet devoid of phytase or TMP supplementation (replaced with sand) served as the negative control and was formulated to be deficient in the aforementioned minerals. The control diet (+TMP) was formulated to be nutritionally adequate. Ca and P levels in diets with added phytase were reduced by 0.1%. Pigs receiving the negative control diet had a 19% reduction in feed intake and weight gain throughout the weaner stage in comparison to those receiving the control treatment. Adding supplementary phytase or both the phytase and TMP improved pig performance back to the level of the control treatment. In addition, the authors observed that 50% of the pigs fed the negative control diet displayed signs of parakeratosis. Interestingly, this effect was not apparent in any of the other treatments. These finding suggests that phytate improves the bioavailability of phytate-bound minerals, and thus has the potential to replace TMP supplementation in corn-SBM based weaner diets without

compromising pig growth performance. In a more recent study, Adedokun et al. (2015) observed quadratic improvements in the AID of N, Ca, P, Mg and K in response to increasing phytase dose in grower pigs. Similarly, Madrid et al. (2013) reported that phytase increased the ATTD of P, Ca, Mg and Cu in growers. It is noteworthy that in the study of Kies et al. (2005) phytase improved the ATTD of the monovalent cation Na. Monovalent cations form very soluble complexes with phytate (Erdman, 1979), therefore it seems unlikely that any improvement in Na digestibility would arise from improved availability through phytate degradation, but rather reduced endogenous losses. This supports the previously described notion that phytate triggers endogenous secretions of Na into the intestines as $NaHCO_3$ in response to phytate-bound protein in the stomach (Section 1.5.3).

As discussed in Section 1.5.1.1, phytate in its fully phosphorylated form can bind with up to 5 Ca atoms in the pig intestine. Therefore, in theory, complete phytate hydrolysis would release 6 phosphates and 5 Ca atoms. Although phytases are more commonly known for their ability to improve P bioavailability, this suggests that their effect on Ca bioavailability may be as significant. Many studies have shown that supplementary microbial phytase enzymes improve Ca bioavailability in pigs (O'Quinn et al., 1997; Traylor et al., 2001; Adedokun et al., 2015). As with P, efforts have been made to determine Ca equivalency values for different phytase products, but this research is predominantly limited to poultry. The generated calcium equivalency values for poultry are variable and range from 0.44 to 0.9 g/kg for 500 FTU/kg. Kornegay et al. (1996) completed two similar Ca equivalency studies in pigs using limestone as the Ca source, and growth rate, Ca ATTD and bone ash percentage as the Ca sensitive parameters. The authors reported two very different equivalency estimates for 500 FTU/kg of phytase of 1.08 g/kg and 0.38 g/kg. It is likely that the use of different phytase enzymes, species, diets, choice of response criteria, Ca levels, P levels and Ca:P ratio have all contributed to the varying results seen in the literature. It has been suggested that Ca equivalency studies are compromised due to the obligatory low dietary Ca levels necessary for equivalency analysis, and that Ca digestibility measurements would be of more use (Selle et al., 2009a).

1.6.2 Effect of phytase on protein digestibility

Most studies investigating the influence of phytase on protein do so by measuring AA digestibility. In general, the amino acids most influenced by phytase appear to be Pro, Gly, Thr and Ser (Adeola and Cowieson, 2011). Selle and Ravindran (2008) presented a summary of 24 studies assessing the influence of phytase on AA ileal digestibility and concluded that the results were variable and inconclusive. Based on their summary, if indeed phytase does influence AA digestibility, its effect is minimal (2.4% rise in median overall AA digestibility; range -2.51 to 15.09%). The authors suggested that the choice of inert dietary marker may in part be responsible for the discrepancies in the results, as it seems that in studies where chromic oxide (Cr_2O_3) was used, the effects of phytase on AA digestibility have been insignificant; however, when acid insoluble ash (AIA) or titanium dioxide (TiO_2) has been used, significant phytase effects are more common. Favero et al. (2014) recently tested this observation, as they assessed the influence of dietary marker (AIA, Cr_2O_3 and TiO_2) on the AA digestibility response in pigs fed phytase supplemented diets with SBM or canola meal (CM) as the main protein source. In this experiment the choice of marker had no influence on the effect of phytase on AA AID when fed SBM as the main protein source. However, when fed CM as the protein source, phytase increased the AID of all AA (except Try) when TiO_2 or Cr_2O_3 was used as the marker, but not when AIA was used. This suggests that the protein source as well as the choice of marker may influence the AA response to phytase.

Table 1.4. Summary of results from selected studies (*n* = 12) assessing the impact of supplementary phytase (250-1,050 FTU/kg) on ileal amino acid digestibility in the growing pig

		Response to phytase treatment (%)[b]			
Amino Acid	No. of studies[a]	Mean	Minimum	Maximum	Median
Indispensable amino acids					
Arginine	12	0.90	-0.50	2.50	1.20
Histidine	12	1.95	-1.50	7.20	2.05
Isoleucine	12	1.60	-0.50	6.30	0.85
Leucine	12	1.51	-1.10	5.50	1.20
Lysine	12	1.67	-1.10	4.20	1.15
Methionine	12	1.47	-1.20	9.50	0.50
Phenylalanine	12	1.88	-0.30	5.30	1.25
Threonine	12	2.82	-0.40	8.00	2.65
Tryptophan	3	4.50	4.30	4.70	4.50
Valine	12	1.97	-0.30	6.80	1.45
Dispensable amino acids					
Alanine	9	2.56	-1.10	7.90	1.70
Aspartic acid	9	2.66	-0.20	5.00	2.80
Cystine	8	2.75	-0.30	8.70	1.70
Glutamic acid	9	1.63	-0.20	3.90	1.40
Glycine	8	3.39	-0.50	8.40	3.80
Proline	8	1.45	-4.10	7.60	0.60
Serine	9	2.54	-0.60	6.00	2.50
Tyrosine	9	2.26	-0.30	6.20	2.40
Overall	12	2.04	-0.73	6.31	1.71

[a] Selected studies include: Nitrayova et al. (2009); Woyengo et al. (2009b); Kiarie et al. (2010); Cervantes et al. (2011); Zeng et al. (2011); Guggenbuhl et al. (2012b); Morales et al. (2012); Yanez et al. (2013); Adedokun et al. (2015); Kahindi et al. (2015); Velayudhan et al. (2015); Zeng et al. (2016).

[b]Values represent absolute change in apparent ileal digestibility (percentage points).

The 24 AA digestibility studies summarised by Selle and Ravindran (2008) were completed between the years of 1993 and 2006. Since then, many more studies assessing the impact of phytase on AA digestibility in the pig have been published, many of which have used newer, more efficacious 2nd and 3rd generation phytases. A summary of 12 selected studies completed between 2009 and 2016 are presented in Table 1.4. These data show that the effect of phytase on AA digestibility in the pig remains both equivocal and minimal (2.04 and 1.71% increase in total AA mean and median respectively). The amino acids that appear to be most influenced by phytase are Try, Gly, Thr, Asp and Ser. Differences in diet composition, substrate level, protein source and solubility, choice of marker, mineral concentration, phytase product, phytase activity and feed processing are all likely contributing factors to variable data seen in the literature (Liao et al., 2005b; Yanez et al., 2013; Favero et al., 2014). Clearly, the effect of phytase on AA digestibility needs to be resolved as any positive effect on protein digestibility would improve the economic and ecological value of phytase significantly. Adeola and Sands (2003) advise that until the factors governing the inconsistencies in the AA response to phytase are determined, AA matrix values for a phytase product must be treated with caution.

1.6.3 Effect of phytase on energy digestibility

It has been suggested that phytase can improve energy availability by a number of different mechanisms, including improved phytate and nutrient solubility, reduced phytate complex formation, improved digestive enzyme efficacy (through improved availability of metallic cofactors or reduced binding to phytate), reduced endogenous secretions and losses, and improved Na-dependent nutrient uptake in enterocytes (Liu et al., 2008; Adeola and Cowieson, 2011). Phytase supplementation consistently improves the apparent metabolisable energy (AME) in poultry; however, the situation in pigs remains unclear (Selle et al., 2009).

Johnston et al. (2004) reported a 6.7% increase in apparent ileal energy digestibility in finisher pigs fed a diet with reduced Ca and P levels and phytase supplemented at 500 FTU/kg. This increase in energy digestibility was attributed to an improvement in both starch and AA digestibility. Similar findings were reported the recent studies of Zeng et al. (2015) and Velayudhan et al. (2015) who found that increasing doses of phytase linearly increased GE ATTD and AID. Conversely, the studies of Kahindi et al. (2015) and Sauer et al. (2003) found that phytase had no effect on

energy digestibility. As with amino acids, it is important that the true effect of phytase on energy availability, and the factors responsible for the inconsistencies in the literature, are elucidated so that accurate energy matrix values can be determined for phytases. Anecdotally, many phytase manufacturers now assign an energy matrix value for their products. Caution must be taken when replacing oil or fat with phytase, as oils and fats possess 'extra-caloric' properties that phytase enzymes do not, such as assisting with the absorption of fat-soluble vitamins, providing essential fatty acids and improving pellet quality (Adeola and Cowieson, 2011).

1.7 Factors influencing phytase efficacy

There are many factors that can influence phytase activity within the GIT of the pig. These factors generally fall into one of two categories: dietary or enzymatic. Significant enzymatic related factors that likely influence *in vivo* phytase efficacy include type of phytase (HAP, BPP, PAP or PTP), source of phytase (plant, fungi, yeast or bacteria) and pH activity profile (as discussed in Section 1.4). Another enzymatic factor that is likely key to the phytase response is the Michaelis constant (K_m). The K_m is the substrate concentration at which the enzyme catalysed reaction is occurring at half its maximum velocity, and thus provides a measure of the ability of the enzyme to operate at low substrate concentrations (Menten and Michaelis, 1913). The K_m of different phytase enzymes are known to vary considerably, for example Greiner et al. (2009) determined the K_m of an *A. niger* (11T53A9) phytase to be 54 µM, whereas Monteiro et al. (2015) reported a K_m of 30.9 mM for an *A. niger* UFV-1 phytase. A phytase with a lower K_m is preferable in monogastric nutrition as it would act on the solubilised phytate faster following ingestion, and continue to work as the substrate levels begin to drop following hydrolysis.

1.7.1 Dietary related factors influencing phytase efficacy

As discussed previously, phytate, particularly the higher phytate esters, have a high affinity for multivalent cations. Maenz et al. (1999) assessed the inhibitory capacity of the divalent cations Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+} on microbial phytase efficacy *in vitro*. The authors found that at pH 7 Zn^{2+} had the strongest inhibitory effect on phytase efficacy, followed by Fe^{2+}, Mn^{2+}, Ca^{2+} and then Mg^{2+}. At pH 4, the inhibitory capacity of the minerals markedly decreased, and this was attributed to improved mineral-phytate solubility. However, as previously mentioned, it is Ca

that has the greatest influence on phytate hydrolysis in the animal as it is the most prevalent dietary cation. Ca is typically supplemented to pig diets as limestone, this is pertinent as limestone has a high acid buffering capacity (Lawlor et al., 2005) and is therefore capable of elevating the pH of the digesta (Shafey et al., 1991). An increase in gut pH has important implications on phytase efficacy as a higher gut pH favours mineral-phytate complex formation and precipitation, and depending on the pH activity range of the enzyme, it could adversely affect phytase activity.

In addition to dietary Ca concentration, the Ca:P ratio is also an important determinant of phytase activity. It is established that wide Ca:P ratios have a negative effect on phytase induced phytate hydrolysis (Qian et al., 1996; Liu et al., 2000; Brady et al., 2002). This effect was first demonstrated in pigs by Lei et al. (1994) as they found that increasing in the Ca:P ratio from 1.6:1 to 3.1:1 in phytase supplemented corn-SBM diets reduced weaner pig growth performance by 32% and plasma P concentration by 41%.

The presence of additional feed additives such as organic acids or other enzymes can also influence phytase activity within the GIT. For example, Kemme et al. (1999) studied the effects of lactic acid and phytase supplementation in growing-finishing pigs using a 2 x 2 x 2 factorial design of treatments. Growing-finishing pigs were fed a corn-SBM diet with or without phytase (900 FTU/kg), with or without Na-phytate (1.5 g P/kg) and with or without lactic acid (30 g/kg). Here, the authors detected a synergistic response between phytase and lactic acid treatments as phytase efficacy (measured as P digestibility) was enhanced by lactic acid. It was postulated that this effect could be due to lactic acid slowing down gastric emptying, thus providing phytase with more time to hydrolyse phytate in the stomach. Nortey et al. (2007) also reported synergy between supplementary phytase and xylanase in grower pigs fed wheat-SBM based diets. Both xylanase (4,375 units/kg) and phytase (500 FTU/kg) improved the ATTD of P by 1.9 and 4.8% respectively; however, when added together a 12.8% improvement in P ATTD was recorded. It is thought that xylanase can increase phytate availability by breaking down the cell wall matrix, thus providing phytase with more substrate to hydrolyse. Several others have also shown that organic acids or other exogenous enzymes can influence phytase efficacy *in vivo* (Boling et al., 2000; Kim et al., 2008; Selle et al., 2009b).

As with other enzymatic reactions, a change in enzyme concentration will likely affect the maximum reaction velocity (Worthington Biochemical Corporation, 1972). Therefore, level of added phytase will have a significant influence on the amount of phytate hydrolysed in the digestive tract. This was demonstrated in the study of Guggenbuhl et al. (2012b), as they found that increasing the phytase inclusion rate of a *C. braakii* derived 6-phytase from 500 to 1,000 FTU/kg increased ileal $InsP_6$ degradability by 7.7 % in growers fed a corn-SBM diet. Similarly, Adeola et al. (2004) reported linear increases in growth rate and P and Ca digestibility in response to increasing levels of phytase.

Other factors that have been shown to influence phytase activity within the GIT include: dietary ingredients (Leske and Coon, 1999), substrate concentration (Selle et al., 2003) and intrinsic phytase activity (Dersjant-Li et al., 2015). Clearly, there are many of factors that can influence phytase efficacy in the animal, many of which are interdependent. This multifactorial nature of the enzyme is likely responsible for the inconsistencies observed in the literature and makes it incredibly difficult to predict the response of an animal to phytase supplementation.

1.8 High phytase dosing

Phytase is typically added to pig diets at a rate of 500 FTU/kg as this is considered an economic dose based on dietary P and sometimes Ca cost savings. At this rate, phytase usually degrades between 40 to 60% of ingested $InsP_6$ to lower phytate esters and MYO at the ileal level (Kies et al., 2006; Adeola and Cowieson, 2011). Therefore, in a diet containing 10 g/kg of phytate it can be assumed that 4 to 6 g/kg of this highly anti-nutritive molecule remains intact within the GIT. As the anti-nutritional effects of phytate and reciprocally the extra-phosphoric effects of phytase have become increasingly apparent, one area that has recently generated much interest is the application of super-doses of phytase. Super-dosing is the use of high levels of phytase (commonly defined as a dose of >1,500 FTU/kg of a 3rd generation phytase; Cowieson et al., 2013b) in an attempt to maximise dietary phytate degradation.

Recent research has shown that super-doses of phytase can markedly improve both pig and poultry growth performance, even when added to P adequate diets (Braña et al., 2006; Cowieson et al., 2006; Zeng et al., 2014). This implies that this super-

dosing response is an extra-phosphoric effect of phytase. Whilst it may be that higher inclusion rates are warranted, the underlying mechanism for the favourable growth response commonly observed in such super-dosing studies remains to be clarified. Several proposed mechanisms have been brought forward, including: (i) improved energy, protein and mineral bioavailability through greater alleviation of the anti-nutritional effects; (ii) increased DM intake; (iii) increased MYO bioavailability; (iv) and a more proportionate Ca:P release (Cowieson et al., 2011; Goncalves et al., 2016).

As discussed in Section 1.5, phytate is a potent anti-nutrient that can impede mineral, protein and energy digestibility. Therefore, it may be that the favourable growth response to super-doses of phytase is the result of improved nutrient bioavailability through greater phytate hydrolysis. Whilst alleviation of the anti-nutritional effects of phytate are likely to have a beneficial effects on the animal, it is thought that the magnitude of the super-dosing response is often greater than expected due to the removal of these anti-nutritional effects (Cowieson et al. 2011).

An alternative suggestion is that super-dosing phytase may improve the feed intake of the animal. Whilst there is little evidence to support this theory, it has been suggested that phytate acts as an ‘appetite suppressant’ (Cowieson et al., 2011). Therefore, improvements in phytate degradation may stimulate feed intake.

More recently, MYO, the product of complete phytate de-phosphorylation has been implicated in the super-dosing response (Cowieson et al. 2011). The biological functions of MYO and its derivatives are numerous and diverse, although its importance in nutrition is obscure. MYO has vitamin like properties and is a requisite for cell signalling, regular plasma membrane function and structure, glycogenolysis and insulin release (Charalampous, 1971; Tolias and Cantley, 1999) McDowell, 2000). In chicks, Hegsted et al. (1941) found that MYO improved growth performance. This finding has since been replicated in the study of Zyla et al. (2013), as they also found that MYO improved chick growth performance.

Higher inclusion rates of phytase likely result in more extensive phytate degradation and thus greater generation of both MYO, and lower phytate esters which can be further degraded by the mucosal phytases to MYO. Therefore, it has been suggested that improved MYO bioavailability may play an important role in the super-dosing

growth response (Cowieson et al. 2011). There is, however, a lack of evidence to support this notion at present and further research in this area could prove worthy.

An additional mechanism for the super-dosing response, as proposed by Cowieson et al. (2011), is a more proportionate release of Ca and P. At conventional inclusion rates, phytase degrades proportionally more of the higher esters of phytate (Wyss et al., 1999), which chelate disproportionally more Ca than the lower esters of phytate (Section 1.5.1). Thus, it can be speculated that at standard phytase inclusion levels, more Ca is released than P. However, at higher inclusion levels, phytase begins to target a greater proportion of the lower phytate esters ($InsP_4$ and $InsP_3$), thus P release continues in a linear manner, as Ca release begins to plateau.

1.9 Concluding remarks

Phytase enzymes are currently added to pig diets across the world to improve P and Ca bioavailability and reduce their excretion. This enables nutritionists to make dietary cost savings through reductions in inorganic Ca and P supplementation, whilst attenuating the contribution of pig production to environmental nutrient pollution. Extensive research over the last two decades has revealed that phytate is a stronger anti-nutrient than originally thought, and that phytase may improve the utilisation of other nutrients such as protein and energy. However, the data show that these extra-phosphoric effects of phytase are inconsistent. Clearly, accurately defining a positive protein or energy effect of phytase presents a tremendous opportunity to further improve the economic and ecological value of phytase, thus further research is this area could prove fruitful.

The anti-nutritional effects of phytate are known to stem from its capacity to bind with positively charged nutrients encountered in the digestive milieu, essentially rendering the complexed nutrients unavailable for digestion. As phytate travels distally through the GIT, it becomes more negatively charged and consequently its chelation potential increases. Notionally, an early and thorough breakdown of phytate to lower, more innocuous phytate esters in the gastric regions of the GIT, where the phytate is most soluble, is necessary to alleviate the anti-nutritive effects of the molecule. Recently, there has been much interest in attempting to achieve this through the use of super-doses of phytase. The effects of super-dosing phytase on

monogastric growth performance appear highly promising, however, studies in the pig are limited.

1.10 Aims and objectives

Given declining enzyme costs together with escalating feed costs, it may be that higher doses of phytase are justified. However, most of research assessing the effects of super-doses of phytase has been performed in poultry. Therefore, the aims of this research are to determine the effects of super-dosing phytase on the growth performance and aspects of nutrient bioavailability in the growing pig. In addition, this work will attempt to shed light on the mechanism for the favourable growth responses commonly observed in other super-dosing studies, paying particular attention to the possible role of MYO.

Specific objectives:

- To determine the effect of super-dosing phytase on the growing pig at different stages of production.
- To determine the effect of super-dosing phytase on ileal phytate ($InsP_6$) degradation and lower inositol phosphate ($InsP_{2-5}$) composition at different stages of production.
- To determine the effect of super-dosing phytase on circulating levels of MYO at different stages of production.
- To determine the effect of super-dosing phytase on different aspects of mineral availability (ileal digestibility, plasma concentration and bone concentration) at different stages of production.
- To determine the effect of different processing methods on phytate degradation and inositol phosphate composition in gastric digesta.

Chapter 2
General Methods

2.1 General methods overview and ethics statement

This chapter has been included to prevent the repetition of methods performed across multiple experiments. All experimental protocols were approved by the University of Leeds Animal Welfare and Ethical Review Body prior to commencement. Pig housing and husbandry practices were compliant with the Council Directive 2008/120/EC standards and the Welfare of Farmed Animals (England) Regulations 2007. The principles of 3 R's, as set out in the Directive 2010/63/EU were applied throughout the experimental design process. Furthermore, all scientific procedures carried out during this research were done so in accordance with the Animals (Scientific Procedures) Act 1986, as amended by the Directive 2010/63/EU.

2.2 Animal husbandry

All experiments used crossbred [(Large White x Landrace) x MAXGRO™] pigs and were conducted at the University of Leeds farm, Spen Farm. Pigs were sourced directly from Spen Farm's breeding herd.

2.2.1 Standard Spen Farm practice

Unless stated otherwise the pigs used in each of the experiments were treated according to standard Spen Farm practice both before and after the experiment. Spen Farm is a 200 sow unit that operates on a 3 week batch farrowing system. Pigs were reared here from birth through to slaughter. After farrowing, sows remained in their gestation crates with their piglets for approximately 4 weeks. Here, the piglets were fed by the sow, but also had access to a standard commercial creep feed and water. All pigs had their teeth clipped, tails docked and ears tagged within the first 24 h post-partum. At ~3 d of age pigs received an oral shot of Baycox Coccidiocide (20 mg/kg BW) and an intra-muscular injection of Fe (200 mg) as gleptoferron. At ~14 d of age an intra-muscular injection of M+PAC (1 ml) and Ingelvac CircoFLEX® (1 ml) was administered. Pigs were weaned at ~28 d of age and moved into a weaner-grower facility where they were mixed with unfamiliar pigs

according to size. In this facility, pigs were fed a standard weaner diet for 3 weeks before moving on to a standard grower diet for the next 5 weeks. After a total of 8 weeks in the weaner-grower facility, pigs were moved into finishing accommodation where they remained until they were sent to the abattoir for slaughter (~110 kg).

2.2.2 Feed and water

In each of the experiments, pigs were provided with feed and water *ad libitum*. Diets were formulated with the assistance of ABN (Peterborough, UK), a leading British pig feed manufacturer and sister company to the industry sponsors of this research. Details of diet manufacture are provided in the relevant chapter. All diets were pelleted through a 3 mm die at Primary Diets (Ripon, UK) at a temperature of 62 ($\pm$2) °C and packaged into bags of 25 kg. Troughs were topped up with a known amount of feed when required. Representative samples of experimental diets were collected weekly and stored at -20 °C pending analyses.

2.2.3 Daily observations

Throughout the experiment pig health and faecal checks were carried out every 24 hours by the same trained individual. Each pen of pigs was assigned a health score ranging from 1 to 4 (1 = no signs of ill health, 2 = few signs of ill health, 3 = signs of ill health, and 4 serious signs of ill health), and a faecal score, which also ranged from 1 to 4 (1 = firm, 2 = soft, 3 = few signs of diarrhoea, and 4 = clear signs of diarrhoea). Any pigs displaying signs of poor health were treated with antibiotics when appropriate, or if necessary taken off trial. Other daily checks included: temperature range, water availability and trough checks.

2.3 Phytase enzyme

The phytase used in each of the experiments was Quantum® Blue (EC. 3.1.3.26; provided by AB Vista, Marlborough, UK). Quantum® Blue is a third generation modified *E. coli* derived 6-phytase that is produced by a modified strain of *Trichoderma reesei*. This phytase enzyme is largely thermo-tolerant, thus precluding the need for enzyme coating prior to feed pelleting. The minimum declared activity of Quantum® Blue is 5,000 FTU/g, whereby 1 FTU/kg is defined as the amount of enzyme activity needed to liberate 1 µmol of Pi/min from an excess of Na-phytate at 37 °C and pH 5.5 (ISO 30024:2009, 2009). The specific form of

the enzyme used in each of the experiments was Quantum® Blue 5G, which is a solid granulated form.

2.4 Sample collection

At the end of the experiment a number of pigs were slaughtered via a Schedule 1 method and dissected to obtain tissue and digesta samples. Selected pigs had a BW that closely matched the pen average, and where possible, the selected pigs across pens within a replicate were from the same litter. Details of the Schedule 1 method and the number of pigs used for sample collection is stated in the relevant chapters.

2.4.1 Peripheral and portal plasma collection

Following the confirmation of death by exsanguination, peripheral blood was collected from the jugular vein directly into a 10 ml heparinised Vacutainer® (BD, Oxford, UK). The Vacutainer® was gently inverted approximately 8 times to ensure thorough mixing of the blood and heparin, and then stored on ice for no longer than 30 min.

In order to obtain a sample of portal blood the abdominal cavity was exposed following an incision along the midline of the body. Portal blood was drawn from the portal vein using a syringe with a 20 G x 2.5 cm needle (Vacuette, Kremunster, Austria), transferred into a 10 ml heparinised Vacutainer®, and treated as above.

Plasma was separated following centrifugation at 1,500 x g for 15 min at 4 °C, apportioned into aliquots, and stored at -80 °C pending analysis.

2.4.2 Ileal Digesta

The GIT was identified, clamps were positioned at the pyloric sphincter and the ileocaecal valve, and the small intestine was removed from the body cavity. Clamps were positioned at various points along the small intestine to prevent excessive mixing of the digesta as it was unravelled on the worktop. The terminal ileum was identified as the distal most section of the intestine containing an abundance of lymphoid tissue aggregations. Digesta were gently squeezed from this section of the tract, mixed, and the pH recorded using a handheld pH meter (HI-99161, Hanna Instruments Ltd, Bedfordshire, UK). The digesta were then apportioned into 30 ml polypropylene screw topped tubes and stored at -20 °C pending analysis.

2.4.3 Bone

The third metatarsal (M3) from the right hind leg was collected for bone mineralisation analysis. A hacksaw was used to separate the foot from the carcass at a point just above the carpus. The foot was stored at -20 °C in a sealed zip-lock bag to prevent desiccation. When required for mineralisation analysis, the foot was thawed and the M3 bone was manually excised from the foot using scalpel blades and forceps. Excised bones were placed in a beaker of boiling water for 1 min to facilitate the removal of adhering tissue prior to ash and mineral analysis.

2.4.4 Intestinal mucosal scrapings

Intestinal mucosa samples were collected for nutrient transporter gene expression analysis. The site of collection is stated in the relevant chapter. A section of intestine (~10 cm) was excised, cut longitudinally to expose the luminal portion and washed with ice-cold PBS. The mucosal surface was gently scraped with a disposable sterile polyethylene spoon to obtain ~100 mg of mucosa, transferred into a cryovial containing 1 ml of TRIzol® Reagent (Thermo Fisher Scientific), snap frozen in liquid nitrogen, and stored at -80 °C pending subsequent processing.

2.5 Laboratory analysis

Unless stated otherwise, the deionised (DI) water used throughout the laboratory analyses was high purity water, with an electrical resistivity of 18 MΩ.

2.5.1 Glassware wash procedure

All glassware underwent a rigorous wash procedure prior to use to minimise contamination. Firstly, glassware were washed in a laboratory glass washer using a standard commercially available glassware detergent. The glassware were then left to soak overnight in a freshly prepared 1% HCl acid bath, rinsed 3 times with DI water, and left to dry at ~40 °C in a drying cupboard. Once dry, clean glassware were covered with cling film and stored in a cupboard until required. Glassware were generally cleaned immediately after use; if this was not possible, glassware were left to soak in a water bath pending cleaning.

2.5.2 Dry matter and ash determination

Both dry matter (DM) and ash were determined gravimetrically. A known amount of sample (~2 g) was accurately weighed out to the nearest thousandth of a gram

into a 28 ml Type 1B neutral glass tube of known weight. The sample was dried at 100 °C for 24 h in a natural convection oven, cooled to room temperature in a closed desiccator for 20 min, and the DM calculated after weighing. The dry sample was then incinerated in a muffle furnace at 550 °C for 16 h, cooled to room temperature in a closed desiccator for 20 min, and weighed for ash content determination. DM and ash content were determined in triplicate for feed and duplicate for digesta.

2.5.3 Phytase activity analysis

Phytase activity in the diets was analysed by Enzyme Services and Consultancy (ESC, Ystrad Mynach, UK) according to an internal method of the enzyme manufacturer (AB Vista Quantum® method). In brief, feed samples were extracted for 30 min in 25 mM borate (pH 10), and the phytase activity assayed at pH 4 at 60 °C using rice as a substrate. P liberation was determined colorimetrically using a molybdate-vanadate system.

2.5.4 Titanium dioxide analysis

Titanium dioxide (TiO_2) was quantified according to a slightly modified method of Short et al. (1996). A known weight (~0.2 g) of dry sample was weighed into a 28 ml Type 1B neutral glass tube of known weight and ashed at 580 °C for 13 h. After cooling, 10 ml of 7.4 M sulphuric acid (H_2SO_4) was added to the ash and a watch glass positioned on top of the glass tube. The tubes were transferred to a hot plate at 150 °C for ~1 h to facilitate ash dissolution. Tubes were allowed to cool before the contents were poured into a small beaker containing 10 ml of DI water. The contents of the beaker were filtered through a Whatman 541 filter paper (GE Medical Systems Ltd, Buckinghamshire, UK) into a 100 ml volumetric flask and 10 ml of hydrogen peroxide (H_2O_2) were added (20 ml used in original method of Short et al., 1996). The solution in the volumetric flask was brought up to 100 ml with DI water. Following the addition of H_2O_2 a deep orange colour was formed, the intensity of which is directly proportional to the amount of dissolved titanium dioxide (TiO_2).

Three 250 µl aliquots of the resulting solution were transferred into a 96 well plate and the absorbance measured at 410 nm using a SPECTRAmax™ 340 (Molecular Devices, California, USA). The concentration of TiO_2 in the sample was calculated using the average absorbance value and plotting it on a calibration curve generated with standards of known concentration as described in Short et al. (1996).

2.5.5 Colourimetric determination of available phosphorus

Available phosphorus content was measured in the diets according to a method described in the K-PHYT assay kit (Megazyme Inc). In brief, 1 g of dried milled material was extracted in 20 ml of 0.66M HCl on a rotary shaker overnight. One ml of the extract was centrifuged at 10,000 x g for 10 min and the resulting supernatant neutralised with NaOH. The P concentration of the neutralised sample was determined using an ammonium molybdate colourimetric system as described in the K-PHYT assay procedure.

2.5.6 Bone measurements

A digital calliper was used to measure M3 length, width at the narrowest point of the midshaft, and width at the widest point of the midshaft. Following this, the fat free dry weight (FFDW) and the ash content of the M3 bone were determined. The bone was placed in a jar of diethyl ether and left to soak for 72 h to extract the fat. Subsequently, the ether soaked bones were left to air dry in a fume hood overnight, oven dried at 100 °C for 24 h, and weighed after cooling in a closed desiccator for the determination of bone FFDW. Each fat free dry bone was placed in a clean glass beaker of known weight, ashed at 600 °C for 24 h, and the resulting ash weighed for bone ash determination. Bone ash content was expressed as a percentage of the FFDW. The collected bone ash was used for bone mineral analysis according to the method described in Section 2.5.7.2.

2.5.7 Sample preparation for mineral analysis

2.5.7.1 Diet and ileal digesta

Dried feed and digesta samples were ground to pass through a 1 mm sieve. A known amount of ground sample (~0.2 g) was weighed out into a 28 ml Type 1B neutral glass tube and ashed at 550 °C for 16 h. After cooling, 10 ml of 5 M high purity hydrochloric acid (HCl) was added and a watch glass was placed on top of the glass tube. Samples were transferred on to a hot plate and gently boiled for 5 min at 100 °C to facilitate ash dissolution. Once cool, the contents of the tube were filtered through a Whatman 42 filter paper (GE Medical Systems Ltd, Buckinghamshire, UK) into a 100 ml volumetric flask, and brought up to 100 ml with DI water. The flask was inverted 8 to 10 times to ensure a homogenous solution, transferred into a screw topped glass tube, and stored at 4 °C until required. A blank sample was

prepared in the same manner. Mineral content was analysed by ICP-OES as described in Section 2.5.8.

2.5.7.2 Bone

Bone ash was dissolved into an aqua regia mixture containing 30 ml of high purity 5M HCl and 3 ml of high purity HNO_3. Samples were gently boiled at 100 °C for 5 min on a hot plate to facilitate dissolution. The solution was filtered through a Whatman 541 filter paper into a 100 ml volumetric flask, brought up to 100 ml with DI water, and then treated as described above (Section 2.5.7.1).

2.5.7.3 Blood

Plasma or serum samples were thawed on ice and vortexed for 15 seconds to ensure a homogenous mixture prior to mineral analysis. One part plasma was added to 4 parts high purity 3.5% HNO_3 (300:1200 µl) in a 1.5 ml polypropylene tube to precipitate plasma proteins. The deproteinised sample was vortexed for 30 seconds, left on ice for 15 min, and then centrifuged at 16,000 x g for 10 min. The supernatant portion was transferred in a clean 1.5 ml polypropylene and stored at 4 °C until required. A blank sample was prepared in the same manner. Mineral content was analysed according the method described in Section 2.5.8.

2.5.8 Mineral analysis by inductively coupled plasma optical emission spectroscopy

When necessary, samples were diluted with DI water to bring the expected mineral concentration to within ~0.1 to 100 µg/ml. Five multi-element standards covering the expected mineral range were freshly prepared on the day of analysis. Mineral concentrations of the blank, standards and samples were analysed by inductively coupled plasma emission spectroscopy (ICP-OES) using a Thermo iCAP 7400 series instrument (Thermo Scientific). The minerals measured (and their analytical wavelengths) included: Ca (370.603 nm), P (178.284 nm), Na (589.592 nm), K (766.490), Mg (285.213 nm), Fe (259.940 nm), Cu (324.754 nm), Mn (257.610 nm), Zn (206.200 nm). All measurements were collected using a radially viewed plasma torch. The unknown mineral concentrations were plotted against the calibration curve generated from the blank and multi-element standards on the spectrometer software (Qtegra™ Intelligent Scientific Data Solution™ Software, Thermo Scientific), enabling the mineral concentrations of the sample to calculated.

2.5.9 Sample preparation for *myo*-inositol analysis

2.5.9.1 Feed and digesta

Freeze-dried and milled samples (~100 mg) were extracted for 60 min at room temperature in 5 ml of solution containing 100 mM sodium fluoride and 20 mM Na_2EDTA (pH adjusted using sodium hydroxide), and filtered through a 0.45 µM polypropylene filter (Kinesis Ltd, UK).

2.5.9.2 Blood

Plasma or serum were thawed on ice and vortexed for 30 seconds to ensure a homogenous mixture prior to MYO analysis. One part plasma was added to 2 parts acetonitrile (100:200 µl) in a 1.5 ml polypropylene tube to deproteinise the sample. Following deproteinisation, the sample was vortexed for 30 seconds, left on ice for 30 min, and then centrifuged at 16,000 x g for 10 min. The supernatant was collected into a 1 ml syringe, passed through a 13 mm 0.45 µM PTFE syringe filter (Kinesis Ltd, Cambridgeshire, UK) into a clean 0.5 ml polypropylene tube, and stored at -20 °C pending MYO analysis as described in Section 2.5.10.

2.5.10 *Myo*-inositol analysis

Myo-inositol was separated from the sample mixture by high-performance liquid chromatography (HPLC) on a Dionex DX600 HPLC system (Thermo Scientific) and detected by pulsed amperometry on a gold electrode. A column switching technique was used to separate inositol from monosaccharides whereby 20 µl of sample was injected on to a 3 x 200 mm Dionex CarboPac PA1 HPLC column (Thermo Scientific) and eluted with 150 mM NaOH at rate of 0.4 ml/min. The eluent was loaded onto a 4 x 50 mm Dionex CarboPac MA1 (Thermo Scientific) guard column and then a 4 x 250 mm analytical column. At 1 min and 30 seconds into the run, the CarboPac PA1 column was diverted out of flow and the mobile phase to this column was switched to 750 mM NaOH to wash the column and elute the sugars to waste. At 11 min 30 seconds, after the MYO peak had eluted from the column, the MA1 mobile phase was switched back to 150 mM NaOH to prepare the column for the next sample.

2.5.11 Inositol phosphate analysis

Freeze dried digesta and feed samples were extracted as in Section 2.5.9.1. $InsP_{2\text{-}6}$ were separated by high-performance ion chromatography (HPIC) and measured by

UV detection at 290 nm following post-column derivatisation. Aliquots of 20 µl were loaded onto a 3 x 300mm Dionex CarboPac PA200 column (Thermo Scientific) fitted with a 3 x 50 mm guard column, and eluted at 0.4 ml/min with a gradient of methane sulfonic acid. This gradient was delivered from two sources containing either water or methanesulfonic acid (600 mM) according to the following schedule: at 0 min = 100% water, 25 min = 100% methanesulfonic acid, 38 min = 100% methanesulfonic acid, 39 min = 100% water, and at 49 min = 100% water. Following elution from the column, a mixture of 0.1% ferric nitrate and 2% perchloric acid were delivered at 0.2 ml/min as a reagent for derivatisation before passing through a knitted reaction coil (200 µl). Peaks were detected at 290 nm using a Jasco UV-2077 Plus UV/Vis detector (JASCO, Japan).

2.5.12 Intestinal mRNA quantification by reverse transcription-quantitative PCR

All RNA work was performed in a designated RNA room using designated RNA only equipment.

2.5.12.1 RNA isolation

Cryovials containing the mucosal samples in Trizol were left to thaw on ice before being transferred into 2 ml polypropylene collection tubes containing one 5 mm stainless steel bead (Qiagen). Mucosal tissue was subject to bead mill homogenisation using a TissueLyser (Qiagen) operating at 40 Hz for 2 min 30 seconds. Samples were then placed on ice for 1 min before the homogenisation step was repeated for a further 2 min 30 seconds at 40 Hz. Particulates were removed from the homogenate following centrifugation at 12,000 x g for 1 min.

Total RNA was isolated from the supernatant fraction of the homogenate using the Direct-Zol™ RNA MiniPrep kit (Cambridge Biosciences, Cambridgeshire, UK), which incorporated an in-column DNase I digestion step to minimise genomic DNA contamination. All centrifugation steps were performed at 12,000 x g using a standard benchtop micro-centrifuge. 350 µl of homogenate was added to an equal volume of 99.5% ethanol and vortexed for 5 seconds. 650 µl of this mixture was loaded on to a Zymo-Spin™ IIC Column and the RNA was isolated according to the manufacturer's instructions. RNA was eluted from the column into 120 µl of nuclease-free water, apportioned into 30 µl aliquots in 0.5 ml nuclease-free polypropylene tubes, and frozen at -80 °C pending analysis.

The quality and quantity of the isolated RNA was assessed using a NanoDrop-ND1000 spectrophotometer (Thermo Scientific). RNA integrity was confirmed following visual inspection of the ribosomal 28S and 18S subunits after gel electrophoresis on a TAE buffer based 1% agarose gel, which contained ethidium bromide at a final concentration of 0.5 µg/ml. Gels were loaded with 5 µl of sample mixture comprising 2.5 µl of eluted RNA sample and 2.5 µl of 1 x Gel Loading Dye (prepared from 6 x Gel Loading Dye; New England Biolabs), and run for 25 min at 85 V. A DNA ladder (1 Kb Plus, Thermo Scientific) was also loaded onto the gel to serve as an electrophoresis positive control. After 25 min of electrophoresis, the RNA were visualised with a UV transilluminator.

2.5.12.2 cDNA synthesis

Isolated total RNA was converted into cDNA using the First Strand cDNA Synthesis Kit (#K1612, Thermo Scientific) according to the manufacturer's instructions. 2 µg of RNA was added to a reaction mixture comprising 5 µM of random hexamer primers, 1 unit of M-MuLV Reverse Transcriptase, 4 µl of Reaction Buffer, 0.5 mM dNTPs, 1 unit of RiboLock RNase Inhibitor in a final reaction volume of 20 µl. The reaction mixture was mixed gently and incubated at 25 °C for 5 min, 37 °C for 1 h, and 70 °C for 5 min. The resulting reverse transcription product was diluted 50-fold with nuclease-free water, apportioned into 30 µl working aliquots, and stored at -80 °C pending quantitative PCR (qPCR) analysis. For each sample reverse transcribed a corresponding minus reverse transcriptase (-RT) control was run in parallel, whereby water was added to the reaction mixture instead of the reverse transcriptase enzyme.

The cDNA synthesis product was assessed on an agarose gel following traditional end point PCR using primers designed to amplify a 205 bp section of the housekeeping gene, beta-actin (*ACTB*). The *ACTB* primer sequences were obtained from a recently published study (Fiesel et al., 2014) and were: forward, 5'-GACATCCGCAAGGACCTCTA-3'; reverse, 5- ACATCTGCTGGAAGGTGGAC-3' (GenBank accession number: XM_003124280.3; synthesised by Sigma Alrdrich). PCR amplification was performed in a total volume of 50 µl comprising 25 µl of GoTaq® G2 Green Master Mix (Promega), 1.5 µl of both the forward and reverse primers (300 nM final concentration), and 20 µl of nuclease-free water. Amplification was run on a Techne TC-512 thermal cycler (Bibby Scientific Ltd) in 0.2 ml PCR tubes. The PCR cycling conditions were: an initial denaturation step at

94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 20 seconds, and extension at 72 °C for 45 seconds. Amplification products were run on a 1% agarose gel in a 1 x TAE running buffer at 75 V for 25 min. Gels contained 0.5 µl/ml ethidium bromide, allowing the DNA to be visualised under UV transillumination.

2.5.12.3 Quantitative PCR

The relative expression of selected genes was measured by qPCR on a CFX-96™ Real Time PCR Detection System (Bio-Rad Laboratories Ltd). 4 µl of cDNA was added to a PCR reaction mix (total volume 20 µl) consisting of 0.5 µl of each primer, 10 µl of SYBR green master mix (SsoAdvanced universal SYBR® Green supermix, Bio-Rad Laboratories Ltd), and 5 µl of nuclease-free water. Amplification took place on white 96-well qPCR plates, sealed with an optically clear adhesive to prevent evaporative losses. PCR amplification cycling conditions were: an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 seconds, and annealing and extension at 60 °C for 30 seconds.

Samples were assayed in triplicate alongside their –RT controls to check for genomic DNA contamination. In addition, a no template control (NTC) was included on every plate for all primer sets being assayed to confirm absence of reagent contamination. NTC and –RT controls were measured in duplicate. The amplification efficiency of each primer set was determined from a standard curve generated from a serial dilution of pooled cDNA (1:10 dilution, 5 standards). Standards were assayed in triplicate. At the end of each PCR run a melt curve analysis (65 to 95 °C, 0.5 °C incremental increase every 5 seconds) was performed to assess primer specificity.

2.5.12.4 qPCR data analysis

All qPCR data analyses were performed on the qbasePLUS software (Biogazelle NV, Zwijnaarde, Belgium). Firstly, the stability of the three candidate reference (housekeeping) genes assayed was evaluated using the geNorm function of the qbasePLUS software. The most stable combination of the three reference genes was identified, and the geometric mean of this combination was used as the normalisation factor for all genes of interest.

The cycle threshold (Ct) values generated by qPCR were converted into normalised relative gene expression values according to the formula described in Hellemans et al. (2007). This formula is an adaptation of the classic $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) that incorporates a PCR efficiency correction and allows the use of multiple reference genes.

2.6 Calculations

Feed conversion ratio (FCR) was calculated as the ratio of feed intake to weight gain. Apparent ileal digestibility (AID) was determined according to the following equation:

$$AID\ (\%) = 100 - \left[\left(\frac{TiO2_{feed,\ \%}}{TiO2_{digesta,\ \%}}\right) \times \left(\frac{Nutrient_{digesta,\ \%}}{Nutrient_{feed,\ \%}}\right) \times 100\right]$$

Chapter 3
The Effects of Super-dosing Phytase on Grower Pig Growth Performance, Ileal Inositol Phosphate Degradation and *Myo*-inositol Generation.

3.1 Abstract

An experiment was conducted to assess the effects of a standard and a super-dose of phytase (Quantum® Blue) on grower pig growth performance, ileal inositol phosphate and MYO concentration, bone mineralisation, mineral availability and serum *myo*-inositol. Subsequently, pigs went on to receive a finisher diet supplemented with either a standard or super-dose of phytase to determine the effects of super-dosing phytase on finisher pig growth performance. A total of 576 mixed sex crossbred pigs (initial BW ± SE = 17.3 ± 0.2 kg) were used in this 49 d study. Treatments were arranged as a 4 x 2 factorial; the first factor (Diet 1) was one of four diets which were fed to grower pigs from 8 to 12 weeks of age (Period 1, d 0 to 30), these included: (PC) a high P positive control (available P at 0.5 g/kg above the BSAS recommendation); (NC) a low P negative control (available P 1.2 g/kg lower than the PC); and the NC diet supplemented with either 500 or 2,000 FTU of phytase/kg. The second factor was a subsequent diet (Diet 2) offered to pigs immediately after Period 1 (Period 2, d 30 to 49 of this trial; initial BW ~44.2 kg), which comprised one of two phytase treatments: a standard dose (500 FTU/kg) or a super-dose (2,500 FTU/kg). All diets were wheat-barley-SBM based and were provided *ad libitum* throughout the study. At the end of Period 1 (d 28 and 29) 32 pigs were euthanised (8/treatment) for collection of ileal digesta, portal and peripheral serum, and the third metatarsal.

Throughout Period 1, pigs fed the low P NC diet had a lower ADFI (P<0.05) and ADG (P<0.01) than those fed the nutritionally adequate PC diet. Phytase supplementation effectively restored the growth performance of pigs fed the NC to the level of the PC (P<0.01). There was a significant reduction in the concentration of $InsP_6$ and $InsP_5$ in the ileal digesta in response to phytase supplementation (P<0.001); however, there was no difference between the 500 and 2,000 FTU/kg treatments. Grower pigs receiving the phytase super-dose treatment had elevated levels of serum MYO in both the portal and peripheral circulations (P<0.05). Supplementing the NC with a super-dose of phytase increased bone Ca, P and Mg

concentration to a level similar to the PC (*P*<0.05). Apparent ileal P digestibility tended to increase with increasing phytase dose (*P*<0.10); whereas the AID of Ca, Mg, K and Fe was not influenced by treatment. In addition, serum mineral concentration was not influenced by dietary treatment.

In the finisher stage, pigs offered the super-dose treatment had a higher ADFI throughout Period 2 than those receiving the standard phytase dose (*P*<0.01). The effect of phytase on finisher pig ADG throughout Period 2 was dependent on the preceding grower treatment resulting in a significant Diet 1 x Diet 2 interaction (*P*<0.05). Pigs initially fed the NC that went on to receive the super-dose (2,140 FTU/kg), or those fed the standard phytase dose throughout had a lower ADG throughout Period 2, and consequently a lower final BW, than those receiving one of the other treatments. This suggests that the effect of phytase on finisher pig performance may be dependent on the animal's existing nutritional status.

3.2 Introduction

Microbial phytase enzymes are routinely added to pig diets at a rate of 500 FTU/kg to improve PP bioavailability and to minimise P levels in the excreta. At this inclusion rate, phytase typically degrades around 50 to 60 % of ingested phytate at the level of the terminal ileum (Adeola and Cowieson, 2011), which suggests scope for improvement. The current industry recommended inclusion rate of 500 FTU/kg for pig diets is used by pig producers, as it is seen as an economic dose based on Pi displacement. However, over the last decade it has become increasingly apparent that phytate has inimical effects on both AA and energy digestibility, endogenous secretions and endogenous enzyme activity (Selle and Ravindran, 2007). Thus, there has been a recent shift in the primary focus of phytase application from P release to alleviation of the anti-nutritional effects of phytate through augmenting phytate degradation.

One area that has generated considerable interest of late is the use of super-doses of phytase (> 1,500 FTU/kg) in an effort to maximise phytate hydrolysis, and thus minimise the associated anti-nutritive impact of the molecule. Recent research has shown that super-dosing phytase can result in improvements in monogastric growth performance, growth efficiency, nutrient digestibility, and bone mineralisation, often to a level that exceeds that of a nutritionally adequate positive control (Cowieson et

al., 2011). Whilst there are studies that have demonstrated the benefits of super-dosing phytase in weaner pigs (Kies et al., 2006b), grower pigs (Brana et al., 2006), turkeys (Pirgozliev et al., 2007) and laying hens (Pace et al., 2014), the majority of the research in this area has been performed in broiler chickens. Shirley and Edwards (2003) were among the first to demonstrate the benefits of unconventionally high doses of phytase. In their study, the authors evaluated the effect of graded phytase inclusion levels from 0 to 12,000 FTU/kg on growth performance, bone ash, and PP disappearance in broilers fed a P deficient corn-based diet. Phytase improved broiler growth rate, feed conversion ratio and bone ash up to the highest dose. In addition, PP disappearance increased from 40.3% in the non-phytase supplemented diet to 94.8% in the 12,000 FTU/kg diet, indicating near complete phytate hydrolysis.

A number of studies have since confirmed the performance benefits of super-doses of phytase in birds (Augspurger and Baker, 2004; Pirgozliev et al., 2008; Walk et al., 2013). Cowieson et al. (2006) reported that 24,000 FTU/kg improved broiler chick feed conversion efficiency, mineral digestibility and bone ash content beyond that of a nutritionally adequate positive control with no added phytase. Similarly, Karadas et al. (2010) found that supplementation of 12,500 FTU/kg of an *E.coli* derived phytase (Quantum®) to a P deficient corn-SBM diet improved broiler FCR in comparison to a standard phytase dose and a nutritionally adequate positive control diet with no added phytase. In this study, it was also reported that the 12,500 FTU/kg treatment improved the anti-oxidant status of the birds, as it restored hepatic coenzyme Q_{10} and vitamin E (α-tocopherol) levels back to the level of the positive control. The mechanism for this response is unclear; however, it is thought that P deficiency restricts α-tocopherol assimilation and storage. It was also suggested that improved nutrient utilisation improves the anti-oxidant status of the animal.

Studies looking into the effects of super-dosing phytase in the pig are relatively scarce particularly in the grower-finisher stages of production. Kies et al. (2006b) assessed the effects of graded phytase inclusion levels (*A. niger* derived enzyme) up to 15,000 FTU/kg on weaner pig growth performance and mineral digestibility. Phytase supplementation up to 15,000 FTU/kg linearly increased pig growth rate, feed conversion efficiency, and apparent Ca, P, Mg, Na, K and Cu total tract digestibility. Similarly, Brana et al. (2006), studied the effects of a super-dosing an

E. coli derived phytase (10,000 FTU/kg) on weaner and grower pig growth performance. Weaners receiving the phytase super-dose had an improved FCR in comparison to those fed standard phytase dose (500 FTU/kg) treatment. In the grower stage, those receiving the super-dose had a higher ADG and a lower FCR than those receiving the standard phytase or positive control treatments. These performance benefits have not been observed by all, for instance Langbein et al. (2013) studied the effects of supplementing three different phytase enzymes (two *E. coli* derived and 1 *C. braakii* derived) at 2,000 FTU/kg to a P adequate corn-SBM diet on finisher pig performance for 78 d. The authors found that none of the enzymes tested influenced finisher pig growth performance or feed efficiency.

The mechanism for the performance benefits often observed in monogastric animals in response to such high doses of phytase requires elucidation. Most super-dosing studies in the literature incorporate a standard 500 FTU/kg P and Ca matrix, therefore, achieving P and Ca adequacy should not take more than 500 FTU/kg. This has led to the consensus that the super-dosing response is an extra-phosphoric effect of phytase. This is supported by the fact that in many of the super-dosing studies, the performance of those receiving the super-dose exceeded that of those receiving a nutritionally adequate positive control. Moreover, the recent findings of Walk et al. (2013) provide further support to this tenet, as they ran a super-dosing study in broilers which incorporated three positive controls: a nutritionally adequate positive control (PC), the PC + 1 g/kg Ca and P in the form of DCP (PC+DCP), and the PC+500 FTU/kg. In addition, phytase was supplemented to a low Ca and P corn-SBM diet (NC) at 500, 1,000 or 1,500 FTU/kg. Phytase improved broiler FCR when added to the NC at 1,500 FTU/kg in comparison to all other treatments. No difference in broiler FCR between the PC and the PC+DCP was observed; however, broilers fed the PC+500FTU/kg had a superior FCR to those fed the PC+DCP treatment. It was therefore concluded that the observed super-dosing response was not due to improved Ca or P availability. The authors proposed that the response may have instead been due to greater alleviation of the ant-nutritional effects of phytate.

As discussed in Section 1.5, phytate is a strong anti-nutritional factor that can bind to and reduce mineral and AA digestibility (Liao et al., 2005a; Woyengo et al., 2009a), stimulate endogenous AA secretions and losses (primarily as pepsin and mucin; Cowieson et al., 2008), reduce digestive enzyme efficacy (Liu et al., 2008;

Woyengo and Nyachoti, 2013), alter the dietary electrolyte balance in the intestines (Ravindran et al., 2006), and interfere with enterocyte Na^+ transport systems (Liu et al., 2008). The capacity of the phytate molecule to chelate with other nutrients diminishes at a disproportionate rate as the phosphates are cleaved from the inositol nucleus; therefore, the lower molecular weight esters of phytate have a reduced anti-nutritional effect (Luttrell, 1993; Persson et al., 1998). Furthermore, lower phytate esters are increasingly soluble in the intestines, and are therefore more available to the animals' endogenous phosphatase enzymes for further degradation.

As super-doses of phytase have been associated with more extensive and quicker phytate hydrolysis in the proximal regions of the digestive tract (Leytem et al., 2008; Walk et al., 2014), it is logical to assume that commonly observed performance benefits were due to alleviation of the anti-nutritional effects of phytate. However, it has been suggested that the reversal of the anti-nutritional effects can not alone account for the magnitude of the growth response observed in such studies, and that MYO, the resulting product of complete phytate hydrolysis, may be involved (Cowieson et al., 2011; Cowieson et al., 2015). Although studies in both rats (Katayama, 1997) and chickens (Hegsted et al., 1941) have demonstrated that supplementation of MYO can improve growth performance, there is limited evidence to support the view that improved MYO bioavailability through greater phytate degradation is responsible for the super-dosing growth response.

Super-dosing phytase focuses on improving animal performance by degrading more ingested phytate and possibly by increasing MYO availability (Cowieson et al., 2011). A reduction in enzyme costs along with an increase in feed prices has made super-dosing an attractive prospect for nutritionists and farmers alike. However, research into the effects of super-dosing is in its relative infancy and more work is needed to determine the true effect in monogastrics at all stages of production. This is particularly true in the pig, especially at the grower and finisher stages, as most research to date has been performed in weaners.

3.2.1 Study Aims

The aim of this experiment was to determine the effect of a standard and a super-dose of phytase on grower pig growth performance, apparent ileal mineral digestibility, bone mineralisation, ileal inositol phosphate ($InsP_{3-6}$) and MYO

concentration, and serum MYO (Period 1). Following on from Period 1, the effects of a standard and a super-dose of phytase on finisher pig growth performance were assessed (Period 2).

3.2.2 Hypotheses

Grower stage (Period 1)

- Super-dosing phytase will improve grower pig growth performance beyond that of a standard phytase dose and a nutritionally adequate positive control diet.
- Phytase will improve mineral AID, with the super-dose having a greater effect than the standard dose.
- Phytase will increase the concentration of minerals in the blood serum, with the super-dose phytase dose having a greater effect than the standard dose.
- Growers fed the phytase super-dose will have a higher bone mineral content than those fed the standard phytase dose.
- Supplementing the diets with phytase will reduce the amount of phytate, particularly $InsP_6$ and $InsP_5$, in the ileal digesta, with the super-dose having a greater effect than the standard dose.
- Supplementing the diets with phytase will increase the levels of MYO in ileal digesta and blood serum, with the super-dose having a greater effect than the standard dose.

Period 2

- Finisher pigs fed the phytase super-dose throughout Period 2 will have superior growth performance to those fed the standard phytase dose.
- There will be no interaction between the Period 1 (grower) and Period 2 (finisher) treatments.

3.3 Materials and methods

3.3.1 Experimental design and dietary treatments

Treatments were arranged as a 4 x 2 factorial in this 49 d feeding experiment. The first factor (Diet 1) comprised one of four grower diets which were fed to pigs for 30 d (Period 1, d 0 to 30) from 56 ± 4 d of age (initial BW ± SE = 17.3 ± 0.2 kg). These included: (PC) a high P positive control, with dig-P formulated at 0.5 g/kg above the BSAS (2003) recommendation; (NC) a low-P negative control, identical to the PC but with Ca and dig-P reduced by 1.6 g/kg and 1.2g/kg respectively; and the NC diet supplemented with 500 (NC+500) or 2,000 (NC+2000) FTU/kg. All of the diets were formulated to meet or exceed the BSAS (2003) recommendations for all other nutrients. The Ca and P reductions in the NC and two test diets were in accordance with the matrix values for 500 FTU/kg of this particular phytase enzyme. Therefore, 500 FTU/kg should liberate enough phytate bound Ca and P to see these diets meet the pigs' requirement for these nutrients. Additional P was added to the PC in the form of DCP. This diet was selected so that any observed super-dosing response could be recognised as an extra-phosphoric effect. Titanium dioxide (TiO_2) was added to all grower diets at 5 g/kg from d 18 to 30 as an indigestible marker.

The second factor (Diet 2) was one of two finisher diets containing either a standard (500 FTU/kg) or a super-dose (2,500 FTU/kg) of phytase, which were fed to the pigs from d 30 to 49 (Period 2). These diets were formulated to meet or exceed BSAS (2003) recommendations for all nutrients with the exception of Ca and P, which were reduced in accordance with the matrix values for 500 FTU/kg, as described above. Diet compositions and calculated nutrient levels for both grower (Period 1) and finisher (Period 2) diets are presented in Table 3.1.

3.3.2 Animals and management

A total of 576 pigs of approximately 8 weeks of age were blocked into mixed sexed pens of nine according to weight, sex and litter. Pens within replicate were randomly allotted to one of the eight dietary treatments (*n* = 8). The experiment was conducted over 3 batches; batches 1 and 3 were each made up of 216 pigs, whereas batch 2 was made up of 144 pigs. Batches were run 3 weeks apart. Throughout the grower stage of the experiment (Period 1, d 0 to 30) pigs were housed in a weaner-grower facility, which consisted of eight identical rooms each containing eight fully

slatted floored pens (269 x 155 cm); four either side of a central passageway. On d 28 and 29, 32 mixed sex pigs (BW 42.9 ± 0.5 kg) were slaughtered via an intracardiac injection of sodium pentobarbitone (1 ml/kg BW) and lignol (5% of mixture), following sedation via an intraperitoneal injection of the same mixture. Death was confirmed by exsanguination.

On d 30, the remaining pigs (initial BW± SE = 44.2 ± 0.3 kg) were moved into an alternative grower facility where they stayed in their original pen groups for the duration of the experiment (Period 2, d 30 to 49). The layout of this facility was identical to that of the weaner-grower facility. Each pen measured (250 x 155 cm) and had a fully slatted plastic floor. Throughout both stages of the experiment rooms were maintained at 22 ± 3 °C, and all pens were equipped with a single spaced trough feeder, two nipple drinkers and a ball on chain. Pigs and troughs were weighed on d 0, 14, 28, 30, 37, 44 and 49 for the determination of pen ADG, ADFI and FCR. The timing of pig mortality or removal from trial was recorded along with the pig BW to allow for ADFI and FCR to be adjusted accordingly. ADFI is presented after accounting for pigs taken off trial (Section 3.4.1 and 3.4.2) according to the following formula:

ADFI (kg) = Total pen feed intake (kg) / $\sum$number of pigs in pen $\times$ days on trial

The adjusted ADFI was used in subsequent determination of the pen average FCR.

3.3.3 Sample collection

Following the confirmation of death, portal and peripheral blood samples were collected as described in Section 2.4.1; however, here the Vacutainers used were heparin free and the blood was allowed to clot for 20 min on ice prior to centrifugation at 1, 500 x g at 4 °C. The resulting serum fraction was obtained and stored at -20 °C pending mineral and MYO analysis. Ileal digesta and the third metatarsal bone were collected as described in Sections 2.4.2 and 2.4.3 respectively.

Table 3.1. Composition and nutrient specifications of experimental diets (%, as-fed basis)

	Period 1[1]		Period 2[2]
	PC	NC	Basal
Ingredient			
Wheat	46.4	47.3	50.48
Soybean meal	25.0	25.0	25.0
Micronised barley	20.0	20.0	20.0
Fish meal	2.50	2.50	--
Soya oil	2.20	2.00	1.34
Dicalcium phosphate	1.55	0.68	0.72
L-Lysine sulphate	0.72	0.72	0.73
Vitamin/mineral premix 1[3]	0.50	0.50	-
Vitamin/mineral premix 2[4]	-	-	0.25
Salt	0.40	0.40	0.49
Limestone flour	0.42	0.42	0.46
Calculated nutrient composition			
Net energy (MJ / kg)	9.64	9.64	9.5
Crude protein	21.1	21.1	19.77
Crude fibre	2.88	2.88	2.96
SID Lysine	1.30	1.30	1.2
SID Methionine + Cystine	0.72	0.72	0.66
SID Threonine	0.84	0.85	0.78
SID Tryptophan	0.26	0.26	0.24
Calcium	0.74	0.59	0.54
Total P	0.69	0.53	0.48
Available P	0.38	0.27	0.26
Ca:P	1.07	1.11	1.13

[1] Period 1 diets offered from d 0 to 30. These diets contained 0.03% trimediazine. Phytase was added to the NC diet at 0.01% and 0.04% at the expense of wheat to create the 500 FTU/kg and 2,000 FTU/kg test diets respectively. Titanium dioxide was added to all Period 1 diets at 0.5 % from days 18 to 30 as an indigestible dietary marker.

[2] Diets fed from d 30 to 49. Phytase was added at 0.01% and 0.05% at the expense of wheat to create the 500 FTU and 2,500 FTU/kg test diets respectively.

[3] Vitamin and trace mineral premix provided per kg of diet: 11,500 IU vitamin A, 2,250 IU vitamin D_3, 75 IU vitamin E, 4 mg vitamin K, 2.5 mg thiamine (B_1), 6 mg riboflavin (B_2), 3.5 mg pyridoxine (B_6), 27.5 µg vitamin B_{12}, 15 mg pantothenic acid, 25 mg nicotinic acid, 150 µg biotin, 1 mg folic acid, 160 mg Cu, 1 mg I, 150 mg Fe, 40 mg Mn, 0.25 mg Se, 110 mg Zn.

[4] Vitamin and trace mineral premix provided per kg of diet : 7500 IU vitamin A, 1650 IU vitamin D_3, 35 IU vitamin E, 2 mg vitamin K, 1.5 mg thiamine (B_1), 3 mg riboflavin (B_2), 2 mg pyridoxine (B_6), 15 µg vitamin B_{12}, 8 mg pantothenic acid, 20 mg nicotinic acid, 50 µg biotin, 0.3 mg folic acid, 15 mg Cu, 1 mg I, 80 mg Fe, 25 mg Mn, 0.25 mg Se, 65 mg Zn.

3.3.4 Laboratory analysis

Subsamples of the dietary samples collected throughout the experiment (as described in Section 2.2.2) were collected, mixed thoroughly and sent to ESC for phytase analysis (Section 2.5.3), and to Sciantec Analytical Services Ltd (Cawood, UK) for crude protein, crude fibre, fat and PP analysis. Triplicate subsamples of the diets and duplicate subsamples of the ileal digesta were analysed for DM and ash (Section 2.5.2), Ca, P, Mg, Na, K, Cu and Zn (Section 2.5.7.1), available P (Section 2.5.5) and TiO_2 (Section 2.5.4). In addition, the concentration of inositol tri- to hexakisphosphate ($InsP_{3-6}$) and MYO in the digesta were analysed according to the method outlined in Section 2.5.11. Serum samples were analysed for minerals by ICP-OES (Section 2.5.8) and MYO by HPLC (Section 2.5.10). Metatarsal size measurements, fat free dry weight and ash, P, Ca, and Mg content were determined according to the methods described in Sections 2.5.6 and 2.5.7.2

3.3.5 Calculations and statistical analysis

Apparent ileal nutrient digestibility was calculated using the analysed nutrient values (Table 3.2). Estimated P intake, apparent absorption and ileal excretion were calculated according to the following calculations:

$$P\ intake\ (g/day) = \frac{Total\ FI\ (kg) \times dietary\ P\ concentration\ (g/kg)}{Total\ no.of\ days}$$

Where P is phosphorus and FI is feed intake.

$$Apparent\ P\ absorption\ (g/d) = \frac{Total\ P\ intake\ (g) \times\ P\ digestibility}{Total\ no.of\ days}$$

$$Ileal\ excretion\ (g/d) = P\ intake\ (g/d) - apparent\ P\ absorption(g/d)$$

Ileal mineral flow was calculated as follows:

$$Ileal\ mineral\ flow\ (g/kg\ DMI) = mineral\ concentration\ (mg/kg)\ \times \frac{Ti_{diet}}{Ti_{ileal}}$$

Where Ti_{diet} is the concentration of TiO_2 in the diet and Ti_{ileal} is the concentration of TiO_2 in the ileal digesta.

Data were analysed by ANOVA using the General Linear Model (GLM) procedure of SPSS Statistics (version 22.0, SPSS Inc., Chicago IL, US) with the pen serving as the experimental unit for all growth performance analyses, and the individual pig for all bone, serum, and ileal inositol phosphate, MYO, and nutrient analyses. Data were first tested for homogeneity of variance and normality using the Levene's test and the Kolmogorov-Smirnov test respectively. Non-normal data or data displaying heteroscedasticity were log transformed prior to statistical analysis, unless it was proportion data, in which case an arcsine transformation was used.

The statistical model for all Period 1 data included the effects of Diet 1 and replicate (block), whereas the model for the performance data throughout Period 2 and overall included the effects of Diet 1, Diet 2 and the associated Diet 1 x Diet 2 interaction. Non-significant interactions were removed from the model and the main effects were analysed individually. Sampling days (d 28 and 29) were not included in the Period 1 or 2 performance analyses, but were included in the total experimental Period (d 0 to 49) performance analyses. Slaughter weight was added to the model as a covariate for bone fat free dry weight, ash weight and measurement analyses. Blood mineral data were analysed as a repeated measures with the blood type (portal or peripheral) serving as the repeated factor. Differences were classed as significant if $P<0.05$ and as trends if $P<0.1$. Significantly different means were separated using the Tukey's post-hoc test. Data are expressed as least-square means along with their pooled standard error of the mean (SEM).

3.4 Results

3.4.1 Period 1: Grower phase

Pigs were generally in good health throughout this stage of the experiment. In total 2 pigs were taken off trial, one for poor health and the other for lameness.

3.4.1.1 Dietary analysis

Table 3.2. Analysed phytase and nutrient composition of the experimental diets offered to grower pigs throughout Period 1 (as-fed basis)

	Treatment			
Item	PC	NC	NC+500	NC+2000
DM (%)	89.5	89.0	89.3	89.4
Total fat (%)	4.90	4.34	4.49	4.34
Crude protein (%)	20.08	20.6	21.3	21.1
Crude fibre (%)	2.40	2.60	2.70	2.60
Ash (%)	5.90	5.40	5.40	5.30
Ca (%)	0.98	0.82	0.85	0.83
Total P (%)	0.71	0.56	0.57	0.57
Available P (%)	0.41	0.28	0.28	0.28
Phytate P (%)	0.24	0.23	0.22	0.21
Ca:P	1.38	1.46	1.49	1.46
Na (%)	0.21	0.21	0.26	0.22
K (%)	0.99	1.03	1.01	0.95
Mg (%)	0.14	0.14	0.14	0.14
Mn (mg/kg)	16	15	17	14
Fe (mg/kg)	300	298	298	308
Cu (mg/kg)	149	174	151	152
Zn (mg/kg)	64	59	57	57
Myo-inositol (g/kg)	0.09	0.09	0.10	0.05
Phytase (FTU/kg)	65	<50	578	1920

The analysed nutrient values for Period 1 diets are presented in Table 3.2. Both total and available P levels were in close agreement with the targeted values. Ca levels, however, were approximately 0.23 % higher than expected in each of the diets. According to the BSAS (2003) recommendations, the Ca requirement for a growing pig of 10 to 30 kg is 0.75 %; therefore, these diets are not considered to be Ca deficient. The analysed phytase activity in each of the test diets were close to targeted. Levels of crude protein and crude fibre were similar across treatments.

3.4.1.2 Growth performance

Table 3.3. Effect of phytase treatment on grower pig growth performance throughout Period 1 (d 0-28)[1]

	Treatment					
Item	PC	NC	NC+500	NC+2000	SEM	*P*-value
Pig BW (kg)						
d 0	17.35	17.33	17.32	17.31	0.11	0.997
d 14	29.82^{a}	28.85^{b}	29.60^{a}	29.67^{a}	0.14	<0.001
d 28	42.94^{a}	41.86^{b}	42.60^{a}	42.84^{a}	0.20	<0.01
ADFI (kg)						
d 0 - 14	1.19^{a}	1.13^{b}	1.18^{a}	1.16^{ab}	0.01	<0.05
d 14 - 28	1.59^{a}	1.54^{b}	1.58^{ab}	1.61^{a}	0.02	<0.05
Overall	1.39^{a}	1.34^{b}	1.38^{a}	1.38^{a}	0.01	<0.05
ADG (kg)						
d 0 - 14	0.89^{a}	0.82^{b}	0.88^{a}	0.87^{a}	0.01	<0.01
d 14 - 28	0.94	0.94	0.93	0.94	0.01	0.914
Overall	0.91^{a}	0.88^{b}	0.90^{a}	0.91^{a}	0.01	<0.01
FCR						
d 0 - 14	1.34	1.38	1.35	1.33	0.02	0.219
d 14 - 28	1.70	1.65	1.71	1.72	0.02	0.105
Overall	1.52	1.52	1.53	1.53	0.01	0.942

[1] Data are means of 16 replicate pens of 9 mixed sex pigs.
[a-b] Means within a row with different superscripts differ ($P<0.05$)

The effect of phytase treatment on grower pig growth performance is presented in Table 3.3. Pigs offered the low P NC diet had an overall 3.9% lower ADFI ($P<0.05$) and a 3.8% lower ADG ($P<0.01$) than pigs offered the nutritionally adequate PC diet. This resulted in these pigs weighing 1.08 kg less than those fed the PC at d 28. Adding phytase to the NC at 500 or 2,000 FTU/kg increased the ADG and ADFI of the grower pigs to a level comparable with those fed the PC. Grower pig FCR was similar irrespective of dietary treatment ($P>0.05$). Increasing the phytase dose from 500 to 2,000 FTU/kg delivered no further improvements in terms of growth performance at this stage of the experiment ($P>0.05$).

3.4.1.3 Ileal inositol phosphate and MYO concentration

Ileal $InsP_{3-6}$ and MYO concentration are presented in Table 3.4. The phytate hydrolysis products $InsP_5$, $InsP_4$ and $InsP_3$ are also presented as a proportion of the sum of the hydrolysis products ($\sum InsP_{3-5}$). As expected, pigs offered diets containing supplementary phytase had lower concentrations of $InsP_6$ and $InsP_5$ in the ileal digesta ($P<0.001$) than those fed diets without supplementary phytase; however, there was no difference between the standard and high phytase treatments. This decrease in ileal $InsP_6$ and $InsP_5$ concentration in pigs fed diets with added phytase was met with a concomitant numerical rise in ileal $InsP_4$ concentration. Ileal MYO concentration was higher in pigs fed the NC+500 treatment than those fed the PC ($P<0.05$).

$InsP_4$ was the predominant hydrolysis product in diets containing added phytase, whereas $InsP_5$ was the predominant hydrolysis product in diets devoid of added phytase ($P<0.001$). $InsP_3$ was a minor hydrolysis product in all treatments, accounting for between 3 and 6% of $\sum InsP_{3-5}$ in grower pig digesta.

3.4.1.4 Serum myo-inositol

The effect of treatment on portal and peripheral serum MYO concentration is presented in Table 3.5. Phytase increased serum MYO concentration in both the portal and peripheral blood when added to the NC at 2,000 FTU/kg ($P<0.05$). Supplementing the NC with 500 FTU/kg resulted in a small increase in portal and peripheral serum MYO concentration to a level not dissimilar to any other treatment.

Table 3.4. Effect of phytase on ileal inositol phosphate ($InsP_{3-6}$) and MYO concentration (nmol/mg TiO_2)[1]

Item	Treatment				SEM	*P*-value
	PC	NC	NC+500	NC+2000		
$InsP_6$	469.5^{a}	582.6^{a}	283.3^{b}	263.6^{b}	52.4	<0.001
$InsP_5$	59.7^{a}	59.4^{a}	28.6^{b}	22.7^{b}	3.7	<0.001
$InsP_4$	49.2	47.6	79.2	71.4	13.6	0.207
$InsP_3$	3.1	8.4	4.8	4.0	1.9	0.219
MYO	106.1^{a}	175.1^{ab}	301.5^{b}	152.8^{ab}	38.9	<0.05
$InsP_5$: $\sum InsP_{3-5}$	0.57^{a}	0.55^{a}	0.27^{b}	0.26^{b}	0.06	<0.001
$InsP_4$: $\sum InsP_{3-5}$	0.40^{a}	0.38^{a}	0.69^{b}	0.70^{b}	0.06	<0.001
$InsP_3$: $\sum InsP_{3-5}$	0.03	0.06	0.04	0.03	0.01	0.165

[1] Data are means of 8 replicate pigs.

[a-b] Means within a row with different superscripts differ ($P<0.05$).

Table 3.5. Effect of phytase on portal and peripheral serum MYO concentration[1]

	Serum MYO (nmol/ml)	
Treatment	Portal	Peripheral
PC	15.9[ab]	13.9[a]
NC	13.5[a]	14.5[a]
NC+500	17.3[ab]	17.5[ab]
NC+2000	26.8[b]	25.3[b]
SEM	2.75	2.92
P-value		
Treatment	<0.05	<0.05
Sample site[2]	0.584	
Treatment x sample site	0.815	

[1] Data are means of 8 replicate pigs.
[2] Paired portal vs peripheral analysis.
[a-b] Means within column with different superscripts differ ($P<0.05$).

3.4.1.5 Metatarsal bone characteristics

Metatarsal data are presented in Table 3.6. Bone fat-free dry weight, length and width measurements did not differ between treatments ($P>0.05$). Pigs receiving the PC or NC+2000 diets had a higher bone ash content than those fed the NC or NC+500 diets ($P<0.01$). Bone P ($P<0.01$) and Ca ($P<0.05$) levels were significantly lower in those receiving the NC than those receiving the PC ($P<0.01$). Supplementing the NC diet with 2,000 FTU/kg increased both bone P and Ca to a level equal to that of the PC. Those receiving the NC+500 treatment had intermediate bone P and Ca levels, not dissimilar from any of the other treatments, including the NC. The bone Mg response to phytase was similar to that of Ca and P, as pigs fed the NC+2000 treatment had higher levels of Mg in the M3 than those fed the NC ($P<0.05$). As with Ca and P, those receiving the NC+500 treatment had intermediate levels of Mg, not dissimilar from any other treatment. The bone Ca:P ratio in the M3 bone was not influenced by treatment ($P>0.05$).

Table 3.6. Effect of phytase treatment on grower pig metatarsal bone traits[1]

Item	Treatment				SEM	*P*-value
	PC	NC	NC+500	NC+2000		
Fat-free dry weight (g)[*]	8.94	8.36	9.13	8.77	0.32	0.389
Bone length (mm)[*]	71.87	71.70	70.78	71.17	0.85	0.789
Narrowest midshaft width (mm)[*]	12.71	12.61	12.49	12.69	0.31	0.954
Widest midshaft width (mm)[*]	20.84	20.70	20.56	20.51	0.40	0.938
Bone ash (%)	37.89[a]	35.65[b]	35.99[b]	37.91[a]	0.45	<0.01
P (%)	6.79[a]	6.43[b]	6.54[ab]	6.92[a]	0.10	<0.01
Ca (%)	12.16[a]	11.29[b]	11.97[ab]	12.42[a]	0.24	<0.05
Mg (%)	0.158[ab]	0.141[b]	0.165[ab]	0.169[a]	0.007	<0.05
Ca:P	1.75	1.76	1.68	1.80	0.057	0.535

[1] Data are means of 8 replicate pigs.
[a-b] Means within a row with different superscripts differ ($P<0.05$).
[*]Slaughter weight covariate significant: fat-free dry weight ($P<0.001$); bone length ($P<0.01$); narrowest midshaft width ($P<0.05$); widest midshaft width ($P<0.01$). The relationship between the covariate and the independent variables was positive in each case.

3.4.1.6 Phosphorus utilisation

The effect of dietary treatment on P utilisation throughout Period 1 is presented in Table 3.7. Pigs receiving the PC had the highest P intake throughout this stage of the experiment, and those fed the NC had the lowest (P<0.001). Supplementing the NC with phytase at either level resulted in an increase in P intake due to an increase in feed intake (P<0.001). There was a tendency (P=0.073) for phytase to increase the AID of P, with the higher dose having a greater effect than the standard dose. Estimates of apparent ileal P excretion were made using the average feed intake, analysed dietary P concentration, and the AID coefficients. Supplementing the NC with phytase at 500 FTU/kg reduced estimated ileal P excretion (P<0.01). This effect was greater when the phytase dose increased from 500 to 2,000 FTU/kg. Grower pigs fed a diet with supplementary phytase had a lower ileal P flow (P<0.05) than either of the controls; however, there was no difference between the standard and high phytase treatments.

3.4.1.7 Nutrient digestibility

The effect of phytase on ileal mineral flow, digestibility and concentration is presented in Table 3.8. Apparent ileal dry matter digestibility was not influenced by dietary treatment. However, there was a trend for phytase supplementation at either level to improve the apparent ash digestibility of the NC back to the level of the PC (P=0.054). Treatment had no effect on ileal Ca flow (P>0.05), and although there were numerical increases in Ca digestibility with increasing phytase dose, this effect was not significant (P>0.05). Pigs fed the NC treatment had a lower AID of Zn than those fed the PC, however, upon the supplementation of phytase at either level, this was restored back to the level of the PC. Adding 500 FTU of phytase/kg or DCP to the NC significantly reduced (P<0.05) Cu digestibility, whereas the addition of 2,000 FTU/kg resulted in a marginal decrease in Cu digestibility, to a level not significantly different from any other treatment. The standard phytase dose had no effect on apparent Na digestibility, whereas 2,000 FTU/kg of added phytase markedly increased apparent Na digestibility (P<0.05). There was no measurable effect of dietary treatment on Mg digestibility.

Table 3.7. Effect of phytase on phosphorus utilisation[1]

	Treatment					
	PC	NC	NC+500	NC+2000	SEM	*P*-value
Phosphorus						
Intake (g/d)	9.9[a]	7.5[b]	7.9[c]	7.9[c]	0.08	<0.001
Ileal flow (g/kg DMI)	3.5[a]	3.2[a]	2.6[b]	2.3[b]	0.28	<0.05
Apparent absorption (g/d)	5.7[a]	3.9[b]	4.7[c]	5.1[d]	0.05	<0.001
Ileal excretion (g/d)	4.2[a]	3.6[b]	3.1[c]	2.8[d]	0.03	<0.001
Ileal concentration (mg/g TiO_2)	679[a]	691[a]	516[b]	469[b]	56	<0.05
Apparent ileal digestibility (%)	57.3	51.9	60.1	64.6	4.0	0.073

[1] Data are means of 8 replicate pigs.

[a-c] Means within a row with different superscripts differ (P<0.05).

Table 3.8. Effect of phytase on ileal mineral flow and dry matter, ash and mineral digestibility[1]

	Treatment					
Item	PC	NC	NC+500	NC+2000	SEM	*P*-value
Dry matter digestibility	73.2	73.6	71.1	76.2	1.9	0.266
Ash digestibility	47.1	40.9	48.6	48.1	2.4	0.054
Calcium						
Digestibility (%)	55.54	52.95	58.36	59.69	5.21	0.657
Ileal flow (g/kg DMI)	4.34	4.15	3.84	3.84	0.45	0.810
Ileal concentration (mg/g TiO_2)	835.0	902.2	753.6	767.5	90.4	0.610
Magnesium						
Digestibility (%)	18.5	12.7	16.8	17.6	6.3	0.911
Ileal flow (mg/kg DMI)	1233	1382	1352	1293	1148	0.672
Ileal concentration (mg/g TiO_2)	237	301	265	259	19	0.127
Sodium						
Digestibility (%)	-1048[a]	-1161[a]	-1118[a]	-708[b]	116	<0.05
Ileal flow (mg/kg DMI)	33192	27513	34507	27426	3206	0.220
Ileal concentration (mg/g TiO_2)	5779	5981	7449	5484	584	0.108
Potassium						
Digestibility (%)	97.9	98.3	97.6	98.3	0.2	0.240
Ileal flow (mg/kg DMI)	238	212	266	169	214	0.073
Ileal concentration (mg/g TiO_2)	45.7	46.1	52.2	33.8	5.1	0.107
Copper						
Digestibility (%)	-0.7[a]	24.2[b]	-1.3[a]	13.7[ab]	7.0	<0.05
Ileal flow (mg/kg DMI)	168	149	172	147	146	0.341
Ileal concentration (mg/g TiO_2)	32.2	32.4	33.7	29.5	2.5	0.649
Iron						
Digestibility (%)	-19.2	-8.4	-16.6	-9.2	4.7	0.240
Ileal flow (mg/kg DMI)	357.6	323.4	348.0	337.3	14.2	0.307
Ileal concentration (mg/g TiO_2)	68.7	70.4	68.4	67.4	2.7	0.843
Zinc						
Digestibility (%)	-277[a]	-325[b]	-229[a]	-246[a]	19	<0.01
Ileal flow (mg/kg DMI)	283	249	250	280	14	0.096
Ileal concentration (mg/g TiO_2)	54.5	54.1	49.0	56.1	2.8	0.248

[1] Data are means of 8 replicate pigs.
[a-b] Means within a row with different superscripts differ ($P<0.05$).

Dietary treatment had no influence on the ileal flow of any of the minerals measured, although there was a tendency for phytase at 2,000 FTU/kg to reduce K flow (*P*=0.073) when compared with the NC+500 treatment.

The concentrations of Ca, Mg, Cu and Zn in the ileal digesta were not affected by treatment (*P*>0.05). This is also true for Na and K, however, there were numerical increases in the ileal concentrations of these minerals when 500 FTU of phytase/kg was added to the diet, but not when 2,000 FTU/kg was added.

3.4.1.8 Serum minerals

The effect of treatment on portal and peripheral serum mineral concentration is presented in Table 3.9. Peripheral serum concentrations of Ca, P, Mg, K and Fe were not influenced by treatment in this experiment, although there were numerical increases in Ca, P and Fe levels when provided with 2,000 FTU/kg. There was a tendency for the high phytase dose to increase Na levels in the peripheral sera (*P*=0.098).

Levels of Ca in the portal sera tended to increase with increasing phytase dose (*P*=0.077). In addition, there was a numerical increase in portal Fe concentration in response to phytase. P, Mg and K levels in the portal circulation were not influenced by dietary treatment (*P*>0.05).

P, Mg and K levels were higher in the portal than in the peripheral blood, whereas Fe levels were higher in the peripheral blood. There was no difference between portal and peripheral concentrations of Na (*P*>0.05). Within sampling site, Ca levels were similar for the PC, NC+500 and NC+2000 treatments; however, in the NC, Ca was significantly higher in the peripheral than in the portal blood thus resulting in a significant treatment x sampling site interaction (*P*<0.05).

Table 3.9. The effect of phytase on portal and peripheral serum mineral concentrations (µg/ml)[1]

Mineral	Ca		P		Mg		K		Na		Fe	
Blood	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral
PC	101.5	103.3	128.1	111.1	26.6	20.9	524.8	244.4	3464	3414	1.20	1.35
NC	94.3	107.3	118.4	109.8	23.7	23.5	498.3	262.6	3460	3491	0.91	1.22
NC+500	103.3	101.0	124.0	110.5	28.4	20.8	525.6	267.5	3509	3356	1.08	1.31
NC+2000	107.7	111.6	122.3	115.8	27.0	22.6	524.8	265.2	3486	3675	1.15	1.44
SEM	3.6	3.5	3.9	3.8	1.6	0.9	29.9	15.4	97.1	89.9	0.12	0.16
P-value												
Treatment	0.077	0.175	0.314	0.682	0.190	0.110	0.851	0.707	0.978	0.098	0.118	0.804
Sample site[2]	<0.05		<0.001		<0.001		<0.001		0.939		<0.001	
Treatment x sample site	<0.05		0.203		<0.01		0.338		0.221		0.846	

[1] Data are means of 8 replicate pigs.
[2]Portal vs peripheral paired analysis.

3.4.2 Period 2: Finisher phase

With few exceptions, the pigs were generally in good health throughout this Period of the experiment. One pig died and 4 pigs were taken off trial, one for a bleeding hernia, two due to prolapse, and another for poor health.

3.4.2.1 Dietary analysis

Table 3.10. Analysed nutrient content and phytase activity of the experimental diets offered to finisher pigs throughout Period 2 (as-fed basis)

	Treatment	
Item	**500**	**2500**
DM (%)	88.50	88.30
Total fat (%)	3.39	3.76
Crude protein (%)	20.90	19.80
Crude fibre (%)	2.80	2.60
Ash (%)	5.30	5.20
Total P (%)	0.54	0.52
Phytate P (%)	0.22	0.21
Available P (%)	0.31	0.30
Ca (%)	0.79	0.83
Phytase (FTU/kg)	566	2140

The analysed nutrient values for Period 2 diets are presented in Table 3.10. Both total and available P levels were slightly higher than formulated (by approximately 0.5 g/kg). As with Period 1, Ca levels were approximately 2.3 g/kg higher than formulated in each of the diets. According to the BSAS nutrient standards, growing pigs of 30 to 60 kg require around 7.2 g Ca/kg feed for optimal growth; therefore, these diets are not considered to be Ca deficient (BSAS, 2003). The measured phytase activity in the 500 FTU/kg treatment was close to formulated (566 FTU/kg), whereas the measured phytase activity in the 2,500 FTU/kg diet was slightly lower than formulated (2,140 FTU/kg), but still a super-dose.

3.4.2.2 Growth performance

The effects of dietary treatments on finisher pig growth performance from d 30 to 49 and overall (d 0 to 49) are presented in Table 3.11. There was no Diet 1 x Diet 2 interaction for grower pig ADFI or FCR throughout Period 2. Pigs receiving the super-dose of phytase throughout this stage of the experiment had a significantly higher ADFI ($P<0.01$) than those receiving the standard phytase dose. However, this only translated into numerical increases in ADG when previously fed the PC or NC+500 treatments. As the effect of phytase treatment throughout this Period was dependent on the previous treatment there was a significant Diet 1 x Diet 2 interaction for ADG ($P<0.05$). Pigs receiving the NC followed by the 2,140 FTU/kg treatment, or the standard phytase dose throughout had a lower Period 2 ADG ($P<0.05$) than those initially fed the PC followed by the 2,140 FTU/kg, and a numerically lower ADG than each of the other treatments. The data also show a trend for pigs receiving the super-dose treatment throughout Period 2 to have a higher FCR ($P=0.068$) than those receiving the standard phytase dose.

There was no Diet 1 x Diet 2 interaction for pig ADFI or FCR throughout the total experimental Period ($P>0.05$). Pigs receiving the high phytase Diet 2 treatment ate more throughout the total experiment than those receiving the standard dose ($P<0.01$; 1.52 vs 1.58 kg/d). There was also a tendency for pigs initially fed the NC Diet 1 treatment to eat less ($P=0.086$) throughout the total experimental Period. As with Period 2, there was a significant Diet 1 x Diet 2 interaction on overall ADG ($P<0.01$), as pigs fed the high phytase diet (2,140 FTU/kg) throughout Period 2 appeared to have a higher ADG than those receiving the standard phytase (566 FTU/kg) Diet 2 treatment, but only when previously fed either the PC or the NC+500 Period 1 treatment. As a result, there was also a significant Diet 1 x Diet 2 interaction on final BW ($P<0.05$).

Pigs offered the NC diet had the lowest growth rate and feed intake throughout Period 1 ($P<0.01$). However, upon the subsequent provision of 500 FTU/kg, these pigs went on to have one of the highest growth rates throughout Period 2, whilst maintaining a relatively low-feed intake. As a result, pigs on this treatment had the numerically lowest FCR throughout Period 2 and the whole experiment. This effect was less apparent when pigs initially fed the NC diet went on to receive the super-dose treatment.

Pigs fed the super-dose throughout Period 2 had a higher overall FCR than those fed the standard phytase dose (P<0.01; 1.73 vs 1.79). This is largely due to the NC:2140 and NC+2000:2140 treatments whereby super-dosing throughout Period 2 increased ADFI, but failed to elicit a corresponding increase in ADG. In addition, the much improved FCR of the pigs exhibiting catch up growth (NC:500) contributed to this effect.

Table 3.11. Effect of phytase treatment on grower pig growth performance throughout Period 2 (d-30-49) and total experimental Period (d 0 to 49)[1]

	Diet 1[2]	PC		NC		NC+500		NC+2000			*P*-value		
Item	Diet 2	566	2140	566	2140	566	2140	566	2140	SEM	D1	D2	D1xD2[3]
Period 2													
ADFI, kg[f]		1.75	1.93	1.77	1.81	1.77	1.90	1.78	1.93	0.06	0.623	<0.01	0.713
ADG, kg		0.83^{ab}	0.91^{b}	0.90^{b}	0.84^{ab}	0.81^{a}	0.87^{ab}	0.84^{ab}	0.84^{ab}	0.02	0.404	0.192	<0.05
FCR		2.12	2.13	1.96	2.16	2.21	2.18	2.12	2.29	0.07	0.120	0.068	0.250
Overall													
ADFI, kg[g]		1.53	1.60	1.50	1.52	1.53	1.60	1.53	1.60	0.03	0.086	<0.01	0.672
ADG, kg		0.87^{ab}	0.91^{a}	0.89^{ab}	0.85^{b}	0.86^{b}	0.89^{ab}	0.89^{ab}	0.88^{ab}	0.01	0.106	0.352	<0.01
FCR[h]		1.75	1.76	1.69	1.78	1.78	1.80	1.72	1.81	0.03	0.265	<0.01	0.228
End weight, kg		60.25^{ab}	61.99^{a}	60.64^{ab}	59.22^{ab}	58.94^{b}	61.39^{ab}	61.15^{ab}	60.31^{ab}	0.78	0.421	0.386	<0.05

[1] Values represent means of 8 replicate pens of 8 mixed sex pigs.
[2] Diet 1 fed from d 0 to 30; Diet 2 fed from d 30 to 49.
[3] D1 = Diet 1, fed from d0 to 30; D2 = Diet 2, fed from d 30 to 49; D1 x D2 = Diet 1 x Diet 2 interaction.
[a-c] Means within a row with different superscripts differ ($P<0.05$).
[f] Effect of diet 2: 566 = 1.77 kg, 2140 = 1.89 kg.
[g] Effect of diet 2: 566 = 1.52 kg, 2140 = 1.58 kg.
[h] Effect of diet 2: 566 = 1.73, 2140 = 1.79.

3.5 Discussion

3.5.1 Period 1: Grower study

3.5.1.1 The effect of phytase on growth performance

Grower pigs fed the low P control diet during Period 1 had a lower growth rate than those fed the high P control throughout this stage of the experiment. This effect was expected and was due to a reduction in feed intake and marginal P deficiency (Ekpe et al., 2002). Supplementing the NC with phytase restored the lost growth performance back to the level of the PC. That phytase improves the performance of pigs offered low P diets is well documented (Cromwell et al., 1993; Harper et al., 1997; Kühn and Männer, 2012) and can be attributed to improved P bioavailability through phytase induced phytate degradation. In support of this are the ileal inositol phosphate, P utilisation and bone data.

The breakdown of the performance data shows that the negative effects of the low P diet on pig performance were expressed in the first two weeks of the study, as those receiving this diet performed similarly to all other treatments in the subsequent two weeks. As the P requirement of the growing pig declines as the pig grows (BSAS, 2003), it may be that the level of P in the NC diet (2.8 g available P/kg) was sufficient for optimal growth throughout the latter stages of the experiment. According to the BSAS Nutrient Requirement Standards (2003), a growing pig of 10 to 30 kg requires around 3.4 g available P/kg feed, whereas a growing pig of 30 to 60 kg requires around 2.5 g available P/kg feed. Therefore, it is likely that NC diet was no longer limiting in available P in the latter stages of this study. In addition, pigs are able to adapt to low dietary P supply by increasing enterocyte absorption efficiency through upregulation of Na^+-phosphate cotransporter proteins (Saddoris et al., 2010). Therefore, it is also possible that pigs fed the NC diet adapted to the suboptimal P supply and began to utilise the available P more efficiently. The numerical drop in grower pig FCR throughout the final two weeks for those fed the NC supports this notion.

It was hypothesised that grower pigs fed the phytase super-dose (2,000 FTU/kg) would have superior growth performance than those fed the standard phytase (500 FTU/kg) or the high P positive control treatments. However, in this study, there was

no difference between the super-dose and standard dose for any of the performance parameters measured. These findings are contrary to the recent study of Santos et al. (2014), who found that supplementing grower diets (~23 to 57 kg BW) with 2,000 FTU/kg of the same phytase enzyme for 43 days improved ADG and FCR when compared to a nutritionally adequate control. In agreement with the findings of Santos et al. (2014), Braña et al. (2006) and Zeng et al. (2014) also reported FCR benefits in pigs through super-dosing phytase. It is possible that the phytase application duration in the present experiment was too short for a favourable FCR response to manifest.

In addition, the higher than expected Ca concentration and Ca:P ratio in the diets used in this experiment are likely to have had a negative impact on both phytase efficacy and P absorption. It is established that high Ca levels suppress P absorption (Vipperman et al., 1974) and that 'wide' Ca:P ratios have a negative impact on phytase efficacy (Qian et al., 1996; Liu et al., 1998; Liu et al., 2000). For example, Adeola et al. (2006), determined the effect of varying Ca:P ratios on phytase efficacy in weaner pigs fed corn-SBM diets, and found that increasing the Ca:P ratio from 1.2:1 to 1.8:1 resulted in a 17.6 % decrease in ADG. The Ca:P ratio in the present study was higher than in each of the aforementioned super-dosing studies (1.46 to 1.49:1 vs 0.88 to 1.35:1), therefore, these differences may also explain the lack of super-dosing growth response in this experiment.

3.5.1.2 Ileal inositol phosphate and MYO concentration

A primary objective of this study was to determine the effect of a standard and a super-dose of phytase on the concentration of phytate ($InsP_6$), phytate hydrolysis products ($InsP_{3-5}$) and MYO in the ileal digesta. Pigs fed the NC diet had similar levels of $InsP_6$ and $InsP_5$ in the ileal digesta as those fed the PC, indicating that lowering the available P concentration in the diet had no effect on phytate hydrolysis. This is in agreement with other studies that have measured phytate hydrolysis in pigs (Rutherfurd et al., 2014; Zeng et al., 2014; Zeng et al., 2016).

Adding phytase to the low P NC was efficacious in reducing the concentration of $InsP_6$ and $InsP_5$ at the terminal ileum; however, in disagreement with the hypothesis, there was no difference between the standard and high phytase treatment. Again, this may attributed to the high dietary Ca content having a negative effect on phytase efficacy by forming insoluble Ca-phytate complexes within the GIT (Wise, 1983).

$InsP_5$ was the predominant lower inositol phosphate measured in the ileal digesta of pigs receiving a non-supplemented diet, accounting for 55 to 57% of $\sum InsP_{3-5}$. Upon the addition of phytase, $InsP_4$ became the predominant lower inositol phosphate ester in the ileal digesta, accounting for 69 to 70 % of $\sum InsP_{3-5}$. This finding suggests that $InsP_4$ is the principal hydrolysis product of Quantum® Blue and supports the conclusions of Wyss et al. (1999) and Greiner and Farouk (2007) in that modern bacterial phytase enzymes preferentially target the higher esters ($InsP_6$ and $InsP_5$) of phytate.

Few studies have investigated the effect of phytase on phytate hydrolysis in the pig. Using a different 6-phytase product (*C. braakii* derived) to the one used in this study, Guggenbuhl et al. (2012b) reported a significant increase in apparent ileal phytate degradation (78.8 vs 86.5%) in growers following an increase in phytase dose from 500 to 1,000 FTU/kg. Similarly, in broilers, Shirley and Edwards (2003) found that increasing the level of phytase continued to improve phytate hydrolysis up to their highest tested dose of 12,000 FTU/kg. These findings are contrary to the results presented in this study, whereby increasing the phytase dose delivered no further improvements in phytate degradation. A similar effect was reported by Rutherfurd et al. (2014), as they found that increasing the level of supplementary phytase (*C. braakii* derived enzyme) from 1,105 to 2,215 FTU/kg in a low P corn-SBM diet had no effect on phytate hydrolysis at the terminal ileal level. Zeng et al. (2016) found that adding 500 FTU/kg of a *Butiauxella* derived phytase to a low Ca and P corn-SBM grower diet reduced ileal $InsP_6$ concentration to a similar extent as the standard dose did in the current experiment (2.25-fold). In their study, increasing the phytase dose to 1,000 FTU/kg delivered no further reductions in $InsP_6$ concentration; however, increasing it to 20,000 FTU/kg resulted in a 9.66-fold reduction in ileal $InsP_6$. Similar findings were reported in an earlier study by the same workers: increasing the phytase (*E.coli* derived enzyme) dose from 500 to 1,000 FTU/kg had no effect on ileal $InsP_6$ hydrolysis (2.07 vs 2.50-fold reduction). However, by increasing the dose to 20,000 FTU/kg, the concentration of $InsP_6$ at the terminal ileum was reduced further (4.65-fold). Therefore, it is plausible that the increase from 500 to 2,000 FTU of phytase/kg in the present experiment was not enough to see significant increases in phytate hydrolysis, particularly as the dietary Ca levels were so high.

The principal site of phytate degradation in the pig is the stomach (Jongbloed et al., 1992; Kemme et al., 2006). In order for phytase to catalyse phytate hydrolysis the phytate must be solubilised. It is possible that in the present study phytate solubility in the stomach, rather than enzyme concentration was limiting the rate of phytase hydrolysis. Moreover, it is well known that there are appreciable differences in the susceptibility of phytate to phytase induced hydrolysis among different feedstuffs (Leske and Coon, 1999; Blaabjerg et al., 2007). Therefore, it is likely that differences in diet composition, nutrient content, and phytase enzymes have all contributed to the differences in phytate degradation observed between the current and previously reported studies.

In this experiment, there was no difference in ileal MYO concentration between the NC, NC+500 or NC+2000 treatments. Pigs receiving the 500 FTU/kg treatment had more MYO at the terminal ileum than those fed the PC treatment. At the time of writing no other published papers have looked into the effects of phytase on ileal MYO generation. Walk et al. (2013) found that phytase increased MYO levels in the gizzard digesta in broilers; unfortunately the MYO concentration in the ileal digesta was not determined. Clements and Reynertson (1977) demonstrated that MYO is readily absorbed in the small intestine, with rats and humans absorbing up to 99.8% of the total dietary MYO content. Therefore, it is likely that in the present study the super-dose treatment did increase MYO levels in the small intestine through phytate degradation (as supported by the serum MYO data); however, the MYO generated was being absorbed prior to the site of digesta collection.

3.5.1.3 The effect of phytase on serum MYO concentration

The final product of complete phytate hydrolysis, MYO, has recently been identified as a possible candidate for stimulating the performance benefits commonly observed in other studies (Cowieson et al., 2011). MYO is a carbocyclic hexitol that is ubiquitously present among eukaryotes (Holub, 1986). The biological functions of this metabolically active organic compound and its derivatives are numerous and diverse. In mammalian cells, MYO can be phosphorylated by endogenous kinase enzymes to form a variety of phosphoinositides and inositol phosphates, many of which have important functions in cell trafficking and signal transduction (Holub, 1986). MYO can also be used to synthesise the important antioxidant inositol hexakisphosphate (phytate). Phytate is typically present in mammalian cells at

concentrations of between 10 and 100 μM (Sasakawa et al., 1995), where it can chelate with free Fe ions, thereby preventing them from catalysing the formation of cell damaging ROS via the Fenton reaction (Graf et al., 1987). Another key function of MYO in eukaryotic cells is as a compatible omsmolyte, protecting cells and cellular components from high or low osmotic environments (Holub, 1986). Cowieson et al. (2013) and Zyla et al. (2013) have both recently shown that MYO supplementation can improve broiler growth and efficiency.

There is a lack of evidence to support the view that MYO is involved in the super-dosing response in pigs; therefore, one of the main objectives of this study was to determine the effect of phytase on circulating levels of MYO in the growing pig. The results presented in this study show that phytase does indeed increase levels of MYO in both portal and peripheral serum. There was a 10.9 nmol/ml increase in portal MYO and a corresponding 11.4 nmol/ml increase in peripheral MYO in response to the super-dosing treatment. An increase in MYO levels in the portal blood demonstrates that the extra MYO liberated from phytate hydrolysis is being absorbed from the GIT, and the increase in peripheral levels shows that the MYO absorbed is largely available for systemic circulation. Interestingly, the levels of blood MYO were similar between pigs fed the standard phytase dose and control treatments. This may in part explain why performance benefits to phytase are not observed at the standard inclusion levels of 500 FTU/kg. This study is the first to demonstrate that phytase super-dosing does indeed improve MYO bioavailability in the pig and are in agreement with the findings of Cowieson et al. (2015), who found that 2,000 FTU/kg increased plasma MYO levels in broiler chickens.

3.5.1.4 The effect of phytase on mineral availability

Phytate has the capacity to bind with multivalent cations within the GIT, forming insoluble complexes that are unavailable for digestion (Section 1.5.1). The current understanding is that phytate-mineral complexes become increasingly insoluble as the pH increases, with precipitation tending to occur at pH ≥ 5 (i.e. in the small intestine). Persson et al. (1998) found that stability of the phytate-mineral chelates decreased as the level of phosphorylation on the phytate molecule decreased, and suggested that degradation to at least $InsP_3$ is necessary to minimise insoluble mineral complex formation at higher pH levels. Therefore, by hydrolysing phytate to lower phytate esters in the gastric regions of the tract, phytase can reduce phytate-

mineral complex formation and thereby improve mineral availability. Studies have shown that phytase can effectively improve Ca, Zn, Fe, Cu, Mg, Na and K digestibility in the pig (Pallauf et al., 1992; Adeola et al., 1995; Kies et al., 2006b). Improved mineral availability may result in improved retention, which in the case of heavy metals such as Cu and Zn will be of benefit to the environment as well as the animal. However, few studies have looked into the effects of super-dosing phytase on mineral availability in the grower pig; therefore, an objective of this study was to determine the effect of super-dosing phytase on mineral availability.

3.5.1.4.1 Bone and P utilisation

The bone is the primary storage organ of the macro-minerals P, Ca and Mg (McDowell, 1992). Bone ash and P concentration are considered a more responsive means of assessing P bioavailability than growth performance (Ketaren et al., 1993). In this study reducing the P content of the diet resulted in a decrease in bone ash content. The super-dose was effective in restoring the ash content back to the level of the PC, whereas the standard phytase dose was not. As pigs offered the high P control or super-dose treatments performed similarly to those fed the standard phytase treatment, these results suggest that P is no longer limiting growth in these pigs, and that the additional available P in the PC and 2,000 FTU/kg treatments has instead been used for bone mineral accrual. This supports the current assessment that the P requirement for maximal bone mineralisation in the pig is ~1 g/kg higher than it is for maximal growth (Hastad et al., 2004). These results are consistent with those of Brana et al. (2006), Veum et al. (2006), Varley et al. (2011) and Yanez et al. (2013) and show that phytase has the capacity to increase the bone ash content of pigs fed a low P diet. As in the present study, Veum et al. (2006) found that 500 FTU/kg of an *E.coli* derived phytase was sufficient in improving the performance of weaners fed a low P diet back to the level of a nutritionally adequate control, but not so for bone ash content. These findings imply that the current industry recommended inclusion rate of 500 FTU of phytase/kg is adequate for improving the performance of pigs fed a low P diet; however, it may be that the bone integrity is compromised, and in order to restore bone mineralisation to the level of those fed a nutritionally adequate diet, higher doses of phytase are necessary.

This is supported by the bone Ca, P and Mg data, which show that supplementing the low P diet with a super-dose of phytase increased bone P, Ca and Mg

concentrations to the level of the PC; however, those receiving the standard phytase dose had intermediate levels of Ca, P and Mg, not dissimilar to any other treatment. Many others have demonstrated that phytase can effectively increase bone Ca and P concentration (O'Doherty et al., 2010; Yanez et al., 2013; Zeng et al., 2015), although few have assessed the effects of phytase on bone Mg concentration. The effect of phytase on bone Mg is unsurprising given that 60 to 70% of the total body Mg is present in the bone (McDowell, 1992) and that phytase has been shown to improve Mg retention (Veum et al., 2006; Madrid et al., 2013). Madrid et al. (2013) also found phytase increased the Mg content of the third metacarpal in growers, though in their study the standard dose of 500 FTU/kg was sufficient.

P utilisation data were in agreement with the bone data. Phytase supplementation to the NC at either level increased P intake and reduced the ileal P flow. That phytase increases P digestibility is the most consistent and well documented effect of phytase in pigs (Simons et al., 1990; Kornegay and Qian, 1996; Harper et al., 1997; Kies et al., 2006b; Zeng et al., 2016). However the magnitude of the response in the literature is highly and this can likely be attributed to differences in phytase source, phytase dose, dietary ingredients, substrate level and nutrient content (most notably Ca, P and the Ca:P ratio; Section 1.7) In the present experiment the extent of the response to phytase was comparatively small (12.7% increase in P digestibility with 2,000 FTU/kg) and was only a trend. This is likely owing to the adverse effect of the relatively high Ca:P ratios (~1.5:1) of the diets on phytase efficacy (Qian et al., 1996; Liu et al., 2000). In grower-finishing pigs fed a low Ca and P corn-SBM diet supplemented with 500 FTU/kg, Liu et al. (1998) demonstrated that increasing the Ca:P ratio from 1.0:1 to 1.5:1 significantly reduced growth performance, P utilisation and bone ash weight. It is thought the adverse effect of wide Ca:P ratios stem from increased Ca-phytate complex formation (Wise, 1983). In addition, high Ca levels have been shown reduce P absorption through the formation of insoluble tricalcium-phosphate within the GIT (Vipperman et al., 1974).

Minimising P output from pig production facilities is central to reducing the damaging environmental impact of pig production. Although faecal P output was not determined in this study, it has been shown that very little P absorption occurs in the hindgut (Liesegang et al., 2002; Rutherfurd et al., 2014a). Therefore, the concentration of P in the ileal digesta and the P ileal flow measurements provide a good indication of P excretion. As phytase treatment at either dose reduced P flow

and digesta concentration at the terminal ileum it is likely that grower pigs fed these treatments had lower levels of P in their excreta. This observation is consistent with the findings of other studies (Lei et al., 1993; Almeida and Stein, 2010).

3.5.1.4.2 Nutrient digestibility and plasma minerals

In this study, phytase had no effect on DM AID but tended to increase ash AID in the grower pig. These findings are in general agreement with the literature as phytase digestibility trials commonly show improvements in ash digestibility in response to phytase (Jongbloed et al., 2000; Kies et al., 2006b; Vigors et al., 2014), whereas responses DM responses are less consistent (Harper et al., 1997; Woyengo et al., 2009b; Madrid et al., 2013).

The impact of phytase supplementation on Ca digestibility in the pig is well documented and the effects appear to be more variable than that of P digestibility. In this study, phytase had no effect on Ca digestibility. Similarly, Harper et al. (1997) reported no effect of phytase on Ca ATTD when supplemented to a low Ca and P corn-SBM based grower diets. In their study, digestibility values for Ca ranged from 54.7 to 58.4 %, which are in line with those obtained in this experiment and others (Johnston et al., 2004; Brana et al., 2006). Likewise, the studies of Han et al. (1997) and Velayudhan et al. (2015) found that phytase had no influence on Ca digestibility. Nevertheless, others have observed significant increases in Ca digestibility in response to phytase, by as much as 27% (Kemme et al., 1999; Igbasan et al., 2001; Jolliff and Mahan, 2012; Madrid et al., 2013; Zeng et al., 2015). It is likely that no Ca digestibility response was observed in this experiment as Ca levels in the diets were already approximately 1 g/kg above the requirement for growing pigs of this size (BSAS, 2003).

Standard doses of phytase have been shown to increase the apparent absorption of the divalent cations Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+} in growing pigs (Brady et al., 2003; Kies et al., 2006b; Madrid et al., 2013; Adedokun et al., 2015). Kies et al. (2006b) reported a dose-dependent effect of phytase supplementation to a Ca and P deficient corn-barley diet on weaner pig apparent mineral (Ca, P, Mg, Na, K and Cu) faecal digestibility up to 15,000 FTU/kg. In the present study, phytase treatment at either level had no influence on the apparent ileal digestibility of Fe, Mg or K. These findings concur with those of Rutherfurd et al (2014a), who also found that phytase (1,107 or 2,215 U/kg) had no effect on the AID of these minerals in grower

pigs when added to a low Ca and P corn-SBM diet. In bioavailability studies it is known that the diet must be deficient in the nutrient under investigation (Augspurger et al., 2003). In this experiment, Fe, Mg and K were provided at approximately 4-fold in excess of the requirement (BSAS, 2003). Therefore, the lack of response of these minerals to phytase supplementation is unsurprising.

Woyengo et al. (2009a) demonstrated that phytate has the capacity to stimulate endogenous Na secretions into the small intestine. It is thought that these secretions occur as $NaHCO_3$ and are in response to the compensatory outputs of HCl associated with refractory protein-phytate complexes in the stomach (Section 1.5.2). Furthermore, Na^+ is a monovalent cation that forms weak complexes with phytate which are soluble across a wide range of pH values (Scheuermann et al., 1988). Therefore, improvements in apparent Na absorption, such as those observed in this study with the phytase super-dose, are not thought to arise from improved availability in the intestine, but rather through a reduction in endogenous Na secretions.

In this experiment, phytase increased Zn AID and decreased Cu AID at 500 FTU/kg. Both Zn and Cu are transition metals that have similar electronic structures and relative atomic weights. Within the GIT, it is thought they act antagonistically to one another, competing for various proteins, such as metallothionein or nutrient transporters (Hill and Matrone, 1970). Therefore, an imbalance of one can have a negative effect on the uptake of the other. This was demonstrated in an early study by Magee and Matrone (1960) as they found that anaemia brought upon by high Zn intake in rats could be reversed by supplementing the diet with additional copper.

Negative AID values were obtained for Na and the trace metals Zn, Cu and Fe, which is indicative of mineral secretion into the gut. Pancreatic and biliary juices are known to have a high Na and Zn content and are thus likely to be the origin of these secreted minerals (Zebrowska et al., 1983; Kelleher et al., 2011). Considerable Na and some Zn reabsorption occurs in the hindgut, therefore, these excreted minerals are not necessarily excreted and lost from the animal (Rutherfurd et al, 2014a). Negative AID values for micro-minerals are common when the variation seen between samples exceeds the mean concentration in the diet (Agudelo-Trujillo, 2005). Although phytase increased the AID of Zn, the

digestibility coefficient was still negative. This suggests that phytase is improving Zn digestibility by reducing endogenous Zn secretions into the digestive tract.

Portal mineral concentrations generally reflected feed intake in that those fed the low P diet had numerically lower concentrations of minerals in the serum than those fed positive control or phytase treatments; however, this effect was not significant (Ca was a trend). The lower uptake of P, Ca and Mg in pigs fed the NC is reflected in the bone mineral data, as discussed above.

From the literature, it is apparent that phytase generally increases circulating levels of P (Gentile et al., 2003; Adeola et al., 2004; Beaulieu et al., 2007; Madrid et al., 2013). Although less common, a small number of studies have reported no effect of phytase on blood P, as is the case in this experiment (Pagano et al., 2007; Kerr et al., 2010). It is possible that differences in blood P were not observed in the present experiment as P was unlikely to be limiting in the latter stages of this experiment. In addition, the majority of phytase studies have been conducted in weaner pigs, and it has been demonstrated that differences in blood P levels in pigs fed sub-optimal and optimal P diets decrease as the pig matures (Jendza et al., 2005; Alexander et al., 2008).

Although phytase increased both Ca and Mg bioavailability in this experiment, as demonstrated with the bone data, the concentration of these minerals in the peripheral serum did not change. This was expected as circulating levels of these minerals are under tight homeostatic regulation, with excess being stored in the bone or excreted in the urine (Mundy and Guise, 1999; de Baaij et al., 2012). The tendency for Na levels in the peripheral serum to increase in response to the super-dose of phytase is in support of the improved Na digestibility result, and shows that the super-dose is likely ameliorating the negative effects of phytate on endogenous Na secretions (Woyengo et al., 2009a).

3.5.2 Finisher phase and total experimental period

Pigs that went on to receive the super-dose finisher treatment had a higher ADFI than those that went on to receive the standard phytase treatment (1.89 vs 1.77 kg). This is in agreement with the work of Kies et al. (2006b) and Veum et al. (2006), as both found that super-dosing phytase increased weaner pig feed intake over those fed a standard dose. It is unclear why pigs fed the super-dose had a higher feed intake, although it has been suggested that phytate is an appetite suppressant

(Cowieson et al., 2011). If this is so, it may be that pig feed intake was stimulated through greater phytate hydrolysis.

This increase in feed intake only translated into an improved rate of gain in pigs previously fed either the positive control or standard phytase dose treatments, resulting in a Diet 1 x Diet 2 interaction. Pigs initially fed the low P negative control had the lowest growth rate and feed intake throughout Period 1. However, upon the subsequent provision of 500 FTU/kg these pigs had a much improved rate of gain, whilst maintaining a comparatively low-feed intake. This response is indicative of compensatory growth: a period of rapid and efficient growth following a period of restricted growth (Mitchell, 2007). Interestingly, this response was less apparent when the pigs went on to receive the super-dose treatment throughout Period 2. Pigs adapt to a sub-optimal P supply by increasing the efficiency of intestinal P absorption through increased expression of the sodium dependent phosphate transporter NaPi-IIb (Saddoris et al., 2010), and increasing the maximal rate of P uptake (V_{max}; Katai et al., 1999). Therefore, when switched to a P adequate diet (500 FTU/kg), it is likely that these pigs are able to capture and utilise the P more efficiently. In this experiment, compensating pigs did not surpass the performance of pigs offered other treatments, thus this response is not considered true compensatory growth. Given more time it would be interesting to see if these pigs could fully compensate. It is unclear why this response was less apparent in those receiving the super-dose; however, it may be due to the potentially toxic effects of excessive P. These pigs have likely adapted to a low P diet and therefore when presented with a high available P diet, it may be that excessive amounts of P are absorbed. Hyperphosphatemia can lead to secondary hyperparathyroidism and increased fibroblast growth factor 23 (FGF23) secretion, both of which can stimulate tissue damage (Calvo and Uribarri, 2013).

The picture was similar for the total experimental period, those fed the super-dose throughout Period 2 had a higher overall feed intake irrespective of the initial grower diet treatment. ADG throughout the experiment was influenced by a significant Diet 1 x Diet 2 interaction, which was similar to that observed in Period 2. Pigs that received a high P diet (in the form of the PC or a super-dose) throughout at least one period of the experiment performed similarly, with the exception of those initially fed the NC. Therefore, those receiving the NC:2140 (Diet 1: Diet 2) or the NC+500:566 treatments had a lower ADG than those fed the

PC:2140 treatment, and a numerically lower ADG than those offered one of the other treatments. Pigs fed the super-dosing diet throughout Period 2 had a higher FCR throughout the experiment than those fed the standard dose. This is somewhat misleading as the FCR of pigs fed the super-dose finisher diet was only higher when it failed to elicit any improvements in ADG (i.e. the NC:2140 and 2000:2140 diets), but when a positive super-dosing response on growth was observed, the FCR was similar to their counterparts that went on to receive the standard phytase dose.

3.5.3 Conclusions

Exogenous microbial phytase effectively restored the growth performance of grower pigs fed low P wheat-barley-SBM based diets. This effect was the result of improved phytate hydrolysis by the terminal ileum and improved P retention. Increasing the phytase dose from 500 to 2,000 FTU/kg delivered no additional benefits in terms of grower performance, but was necessary to maintain bone mineralisation levels equal to those fed a P adequate diet.

Phytase effectively hydrolysed phytate in the GIT as demonstrated by the reduced concentration of $InsP_6$ in the ileal digesta. Although there was no significant differences in the ileal $InsP_{3-6}$ and MYO content between pigs fed the standard or high phytase dose, super-dosing phytase increased circulating serum MYO, which would suggest increased phytate hydrolysis in these pigs. The phytase used preferentially targeted the higher phytate esters $InsP_6$ and InP_5. This is pertinent as it these phytate esters that have a higher chelating capacity and thus a greater anti-nutritive effect. A central finding of this study was that super-dosing phytase effectively increased grower pig serum MYO concentration.

Phytase tended to improve apparent ileal P digestibility, with the super-dose having a greater effect than the standard dose. The AID of Ca, Mg, K and Fe were not influenced by phytase at either level. Other studies have reported improved mineral digestibility coefficients, particularly with higher doses of phytase; however, it is likely that in the present experiment the high dietary concentration of these minerals, particularly Ca, prevented a detectable phytase response.

The effect of phytase supplementation (566 vs 2140 FTU/kg) on finisher pig ADG throughout the subsequent phase of the experiment was dependent on the preceding grower treatment (Diet 1 x Diet 2 interaction). Pigs performed similarly throughout the experiment unless fed either the low P negative control followed by the super-

dose, or the standard phytase dose throughout both stages. It seems that pigs that received a high P diet (be it the PC or phytase super-dose) for at least one stage of the experiment performed similarly overall, with the exception of the negative control followed by the super-dose treatment. It may be that these pigs were unable to cope with excessive amounts of available P following a period of P restriction. The Diet 1 x Diet 2 interaction observed in this study is difficult to interpret but suggests that the effect of phytase may be dependent on the pigs' existing nutritional status. More research is required to confirm and clarify this interaction.

Chapter 4
The Effects of High Levels of Phytase on Pig Performance at the Early Finishing Stage of Production

4.1 Abstract

An experiment was conducted to evaluate the effects of supplementing a low Ca and P diet with high levels of phytase on finisher pig growth performance, phytate degradation, MYO availability, AA digestibility, mineral availability and intestinal nutrient transporter gene expression. A total of 288 mixed sex crossbred pigs (initial BW ~37 kg) were used in this 28 d study. Pigs were fed one of the following six dietary treatments: (PC) a positive control formulated to meet or exceed the BSAS (2003) nutrient recommendations for all nutrients; (NC) a negative control similar to the PC but with reductions in Ca (reduced by1.6 g/kg), P (reduced by 1.24 g/kg), and ME (0.217 MJ/kg); and the NC diet supplemented with phytase at 500, 1,000, 2,000 or 8,000 FTU/kg. All diets were wheat-barley based and were provided *ad libitum* throughout the study. At the end of the study (d 28 and 29) 60 pigs were euthanised for the collection of ileal digesta, portal and peripheral plasma, the third proximal phalanx and jejunal mucosa.

Phytase improved the ADG ($P<0.05$) and FCR ($P<0.001$) of finisher pigs fed the nutritionally inadequate NC diet to the level similar to the PC; however, there was no difference between the standard and higher phytase treatments. There were linear increases in ileal phytate degradation in response to increasing phytase dose ($P<0.01$) and this was met with a linear increase in ileal MYO concentration ($P<0.001$). Plasma data suggest that this MYO was largely available for absorption as phytase linearly increased both portal ($P<0.05$) and peripheral ($P<0.05$) plasma MYO concentrations.

There were quadratic increases in bone ash, Ca, P and Mg content in response to phytase supplementation ($P<0.01$). In addition, phytase linearly improved apparent ileal P (P<0.01) and Cu ($P<0.05$) digestibility. With the exception of P, phytase had no effect on portal or peripheral plasma mineral concentrations. Phytase supplementation prevented the up-regulation of jejunal calbindin D9k gene expression observed in pigs fed the NC diet, but had no effect on NaPi-IIb, PepT1, SGLT1, CD36 or SMIT2 expression. In conclusion, adding 500 to 8,000 FTU of

phytase/kg to a wheat-barley based diet can allow for reductions in Ca, P and energy to be made without compromising finisher pig growth performance. In addition, although the higher doses of phytase were associated with improved phytate hydrolysis and MYO generation, they delivered no further improvements in finisher pig growth performance.

4.2 Introduction

Microbial phytase enzymes have been commercially available since 1991 and although adaptation of this technology in monogastric nutrition was initially relatively slow, it is now the thought that an estimated 70% of all pig diets globally contain an exogenous phytase (Plumstead, 2013). These enzymes are most commonly used as an economic substitute for inorganic P and Ca supplements, and as a means of reducing P outputs from intensive pig production facilities. However, since their introduction some 25 years ago, phytase has been the subject of intense study, and it has become increasingly clear that this enzyme has effects that extend beyond improving Ca and P availability. These extra-phosphoric effects include improved protein, mineral and energy digestibility (Selle and Ravindran, 2008). Moreover, super-doses of phytase have been shown to improve monogastric growth performance (Santos et al., 2014; Cowieson et al., 2011) to levels beyond expected based on improved Ca and P retention.

However, despite a great deal of research, these extra-phosphoric effects remain largely ambiguous, particularly in relation to improved protein digestibility. As discussed in Section 1.5.2, the phytate molecule has the capacity to form *de novo* phytate-protein complexes within the GIT, and in doing so reduce protein digestibility (Selle et al., 2012). In weaner pigs, Zeng et al. (2011) demonstrated that supplementation of low P corn-SBM diet with 1,000 FTU of a *Trichoderma Reesei* derived phytase/kg improved the AID of leucine, lysine, alanine, cysteine and phenylalanine, with tendencies for improved CP and overall AA digestibility. Similar findings were reported in the study of Guggenbuhl et al. (2012b), as they also found that the supplementation of a low P corn-SBM with 1,000 FTU/kg (*C. braakii* derived phytase) improved both total indispensable AA and total AA AID in grower pigs.

However, these findings are not shared by all; Adeola and Sands (2003) presents a body of literature that show no effect of phytase on AA digestibility. More recent studies such as Woyengo et al. (2009b) and Morales et al. (2012) attest to this view. Liao et al. (2005b) completed an informative study in which they found that phytase had no effect on AA digestibility in weaners fed corn-SBM, wheat-SBM or barley-pea based diets, but phytase did increase, or tended to increase, the AID of CP and AA in weaners fed a wheat-SBM-canola based diet. This suggests that the effects of phytase on AA digestibility are diet dependent. In a recent survey of the literature (Section 1.6.2), it was surmised that the overall net effect of phytase on protein availability is a 2% increase in AA (total) digestibility.

Although phytase appears to consistently improve energy digestibility in broilers, the situation in pigs is uncertain (Selle and Ravindran, 2008). Several authors have reported positive effects of phytase on energy digestibility in pigs (Brady et al., 2003, Johnston et al., 2004; Zeng et al., 2016), whereas many others have reported no effect (Liao et al., 2005b; Zeng et al., 2011; Guggenbuhl et al., 2012b). It is thought that improvements in energy digestibility would largely stem from increases in protein digestibility (Selle and Ravindran, 2008); however, some studies have demonstrated that phytase can improve both glucose and lipid digestibility (Johnston et al., 2004; Zaefarian et al., 2013).

Another ambiguous effect of phytase is the influence of super-doses on pig growth performance. As discussed in Chapter 3, several studies have reported that super-doses of phytase can enhance pig growth rate and FCR, beyond that of a P adequate control (Brana et al., 2006; Kies et al., 2006; Santos et al., 2015), whilst others have found no such effects (Langbein et al., 2013; Zeng et al., 2014; and the data presented in Chapter 3). The current understanding is that the performance benefits commonly observed in response to super-doses of phytase are mediated through an increase in phytate hydrolysis, and thereby improvements in mineral, AA, energy and MYO availability (Adeola et al., 1995; .Kies et al., 2006b; Cowieson et al., 2011). A recent *in vitro* study demonstrated that degradation of phytate to lower $InsP_x$ esters significantly improved soya protein solubility, thus demonstrating that the binding capacity of phytate to proteins diminishes as the level of phosphorylation of the phytate molecule decreases (Yu et al., 2012).

Reasons for the apparent inconsistent extra phosphoric effects of phytase observed in the literature are unclear. However, as discussed in Section 1.7, exogenous phytase efficacy within the GIT is influenced by a multitude of animal related (species, gut pH, intestinal transit time), enzyme related (pH optima, K_m, gastric stability), and dietary related factors (ingredients, substrate accessibility, mineral concentration and the intrinsic phytase activity).

It is evident that microbial phytase supplementation improves the availability of phytate bound minerals, protein and starch in the intestines; however, the molecular mechanisms underlying improved nutrient uptake are unclear. Enterocytes are exposed to a dynamic luminal environment in which nutrient availability is highly dependent on diet. In order to maintain their absorptive capacity, enterocytes are able to respond to luminal nutrient fluctuations by modulating the expression of nutrient transporters (Saddoris et al., 2010; Wang et al., 2012). This occurs through an enteroendocrine chemosensory mechanism, in which chemical components of nutrients are detected by mucosal or luminal chemosensors, triggering a cascade of down-stream signalling events that relay information back to the enterocytes (Dyer et al., 2005; Liu et al., 2013). Therefore, by altering nutrient availability in the gut, it may be that phytase influences nutrient transporter expression on the apical surface of enterocytes.

4.2.1 Study Aims

At present there is a lack of available information on the effects of super-dosing phytase in pigs at the finisher stage of production, particularly when fed wheat based diets. Therefore, this study set out to evaluate the effects of increasing levels of phytase on pig growth performance at the early finisher stage of production (40 to 60 kg) in wheat-barley based diets. Furthermore, as the mechanism underlying the super-dosing response requires clarification, this study also set out to determine the effect of increasing levels of phytase on the pattern of phytate degradation, MYO availability, bone mineralisation, and nutrient (mineral and AA) digestibility. Particular emphasis will be placed on the effect of phytase on intestinal nutrient transporter expression at the transcriptional level.

4.2.2 Hypotheses

- Phytase will improve finisher pig performance; those fed a standard dose will perform similarly to those fed the positive control, whereas those fed a super-dose will outperform those fed a nutritionally adequate control or standard phytase diet.
- Phytase will increase ileal phytate ($InsP_6$) degradation in a dose-dependent manner.
- Increasing levels of phytase will reduce the concentration of $InsP_5$, $InsP_4$, $InsP_3$ and $InsP_2$ in the digesta at the terminal ileum.
- Increasing levels of phytase will increase the levels of MYO in ileal digesta and blood plasma.
- Increasing levels of phytase will increase bone mineral content.
- Increasing levels of phytase will increase mineral and AA AID.
- Phytase will reduce NaPi-IIb and calbindin D9k, and increase PepT1, SGLT2, SMIT2 and CD36 gene expression in the jejunal mucosa.

4.3 Methods

4.3.1 Experimental design and dietary treatments

Finisher pigs were offered one of six dietary treatments throughout this 28 d study. The treatments included: (PC) a positive control formulated to meet or exceed the BSAS (2003) nutrient recommendations for all nutrients; (NC) a negative control similar to the PC diet but with reductions in Ca (1.6 g/kg), P (1.2 g/kg), Lys (0.17 g/kg), Thr (0.33 g/kg) and ME (0.217 MJ/kg); and the NC diet supplemented with 500 (NC+500), 1,000 (NC+1000), 2,000 (NC+2000) or 8,000 (NC+8000) FTU/kg. Diets NC+500 and NC+1000 are considered standard phytase inclusion rates (STD), while the NC+2000 diet is considered a phytase super-dose (SD), and the NC+8000 a very high super-dose. Diets were selected to cover a wide range of doses in order to obtain a more accurate estimation of the pattern of the response curve.

Phytase was added to the test diets based on the declared activity of 5,000 FTU/g. The nutrient reductions in the NC and test diets were in accordance with the matrix values for 500 FTU/kg of this particular phytase. Titanium dioxide was added to all

diets at 5 g/kg as an inert marker for nutrient digestibility calculations. Diet composition and calculated nutrient levels are presented in Table 4.1.

4.3.2 Animals and management

A total of 288 pigs of approximately 12 weeks of age (initial BW ± SE = 36.7 ± 0.3 kg) were blocked into mixed sex pens of 4 balancing for weight, sex and litter of origin. Pens within a replicate were randomly allotted to one of the six dietary treatments (n = 12). The experiment was conducted over two batches of equal size, which were run 3 weeks apart. Pigs were housed in a finisher facility consisting of six identical rooms, each comprising 24 fully slatted floored pens (230 x 220 cm); 12 either side of a central passageway. Throughout the experiment room temperature was maintained at 21 ± 2 ºC. Each pen was equipped with a single spaced trough feeder, two nipple drinkers and a ball on chain.

Pig BW was recorded on d 1, 7, 14, 21 and 28 for the determination of pen ADG. Troughs and feed refusals were weighed at the end of the experiment (d 28) for pen ADFI and FCR determination. Both ADFI and FCR were adjusted for pigs taken off trial (due to ill health) as described in Section 3.3.2.

Health and faecal scores were recorded daily as described in Section 2.2.3. At the end of the four week experiment 60 mixed sex pigs (n = 10; BW ± SE = 59.8 ± 0.6 kg) were slaughtered (captive bolt penetration followed by exsanguination) over two consecutive days for sample collection. Pigs were selected for slaughter according to the criteria discussed in Section 2.4. The 10 replicates from which a pig was slaughtered (1 pig per pen) were selected from the 12 replicates randomly.

4.3.3 Sample collection

Following the confirmation of death, portal and peripheral blood were collected into heparinised Vacutainers and plasma was obtained according to the procedure outlined in Section 2.4.1. Digesta were collected from the terminal ileum and frozen at -20 °C (Section 2.4.2). Mucosal scrapings from non-gut associated lymphoid (GALT) jejunal tissue (mid-way point of small intestine) were collected into Trizol and snap frozen in liquid nitrogen (Section 2.4.4). The M3 and third proximal phalanx bones were collected from the right foot as described in Section 2.4.3.

Table 4.1. Composition and nutrient specifications of experimental diets (%, as-fed basis)

Diet	PC	NC[1]
Ingredient		
Wheat	48.1	48.5
Barley	15.0	15.0
Wheat feed	10.3	12.0
Rapeseed meal	10.0	10.0
Sunflower seed extract	7.0	7.4
Soybean meal	3.6	2.7
Soya oil	2.7	1.9
Dicalcium phosphate	0.99	-
Limestone flour	0.62	0.91
Vitamin-mineral premix[2]	0.25	0.25
Salt	0.33	0.33
L-Lysine	0.56	0.55
L-Threonine	0.09	0.06
Titanium dioxide	0.50	0.50
Calculated nutrient composition		
Net energy (MJ/kg)	9.30	9.13
Crude protein (%)	16.0	16.0
Crude fibre (%)	5.32	5.50
SID Lysine (%)	0.81	0.79
SID Methionine + Cystine (%)	0.50	0.50
SID Threonine (%)	0.53	0.49
SID Tryptophan (%)	0.16	0.16
Calcium (%)	0.72	0.56
Total P (%)	0.61	0.45
Available P (%)	0.25	0.13
Ca:P	1.18	1.24

[1] Phytase was added to the NC diet at 0.01, 0.02, 0.04 and 0.16% at the expense of wheat to create the 500, 1,000, 2,000 and 8,000 FTU/kg test diets respectively.

[2] Vitamin and trace mineral premix provided per kg of diet : 7500 IU vitamin A, 1650 IU vitamin D_3, 35 IU vitamin E, 2 mg vitamin K, 1.5 mg thiamine (B_1), 3 mg riboflavin (B_2), 2 mg pyridoxine (B_6), 15 µg vitamin B_{12}, 8 mg pantothenic acid, 20 mg nicotinic acid, 50 µg biotin, 0.3 mg folic acid, 15 mg $CuSO_4$, 1 mg iodine, 80 mg $FeSO_4$, 25 mg manganese, 0.25 mg selenium, 65 mg $ZnSO_4$.

4.3.4 Laboratory analysis

Dietary samples were sent to ESC for phytase and phytate analysis (Section 2.5.3), and to Sciantec Analytical Services Ltd (Cawood, UK) for AA, crude protein, crude fibre, and total fat analysis. In addition, a dried subsample of ileal digesta was also sent to Sciantec Analytical Services for AA analysis. Duplicate digesta and triplicate dietary samples were analysed for DM (Section 2.5.2), Ca, P, Mg, Na, K, Mn, Cu, Fe and Zn (Section 2.5.7 and 2.5.8), $InsP_{2-6}$ (Section 2.5.11), MYO (2.5.10), and TiO_2 (section 2.5.4).

Plasma were analysed for minerals by ICP-OES (Section 2.5.8) and MYO by HPLC (Section 2.5.10). Bone (M3 and proximal phalanx) measurements, fat free dry weight and ash, P, Ca and Mg content were determined according to the methods described in Sections 2.4.3 and 2.5.6.

The relative gene expression of selected nutrient transporters (Table.4.2) in the jejunal mucosa was determined by qPCR. Total RNA extraction, cDNA synthesis, and qPCR were performed as described in Section 2.5.12. The stability of three commonly used reference genes (*ACTB, HMBS* and *HPRT*) was assessed using the geNorm function of qbasePLUS (Biogazelle); a stability value (M) of <0.5 was considered stable. The most stable combination of reference genes tested was identified as *HMBS* and *HPRT* (M=0.5, CV=0.18), therefore, the geometric mean of these two reference genes was used as the normalisation factor for all genes of interest (GOI) in this experiment. All primers were purchased from Bio-Rad Ltd as predesigned PrimePCR™ Assays, with the exception of *SMIT2*, for which primers were designed using the NCBI Primer-Blast tool (Ye et al., 2012) and purchased as a custom PrimePCR™ Assay. Primer efficiency was calculated for all PrimePCR™ Assays from a standard curve generated from a serially diluted (1:10) pooled cDNA mix as described in Section 2.5.12.3.

4.3.5 Calculations and statistical analysis

Apparent ileal nutrient digestibility was calculated using the analysed nutrient values (Table 4.3) according to the formula outlined in Section 2.6.

Data were analysed as a randomised complete block design using the GLM procedure of SPSS Statistics (Version 22) with the pen mean serving as the experimental unit for the growth performance data, and the individual pig serving as

the experimental unit for all other analyses. Data were first tested for homogeneity of variance and normality using the Levene's test and the Kolmogorov-Smirnov test respectively. Non-normal data or data displaying heteroscedasticity were log transformed prior to analysis, unless it was proportion data, in which case an arcsine transformation was used. Transformed data were back transformed before inclusion in the respective tables.

Polynomial contrasts for unequally spaced increments were conducted for diets NC, NC+500, NC+1000, NC+2000 and NC+8000 to test for linear and quadratic responses to phytase. In addition, treatment means were compared using the following single degree-of-freedom contrasts: PC vs NC, NC vs NC+500 and NC+1000 (NC vs STD), PC vs STD, STD vs NC+2000 (STD vs SD), and NC+2000 vs NC+8000 (SD vs 8000). Slaughter weight was added to the model as a covariate for bone analyses. Plasma mineral and MYO data were analysed with repeated measures with the blood type (portal or peripheral) serving as the repeated factor. Differences were classed as significant if $P<0.05$ or as a trend if $P<0.1$. Data are expressed as least-square means along with the pooled standard error of the mean (SEM).

Table 4.2. Selected genes for nutrient transporter gene expression analysis

Gene name	Symbol	Function	Unique Assay ID	Amplicon size (bp)	Efficiency (%)
Reference genes					
Beta-actin	*ACTB*	Actin synthesis	qSscCED0016579	110	100
Hydroxymethylbilane synthase	*HMBS*	Porphyrin metabolism	qSscCID001281	116	99
Hypoxanthine phosphoribosyltransferase 1	*HPRT1*	Purine synthesis	qSscCID0002342	100	101
Nutrient transporter GOI					
Na-dependent phosphate transporter 2b	*NaPi-IIb*	Phosphate transporter	qSscCID0011723	108	103
Calbindin D9k	*S100G*	Calcium binding protein D9k	qSscCED0015930	113	110
Peptide transporter 1	*PepT1*	Oligopeptide transporter	qSscCID0005181	69	105
Na/glucose transporter 1	*SGLT1*	Glucose transporter	qSscCID0005022	75	109
Cluster of differentiation 36	*CD36*	Fatty acid transporter	qSscCED0020140	110	100
Na/myo-inositol transporter 2	*SMIT2*	MYO transporter	N/A[1]	88	107

[1] *SMIT2* primer sequence as follows: forward (5'to 3') GTTTACTCGCCATGACCCCA, reverse (5' to 3') TGGTGTCCCGTTCTGAGAGA.

4.4 Results

Overall, the pigs were generally in good health throughout the experiment. Although there were a couple of exceptions: one pig receiving the NC+2000 diet was taken off trial following a prolapse, and another from diet NC+8000 due to lameness.

4.4.1 Dietary analysis

The analysed nutrient content of the experimental diets is presented in Table 4.3. Calcium, total P, and available P content were in close agreement with the targeted values. Although less crystalline lysine and threonine were added to the NC basal diet, dietary analysis revealed that levels of these amino acids were higher than anticipated and equal across treatments. Therefore, the planned reductions of these nutrients in the NC and phytase test diets were not successful. However, as AA analysis is inherently variable (Fontaine and Eudaimon, 2000), it is possible that the deviations from the formulated values are due to analytical error. Fat content was higher in PC than in the other treatments which can be attributed to the planned ME reduction in the NC and phytase test diets. The analysed phytase content in each of the test diets was approximately 1.3-fold higher than expected. This is most likely the result of the phytase product having a greater activity than anticipated. Nevertheless, the relative difference between the phytase treatments is as expected and thus the treatments were considered satisfactory for use.

Table 4.3. Analysed phytase, nutrient and inositol phosphate composition of the experimental diets (as-fed basis).

Treatment	PC	NC	NC+500	NC+1000	NC+2000	NC+8000
DM (%)	89.40	89.40	89.30	89.30	89.50	89.10
Total fat (%)	4.97	4.38	4.09	3.99	4.09	4.34
CP (%)	16.50	16.40	16.20	16.30	16.40	17.40
CF (%)	6.10	6.40	6.50	5.80	5.60	6.40
Ca (%)	0.71	0.58	0.57	0.56	0.61	0.59
Total P (%)	0.60	0.43	0.41	0.41	0.43	0.44
Available P (%)	0.29	0.16	0.13	0.14	0.15	0.15
Phytate P (%)	0.29	0.27	0.29	0.27	0.28	0.28
Ca:P	1.19	1.36	1.38	1.38	1.41	1.34
Na (%)	0.21	0.22	0.20	0.18	0.23	0.22
K (%)	0.59	0.56	0.55	0.55	0.59	0.59
Mg (%)	0.16	0.17	0.17	0.16	0.17	0.17
Mn (mg/kg)	18	17	17	16	19	19
Fe (mg/kg)	122	145	134	130	146	146
Cu (mg/kg)	27	21	24	16	25	27
Zn (mg/kg)	150	120	117	132	127	119
Phytase (FTU/kg)	85	<50	751	1,240	2,420	11,000
Inositol phosphate (nmol/g)						
$InsP_6$	9,532	10,748	10,565	10,094	10,000	8,811
$InsP_5$	1,464	1,782	2,047	2,179	2,284	2,513
$InsP_4$	145	228	259	287	290	625
$InsP_3$	154	189	263	224	294	373
$InsP_2$	1,205	1,662	1,743	1,840	1,713	1,643
MYO	488	483	566	513	572	559
Indispensable AA (%)						
Arginine	0.92	0.92	0.96	0.93	0.96	0.97
Histidine	0.39	0.39	0.41	0.40	0.40	0.41
Isoleucine	0.58	0.59	0.60	0.59	0.59	0.62
Leucine	1.06	1.07	1.10	1.09	0.99	1.12
Lysine	1.03	1.09	1.15	1.12	1.06	1.08
Methionine	0.27	0.27	0.28	0.27	0.28	0.29
Phenylalanine	0.67	0.73	0.74	0.74	0.73	0.77
Threonine	0.64	0.62	0.64	0.63	0.63	0.64
Tryptophan	0.18	0.18	0.17	0.19	0.18	0.17
Valine	0.74	0.74	0.75	0.74	0.75	0.77
Dispensable AA (%)						
Alanine	0.64	0.64	0.66	0.64	0.65	0.67
Aspartic acid	1.10	1.11	1.15	1.12	1.12	1.17
Cystine	0.34	0.35	0.36	0.35	0.35	0.36
Glutamic acid	3.71	3.66	3.67	3.76	3.71	3.82
Glycine	0.73	0.73	0.75	0.75	0.75	0.77
Proline	1.15	1.14	1.16	1.22	1.17	1.29
Serine	0.73	0.73	0.75	0.74	0.74	0.76
Tyrosine	0.31	0.30	0.32	0.32	0.32	0.31

4.4.2 Growth performance

The effect of phytase on finisher pig growth performance is presented in Table 4.4. Treatment had no effect on ADFI (*P*>0.05) throughout this study. Pigs fed the NC diet had a 14.3% lower ADG (*P*<0.001) and a proportionally worse FCR (*P*<0.001) than those fed the PC. Adding phytase to the NC restored performance back to the level of the PC; however, there was no difference between the different doses tested.

Table 4.4. Effect of phytase treatment on finisher pig growth performance[1]

Treatment	Start BW (kg)	ADFI (kg)	ADG (kg)	FCR	End BW (kg)
PC	36.79	1.80	0.825	2.20	59.86
NC	36.71	1.77	0.707	2.51	56.51
NC+500	36.80	1.85	0.813	2.28	59.55
NC+1000	36.58	1.80	0.792	2.27	58.79
NC+2000	36.74	1.79	0.818	2.23	58.65
NC+8000	36.72	1.87	0.809	2.30	59.28
SEM	0.73	0.03	0.150	0.04	0.45
			P-value		
Linear	0.896	0.113	<0.05	0.189	<0.05
Quadratic	0.828	0.701	<0.05	<0.001	<0.01
			Pre-determined contrasts		
PC vs NC	0.513	0.514	<0.001	<0.001	<0.001
NC vs STD	0.881	0.219	<0.001	<0.001	<0.001
PC vs STD	0.366	0.631	0.226	0.128	0.214
STD vs SD	0.664	0.427	0.944	0.365	0.816
SD vs 8000	0.902	0.113	0.671	0.227	0.704

[1] Data are means of 12 replicate pens of 4 mixed sex pigs.

4.4.3 Ileal phytate hydrolysis and inositol phosphate and MYO concentration

There was no difference in ileal $InsP_6$ degradation (P>0.05) between the PC and NC diets (Figure 4.1). Phytase supplementation increased ileal $InsP_6$ degradability in a quadratic manner (P<0.01). $InsP_6$ degradability in finishers receiving the NC+2000 treatment was similar to those receiving the standard phytase doses (500 and 1,000 FTU/kg) or the very high super-dose (NC+8000) treatments (P>0.05).

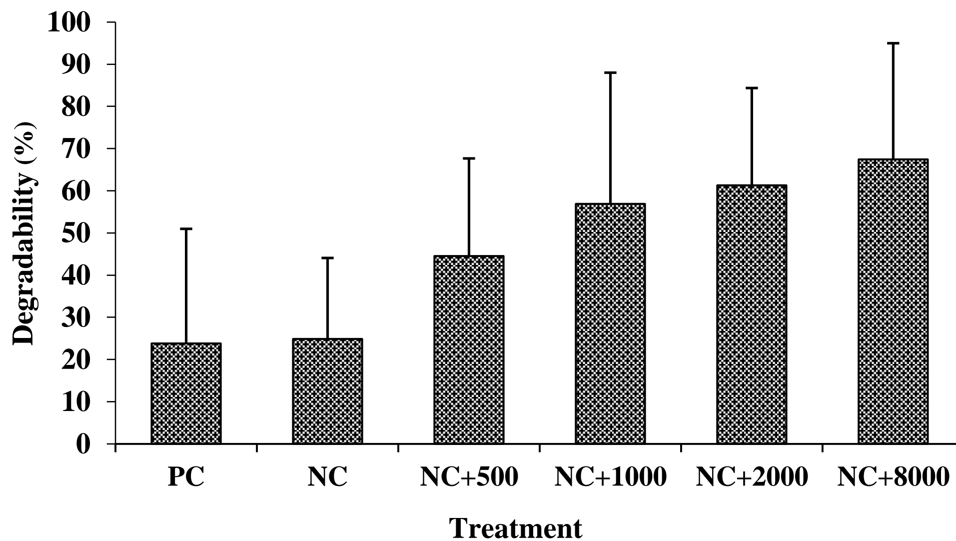

Figure 4.1 Effect of phytase on ileal $InsP_6$ degradability

Bars represent the mean of 10 replicate pigs + standard deviation. Trend analysis: Linear P=0.002, quadratic P=0.013. Specific orthogonal contrasts: PC vs NC P = 0.929, NC vs STD P<0.05, PC vs STD P<0.05, STD vs SD P = 0.312, SD vs 8000 P = 0.604. STD = NC+500 & NC+1,000 treatments. SD = NC+2,000 treatment.

Ileal inositol phosphate and MYO concentration data are presented in Table 4.5. Reducing the Ca and P content of the diet had no effect on the concentration of $InsP_2$ through $InsP_6$ or MYO in the ileal digesta. In agreement with the $InsP_6$ degradability data are the ileal $InsP_6$ concentration data, which show that phytase reduced ileal $InsP_6$ levels in a quadratic fashion (P<0.01); those fed the NC+8000 treatment had 66% less $InsP_6$ in the digesta than those fed the non-supplemented NC. Similarly, phytase reduced the amount of $InsP_5$ reaching the terminal ileum in a quadratic fashion (P<0.01). Although numerically lower, the concentration of $InsP_6$ and $InsP_5$ in the ileal digesta of pigs consuming the NC+8000 treatment was not significantly different from those fed the NC+2000 treatment. The reductions in $InsP_6$ and $InsP_5$

concentrations with phytase treatment were met with concurrent increases in $InsP_4$ and $InsP_3$, particularly at the lower phytase doses; however, the build-up of these esters began to diminish when the phytase concentration was increased further, thus resulting in a linear effect on $InsP_4$ ($P<0.05$) and a quadratic effect on $InsP_3$ ($P<0.01$). Pigs fed the NC+8000 treatment had 82% less $InsP_4$ and 58% less $InsP_3$ in the ileal digesta than those fed the NC+2000. Levels of $InsP_2$ in the ileal digesta were not influenced by treatment ($P>0.05$).

There was a quadratic reduction in the proportion of $InsP_5$: $\sum InsP_{2-5}$ with increasing phytase dose ($P<0.001$). Supplementing the NC diet with phytase at 500, 1,000 or 2,000 FTU/kg increased the proportion of the hydrolysis products $InsP_4$ and $InsP_3$ in the digesta ($P<0.01$); this effect was not apparent when supplemented with 8,000 FTU/kg. $InsP_2$ was the predominant phytate hydrolysis product in the digesta irrespective of treatment, although the proportion of this inositol phosphate increased in finishers offered a super-dose, with the NC+8000 having a greater effect than the NC+2000 treatment ($P<0.001$).

There were linear increases in ileal MYO concentration with increasing dietary concentration of phytase ($P<0.001$); supplementing the NC with 8,000 FTU/kg increased ileal MYO levels 4-fold.

Table 4.5. The effect of phytase on ileal inositol phosphate ($InsP_{2-6}$) and MYO concentration (nmol/mg Ti)[1]

Treatment	$InsP_6$	$InsP_5$	$InsP_4$	$InsP_3$	$InsP_2$	MYO	$InsP_5$: $\sum InsP_{2-5}$	$InsP_4$: $\sum InsP_{2-5}$	$InsP_3$: $\sum InsP_{2-5}$	$InsP_2$: $\sum InsP_{2-5}$
PC	1499	216	98	53	363	261	0.29	0.16	0.08	0.46
NC	1566	191	54	33	317	234	0.31	0.11	0.06	0.51
NC+500	902	125	171	80	307	435	0.17	0.22	0.12	0.49
NC+1000	886	90	133	69	324	656	0.14	0.20	0.14	0.52
NC+2000	695	63	109	72	358	787	0.10	0.17	0.12	0.61
NC+8000	538	39	20	30	343	945	0.09	0.04	0.07	0.80
SEM	164.6	19.5	20.9	8.1	40.3	90.2	0.02	0.03	0.01	0.04
					P-value					
Linear	<0.01	<0.001	<0.001	<0.01	0.573	<0.001	<0.001	<0.001	<0.05	<0.001
Quadratic	<0.01	<0.01	<0.05	<0.01	0.477	<0.001	<0.001	<0.05	<0.01	0.598
					Pre-determined contrasts					
PC vs NC	0.762	0.334	0.075	0.086	0.398	0.877	0.552	0.192	0.313	0.348
NC vs STD	<0.01	<0.01	<0.01	<0.001	0.976	<0.001	<0.001	<0.01	<0.001	0.985
PC vs STD	<0.01	<0.001	0.516	<0.05	0.319	<0.001	<0.001	0.072	<0.05	0.288
STD vs SD	0.319	0.063	0.545	0.805	0.388	<0.05	0.093	0.212	0.574	<0.05
SD vs 8000	0.494	0.393	<0.001	<0.001	0.785	0.364	0.617	<0.001	<0.05	<0.001

[1] Data are means of 10 replicate pigs.

4.4.4 Plasma *myo*-inositol

Table 4.6. Effect of phytase on portal and peripheral plasma MYO concentration[1]

	Plasma MYO (nmol/ml)	
Diet	Portal	Peripheral
PC	47.8	37.9
NC	47.4	37.8
NC+500	54.2	37.5
NC+1000	62.6	40.6
NC+2000	66.1	42.9
NC+8000	77.6	50.3
SEM	7.6	4.5
	P-value	
Sampling site[2]	<0.001	
Linear	<0.05	<0.05
Quadratic	0.217	0.646
Treatment x sampling site	0.710	
	Pre-determined contrasts	
PC vs NC	0.971	0.993
NC vs STD[a]	0.223	0.829
PC vs STD	0.256	0.837
STD vs SD[b]	0.393	0.509
SD vs 8000	0.276	0.276

[1] Data are means of 10 replicate pigs.
[2] Paired portal vs peripheral analysis.
[a] STD = NC+500 and NC+1,000.
[b] SD = NC+2,000.

The effect of treatment on portal and peripheral plasma MYO concentration is presented in Table 4.6. There was no difference in plasma MYO levels between the PC and NC. Adding phytase to the NC linearly increased plasma MYO concentration in the portal blood ($P<0.05$). This was also true for MYO levels in the peripheral blood ($P<0.05$); however, here the effects were less apparent, particularly with the standard (500 or 1,000 FTU/kg) phytase inclusion rates. The concentration of MYO in the portal circulation was higher than that in the peripheral circulation ($P<0.001$).

4.4.5 Proximal phalanx bone characteristics

Due to unforeseen circumstances 18 of the 60 M3 bones were not suitable for subsequent ash and mineral analysis. Therefore, the third proximal phalanx bone from the same foot was obtained ($n = 60$). Ash and mineral analyses were conducted on the incomplete M3 set and the complete proximal phalanx set. Correlation analysis of the paired bones from the same pig ($n = 42$) revealed that the proximal phalanx was a suitable replacement for the M3 (ash content Pearson's correlation = 0.866, $P = <0.001$; P content Pearson's correlation = 0.902, $P = <0.001$; Ca content Pearson's correlation = 0.882, $P = <0.001$; and Mg content Pearson's correlation = 0.925, $P = <0.001$). Therefore, the proximal phalanx data are presented in Table 4.7. Bone weight and size measurements did not differ between treatments ($P>0.05$). There was a quadratic increase in bone ash weight and bone P, Ca and Mg concentration in response to phytase supplementation ($P<0.01$). Bone ash weight tended to be lower in pigs fed the NC than those fed the PC (1.87 vs 1.72; $P=0.084$). This was restored back to the level of the PC upon the supplementation of a standard phytase dose ($P<0.01$). Bone Ca, P and Mg concentrations were lower ($P<0.05$, $P<0.05$ and $P<0.001$ respectively) in pigs fed the NC than the PC. Again, supplementing the NC with a standard dose of phytase was sufficient in restoring these back to the level of the PC ($P<0.05$). There were no differences between the standard phytase doses and the NC+2000, or the NC+2000 and the NC+8000 treatments for any of the bone variables measured ($P>0.05$).

Table 4.7. Effect of phytase on finisher pig proximal phalanx characteristics[1]

Treatment	Fat-free dry weight[*]	Bone length (mm)[*]	Bone ash (g)[*]	Bone ash (%)	P (%)	Ca (%)	Mg (%)
PC	4.77	31.04	1.87	39.12	7.63	15.66	0.254
NC	4.64	31.57	1.72	37.80	7.03	14.38	0.211
NC+500	4.98	31.16	1.98	39.54	7.56	15.51	0.247
NC+1000	4.92	31.06	1.97	39.98	7.71	15.78	0.264
NC+2000	5.03	31.98	2.06	41.29	7.83	16.05	0.268
NC+8000	5.01	31.40	2.02	40.63	7.71	15.82	0.266
SEM	0.15	0.468	0.06	0.86	0.18	0.38	0.007
				P-value			
Linear	0.322	0.924	<0.05	0.131	0.154	0.094	<0.001
Quadratic	0.155	0.523	<0.01	<0.05	<0.01	<0.01	<0.001
				Pre-determined contrasts			
PC vs NC	0.552	0.430	0.084	0.288	<0.05	<0.05	<0.001
NC vs STD	0.104	0.435	<0.01	0.071	<0.05	<0.01	<0.001
PC vs STD	0.310	0.901	0.141	0.545	0.972	0.981	0.853
STD vs SD	0.679	0.131	0.243	0.155	0.385	0.355	0.126
SD vs 8000	0.907	0.375	0.648	0.592	0.618	0.640	0.830

[1] Data are means of 10 replicate pigs.
[*]Slaughter weight covariate significant: fat-free dry weight ($P<0.001$); bone length ($P<0.01$); bone ash weight ($P<0.001$). The relationship between the covariate and the independent variables was positive in each case.

Table 4.8 Effect of phytase on ileal mineral concentration (mg/g TiO_2) and apparent ileal mineral digestibility (%)[1]

Item	Treatment							P-value						
	PC	NC	NC+500	NC+1000	NC+2000	NC+8000	SEM	Linear	Quadratic	PC vs NC	NC vs STD	PC vs STD	STD vs SD	SD vs 8000
DM digestibility	58.80	62.57	59.33	56.40	61.62	60.89	2.65	0.841	0.632	0.292	0.136	0.763	0.251	0.846
P														
Digestibility	40.42	34.29	40.54	43.84	43.80	55.88	5.36	<0.01	0.580	0.351	0.188	0.759	0.787	0.077
Ileal concentration	651	524	404	451	399	314	40	<0.01	0.242	<0.05	<0.05	<0.001	0.564	0.139
Ca														
Digestibility	43.87	40.76	45.00	45.12	42.06	42.89	6.01	0.986	0.964	0.687	0.535	0.857	0.677	0.920
Ileal concentration	727	626	512	563	618	542	59	0.620	0.762	0.217	0.214	<0.01	0.260	0.365
Mg														
Digestibility	-2.20	1.50	-4.31	-13.33	-6.17	-5.90	7.17	0.845	0.371	0.700	0.192	0.442	0.746	0.978
Ileal concentration	339	328	302	346	335	339	22	0.697	0.570	0.743	0.859	0.592	0.659	0.904
Na														
Digestibility	-427.0	-309.1	-333.0	-411.1	-315.4	-410.2	42.7	0.137	0.998	0.090	0.299	0.348	0.350	0.170
Ileal concentration	2247	1595	1585	1946	1449	2042	167	<0.05	0.420	<0.01	0.404	<0.05	0.145	<0.05
K														
Digestibility	86.24	87.55	89.71	84.74	89.03	86.60	1.72	0.553	0.855	0.580	0.878	0.622	0.392	0.325
Ileal concentration	165	140	101	166	118	144	18	0.518	0.773	0.200	0.826	0.205	0.387	0.323
Cu														
Digestibility	22.28	-6.65	6.99	6.73	12.51	19.87	7.09	<0.05	0.200	<0.01	0.099	0.053	0.504	0.453
Ileal concentration	4.2	4.5	3.8	4.1	4.0	3.9	0.3	0.383	0.562	0.554	0.199	0.520	0.983	0.808
Fe														
Digestibility	-35.01	-30.23	-23.58	-25.91	-33.31	-22.45	5.43	0.419	0.407	0.502	0.386	0.097	0.178	0.148
Ileal concentration	33.6	32.4	32.9	33.3	35.4	33.4	1.6	0.797	0.176	0.570	0.716	0.770	0.250	0.391
Zn														
Digestibility	-37.85	-42.36	-44.67	-43.26	-57.09	-54.13	9.31	0.367	0.356	0.703	0.879	0.563	0.233	0.818
Ileal concentration	29.5	21.6	23.3	20.9	23.4	23.6	1.8	0.465	0.779	<0.01	0.828	<0.01	0.546	0.931

[1] Data are means of 10 replicate pigs.

4.4.6 Apparent DM and mineral digestibility

The effects of phytase on apparent DM and mineral ileal digestibility are presented in Table 4.8. Treatment had no effect on apparent ileal dry matter digestibility ($P>0.05$). There was no difference in apparent ileal P digestibility between the PC and NC treatments ($P>0.05$). Adding phytase to the NC resulted in linear increases in apparent P digestibility ($P<0.01$) up to 8,000 FTU/kg. Finishers receiving the NC treatment had a 28.9% lower apparent ileal Cu digestibility than those receiving the PC ($P<0.01$). Adding phytase to the NC saw Cu digestibility linearly increase ($P<0.05$), with the 8,000 FTU/kg treatment necessary to bring it back to the level of the PC. Phytase had no effect on apparent Ca, Mg, Na, Fe K or Zn ileal digestibility ($P>0.05$). Finishers fed a standard phytase (500 to 1,000 FTU/kg) diet had significantly less Ca, P, Zn ($P<0.01$) and Na ($P<0.05$) in the digesta at the terminal ileum than those fed the PC.

4.4.7 Plasma minerals

The effect of treatment on portal and peripheral plasma mineral concentration is presented in Table 4.9. Pigs fed the NC had a lower P concentration in both portal and peripheral plasma ($P<0.001$). Supplementing the NC with increasing doses of phytase increased portal ($P<0.01$) and peripheral ($P<0.001$) plasma P in a quadratic manner, with the standard dose sufficient in elevating levels back to that of the PC. Ca, Mg, Na, K or Fe levels in the portal or peripheral plasma were not influenced by phytase treatment ($P>0.05$). Those fed the nutritionally adequate PC diet had greater levels of Na ($P>0.05$) in the portal plasma than those fed the NC, and greater levels of Mg ($P>0.05$) in the portal plasma than those fed a standard phytase dose.

The concentration of Ca, P, Mg, K and Fe was higher in the portal than in the peripheral plasma ($P<0.001$), whereas Na levels were higher in the peripheral plasma ($P<0.001$).

Table 4.9 Effect of phytase on portal and peripheral plasma mineral concentrations (μg/ml)[1]

Mineral	Ca		P		Mg		K		Na		Fe	
Blood	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral
PC	133.4	104.4	138.6	120.4	41.4	20.8	490.2	461.3	3442	3459	1.02	0.81
NC	127.0	112.2	108.0	98.5	39.9	20.3	496.8	416.7	3182	3471	0.99	0.91
NC+500	123.8	106.4	126.3	116.8	33.9	21.2	499.6	484.8	3321	3552	0.85	0.69
NC+1000	116.3	102.9	131.2	119.3	35.8	20.2	536.1	411.1	3158	3542	0.80	0.73
NC+2000	123.7	105.1	136.0	121.4	38.9	20.9	516.4	475.6	3334	3527	0.99	0.81
NC+8000	119.9	103.0	129.0	120.2	35.8	20.6	527.2	449.3	3186	3418	0.97	0.77
SEM	4.4	3.4	5.4	2.9	2.6	0.7	31.0	19.5	84	61	0.140	0.108
						P-value						
Blood	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
Linear	0.521	0.195	0.209	<0.01	0.661	0.934	0.526	0.660	0.664	0.170	0.673	0.889
Quadratic	0.581	0.192	<0.01	<0.001	0.909	0.744	0.580	0.177	0.320	0.391	0.967	0.611
Treatment x blood	0.531		0.808		0.196		0.169		0.379		0.597	
						Pre-determined contrast						
PC vs NC	0.296	0.089	<0.001	<0.001	0.672	0.615	0.877	0.103	<0.05	0.890	0.862	0.488
NC vs STD	0.193	0.062	<0.01	<0.001	0.104	0.648	0.555	0.189	0.570	0.316	0.312	0.120
PC vs STD	0.056	0.944	0.139	0.507	<0.05	0.907	0.461	0.557	0.052	0.246	0.245	0.436
STD vs SD	0.514	0.906	0.251	0.323	0.191	0.829	0.965	0.226	0.334	0.791	0.312	0.413
SD vs 8000	0.534	0.641	0.343	0.758	0.384	0.791	0.781	0.318	0.192	0.214	0.894	0.795

[1] Data are means of 10 replicate pigs.

4.4.8 Apparent ileal AA digestibility

Phytase treatment had no effect on the apparent ileal amino acid digestibility (*P*>0.05) of any of the amino acids measured (Table 4.10). There was, however, a small increase in tyrosine digestibility following an increase in phytase concentration from a standard (500 and 1,000 FTU/kg) dose to a 2,000 FTU/kg, although this was only a trend (*P*<0.099).

Table 4.10. Effect of phytase on apparent ileal digestibility of amino acids (%)

Amino acid	Treatment						SEM	*P*-value						
	PC	NC	NC+500	NC+1000	NC+2000	NC+8000		Linear	Quadratic	PC vs NC	NC vs STD	PC vs STD	STD vs SD	SD vs 8000
Indispensable														
Arginine	77.78	76.63	75.93	72.13	76.88	76.70	1.74	0.574	0.710	0.620	0.199	0.062	0.139	0.939
Histidine	74.59	73.32	73.34	68.70	72.33	73.88	1.93	0.446	0.323	0.621	0.304	0.114	0.539	0.549
Isoleucine	71.77	71.24	69.99	65.88	71.47	71.88	2.18	0.854	0.192	0.854	0.192	0.124	0.143	0.889
Leucine	72.65	72.18	71.49	67.37	69.95	72.28	2.23	0.632	0.227	0.874	0.286	0.204	0.831	0.435
Lysine	78.13	78.93	78.04	75.44	77.14	77.85	1.70	0.962	0.286	0.725	0.267	0.478	0.832	0.756
Methionine	78.32	78.15	76.00	73.64	77.55	77.81	1.92	0.606	0.592	0.945	0.117	0.111	0.196	0.917
Phenylalanine	71.43	74.51	72.24	68.01	73.00	74.00	2.24	0.565	0.311	0.284	0.079	0.607	0.243	0.739
Threonine	64.64	64.86	63.78	58.77	63.76	63.08	2.81	0.959	0.533	0.949	0.246	0.292	0.419	0.855
Tryptophan	69.92	69.88	66.59	65.88	68.13	67.46	2.24	0.894	0.637	0.990	0.175	0.187	0.478	0.837
Valine	68.96	68.40	67.42	62.20	68.49	68.77	2.30	0.500	0.613	0.855	0.179	0.115	0.149	0.928
Average	72.99	73.43	71.79	68.19	71.68	72.57	2.09	0.683	0.311	0.870	0.143	0.217	0.468	0.751
Dispensable														
Alanine	63.70	65.53	62.61	56.19	62.52	64.03	2.82	0.645	0.231	0.617	0.059	0.190	0.321	0.691
Aspartic acid	63.31	64.59	63.06	58.19	62.02	65.13	2.50	0.348	0.177	0.693	0.157	0.353	0.614	0.357
Cysteine	61.54	63.71	62.34	55.67	61.09	64.84	2.85	0.271	0.159	0.551	0.135	0.431	0.503	0.327
Glutamic acid	84.34	82.93	81.55	79.90	81.20	82.71	1.66	0.527	0.245	0.243	0.065	0.483	0.579	0.429
Glycine	49.67	48.82	42.26	40.50	47.51	46.32	4.77	0.834	0.750	0.889	0.158	0.129	0.243	0.852
Proline	74.74	76.67	74.76	73.81	75.56	77.58	2.50	0.501	0.508	0.546	0.384	0.873	0.657	0.561
Serine	68.09	70.40	68.46	64.29	68.96	67.90	2.41	0.834	0.479	0.456	0.132	0.528	0.329	0.742
Tyrosine	61.21	61.33	59.55	55.44	63.12	61.94	3.07	0.578	0.935	0.975	0.256	0.286	0.099	0.774
Average	65.48	66.56	64.61	60.95	64.80	66.69	2.54	0.429	0.326	0.744	0.185	0.359	0.475	0.580

[1] Data are means of 10 replicate pigs.

4.4.9 Nutrient transporter gene expression

Table 4.11. Effect of phytase on the normalised relative abundance of jejunal nutrient transporter mRNA[1]

Treatment	NaPi-IIb	Calbindin D9k	PepT1	SGLT1	CD36	SMIT2
PC	3.81	1.91	1.17	1.10	1.87	1.28
NC	2.53	2.86	0.90	1.11	1.07	1.02
NC+500	2.94	1.65	1.14	1.18	1.22	1.06
NC+1000	2.84	1.13	1.14	1.01	1.38	1.06
NC+2000	2.61	1.14	1.12	1.17	1.28	0.99
NC+8000	2.80	1.23	0.95	1.05	0.99	1.00
SEM	0.51	0.33	0.16	0.13	0.22	0.09
			P-value			
Linear	0.480	<0.05	0.535	0.732	0.402	0.480
Quadratic	0.238	0.01	0.326	0.763	0.753	0.842
			Pre-determined contrasts			
PC vs NC	0.167	0.323	0.250	0.953	0.091	0.180
NC vs STD	0.449	<0.05	0.245	0.955	0.536	0.876
PC vs STD	0.387	0.133	0.878	0.991	0.162	0.070
STD vs SD	0.973	0.234	0.824	0.646	0.378	0.639
SD vs 8000	0.481	0.887	0.460	0.560	0.800	0.870

[1]Means are relative expression of target gene normalization to geometric mean of selected housekeeping genes.

Calbindin D9k gene expression was up-regulated in finishers fed the low Ca and P NC in comparison to those fed the nutritionally adequate PC ($P<0.05$). Adding supplementary phytase to the NC brought calbindin D9k expression back to the level of the PC. The mRNA expression of NaPi-IIb, PepT1, SGLT1 and SMIT2 did not differ between treatments. There was a numerical decrease in CD36 expression in pigs fed the NC in comparison to those fed the PC ($P<0.10$).

4.5 Discussion

4.5.1 The effect of phytase on finisher pig growth performance

Phytase was effective in restoring the performance of finisher pigs fed the NC to the level of those fed a nutritionally adequate positive control diet. This effect can be attributed to an increase in phytate hydrolysis and the concomitant improvements in Ca, PP and potentially fat bioavailability, as supported by the bone, $InsP_6$ hydrolysis, and nutrient transporter data. Similar restorative effects of phytase in finishers have been reported by Harper et al. (2007), Brana et al. (2006) and Kuhn and Manner (2012).

A primary objective of this study was to determine the effect of graded levels of phytase above current industry standards on finisher pig performance. It was hypothesised that performance would improve with increasing phytase dose, with those receiving a super-dose outperforming those fed the standard dose. However, results of the current experiment show that there were no differences between any of the phytase doses tested in terms of growth performance. Therefore, supplementation of 500 FTU/kg was sufficient to achieve maximal growth performance. These results are in agreement with those of the previous study, in which increasing the dose from 500 to 2,000 FTU/kg had no effect on grower pig (17 to 40 kg) growth performance.

At present, there is a dearth of peer reviewed literature that has studied the effects of high phytase dosing on finisher pigs. Langbein et al. (2013) reported that supplementing P adequate corn-SBM diets with a super-dose (2,000 FTU/kg) for 78 d had no effect on finisher pig growth performance. This was tested using three different phytase products, none of which influenced pig ADG, ADFI or FCR. Using the same enzyme as that in the present study, Holloway et al. (2015) recently reported that increasing levels of phytase up to 2,500 FTU/kg had no effect on energy, CP or DM digestibility in finishers at 40, 60 or 80 kg when added to P adequate corn-SBM diets. These results differ from those of Brana et al. (2006), Kies et al., (2006b), Zeng et al. (2014) and Zeng et al. (2015), all of who have reported continuing improvements in pig performance in response to increasing levels of phytase.

Based on the results presented herein and the previously reported studies, it is clear that the performance benefits of super-dosing phytase are inconsistent. Reasons for these variable outcomes are unclear; however, it is noteworthy that in each of the

aforementioned studies in which a beneficial super-dosing growth response was observed, younger pigs were used. Whilst largely speculative, it may be that as the pig matures so too does the capacity of the GIT to digest phytate. In poultry, it has been suggested that endogenous phytase production increases as the bird ages (Ravindran et al., 1995; Marounek et al., 2010); however, this remains to be tested in pigs. Moreover, it is known that the capacity of the pig to digest protein increases as the animal ages (Caine et al., 1997; Stein et al., 1999). The reasons for this ontogenetic change remains uncertain, although it has been suggested that longer intestinal transit time and greater microbial activity along the small intestine of the mature animal may play a part. Such factors would also likely influence phytate digestibility. Thus, if this proposition is true, it may be that younger pigs are increasingly sensitive to the anti-nutritional effects of phytate and hence more responsive to the beneficial effects of microbial phytase supplementation.

Furthermore, it appears that the beneficial response to high phytase dosing is more consistent in trials where corn has been used as the principal dietary ingredient. Research has demonstrated that standard doses of phytase are more efficacious in corn based diets than in wheat based diets (Jongbloed et al., 2000; Johansen and Poulsen, 2003). In corn, most of the phytate is found in the germ cells, whereas in wheat, it is largely concentrated in the aleurone layer where its accessibility is thought to be largely restricted by the non-starch polysaccharides of the surrounding cell wall (Kim et al., 2005). Thus, it is possible that responses to high doses of phytase are less apparent in wheat based diets due to limited substrate accessibility.

4.5.2 Ileal inositol phosphate and MYO concentration

Phytate is an anti-nutrient that is capable of binding with cationic minerals such as Ca, Fe, Zn and Cu, inhibiting gastric proteolysis, and increasing AA secretions and losses. Super-dosing aims to enhance animal performance by alleviating the anti-nutritional effects through more extensive phytate degradation (Cowieson et al., 2011). However, few studies have investigated the impact of high doses of phytase on phytate degradation, particularly in the finisher pig. Most studies that have assessed the effect of phytase on phytate degradation have done so by quantifying $InsP_6$ degradation or PP digestibility. Very few have looked at the impact of phytase on the degradation of the lower phytate esters ($InsP_{1-5}$); however, this is appropriate as these lower esters, particularly $InsP_5$ and $InsP_4$, are still anti-nutritive. For example, using a gastric

simulation *in vitro* study Yu et al. (2012) showed that degradation of phytate to at least $InsP_2$ is required to completely mitigate the inhibitory effect of phytate on pepsin activity. Likewise, Sandberg et al. (1999) found that phytate esters down to (and including) $InsP_3$ had an inhibitory effect on Fe absorption in humans.

In this study phytase continued to increase $InsP_6$ hydrolysis in a linear manner up to the highest tested dose of 11,000 FTU/kg (25% to 67%). Guggenbuhl et al. (2012b) found that 1,000 phytase units/kg increased ileal $InsP_6$ degradation from 46 to 87% in grower pigs fed a low P corn-SBM diet. Whereas, Rutherfurd et al. (2014b) reported an increase in ileal $InsP_6$ degradability from 39 to 71% in response to 2,215 phytase units/kg. The phytase induced improvements in $InsP_6$ degradation were somewhat higher in these studies than in the present study; however, drawing comparisons between studies that have used different enzymes and different diets should be done with caution, as both are known to influence phytase efficacy (Angel et al. 2002).

As with $InsP_6$, phytase continued to hydrolyse $InsP_5$ with increasing phytase dose, resulting in a linear decrease in ileal $InsP_5$ concentration. At standard inclusion rates, phytase increased the levels of $InsP_4$ and $InsP_3$ in the digesta. However, the build-up of these was effectively diminished with incremental increases in phytase activity. These findings are in agreement with ileal phytate degradation pattern observed by Holloway et al. (2016) who conducted an experiment in growing-finishing gilts using the same phytase as that used in the present study. The authors also reported that levels of $InsP_4$ and $InsP_3$ increased with standard levels of phytase and then decreased with further incremental increases in phytase concentration.

The primary site of exogenous phytase activity is the stomach (Yi and Kornegay, 1996; Rutherfurd et al., 2014b), which is also the site of maximal substrate solubility (Campbell and Bedford, 1992). Blaabjerg et al. (2011) found that the greatest opportunity for exogenous phytase to hydrolyse phytate is within the first hour after feeding. The K_m of phytase increases as the level of phosphorylation of phytate decreases (Greiner et al., 1993), therefore, phytase has a preference for the higher molecular weight esters of phytate. This in combination with the time constraints of gastric digestion is likely why we see an accumulation of the intermediate esters ($InsP_4$ and $InsP_3$) with standard inclusion levels of phytase. Given the higher K_m of phytase for these phytate esters, it seems likely that further dephosphorylation at standard phytase inclusion rates is limited by substrate concentration. The findings of this study

show that higher doses of phytase begin to target these intermediate phytate esters, allowing for more effective phytate dephosphorylation to take place in the relatively constrained conditions of the GIT of the pig. Interestingly, the concentration of $InsP_2$ did not differ between treatments, suggesting that the rate of formation of this inositol phosphate is equal to the rate of degradation/absorption.

MYO has been implicated as a candidate for eliciting the favourable growth response to phytase; however, definitive evidence for complete phytate hydrolysis in the intestines is lacking (Cowieson et al., 2013a). The results of this study suggest that complete phytate hydrolysis in the GIT is achieved, as phytase effectively increased MYO concentration at the terminal ileum (234 to 945 nmol/mg TiO_2). However, whether this was achieved by exogenous phytase directly, or rather the exogenous phytase presenting the endogenous mucosal phosphatases with a soluble substrate, or a combination of both, is uncertain.

4.5.3 The effect of phytase on plasma MYO concentration

MYO is the most prevalent and biologically active isomeric form of inositol in plants and animals (Clements and Darnell, 1980; McDowell, 2000), where it serves as a component of inositol lipids and inositol phosphates and is thus essential for regular cell growth and metabolism (Michell, 2011). MYO was once classed as a member of the B vitamin family; however, following the discovery that most animals (including pigs) can synthesise this nutrient *de novo* from glucose, this is no longer the case (Murthy, 2006). Therefore, MYO is not considered an essential dietary nutrient for pigs.

However, the role of MYO in nutrition is obscure and warrants further research. Although most animals can synthesise MYO endogenously, the literature suggests that a high dietary MYO supply is important for developing neonates. It is established that the concentration of MYO in the tissue of developing foetuses and juveniles is relatively high, and as the animal ages, the concentration begins to decrease (Lewin et al., 1978; Quirk and Bleasdale, 1983; Pereira et al., 1990). Moreover, the concentration of MYO in human breast milk is particularly high (~1.2 mmol/L), implying that this this nutrient has an important function in babies (Brown et al., 2009). It may be that younger, faster growing animals have a higher requirement than mature animals, or rather, juveniles are unable to synthesise MYO at a rate sufficient

to meet their metabolic requirement. This is true with common carp, as the intestinal cells of these fish can produce MYO from glucose, but not at an adequate rate for the normal growth of juveniles (Lall, 2010). The work of Zyla et al (2013) provides strength to this notion as they found that supplementation of 1 g/kg of MYO improved broiler growth efficiency, but it had a greater effect in the starter phase (d 1 to 21) than in the grower (d 22 to 42) phase. Therefore, it is possible that the dietary importance of MYO, particularly in juveniles, has been underestimated.

The growth promoting properties of MYO have been previously demonstrated in poultry (Cowieson et al., 2013a; Zyla et al., 2013). These findings have prompted speculation that the beneficial effects of high doses of exogenous phytase are in part mediated through an increase in MYO bioavailability (Cowieson et al., 2011; Walk et al., 2014). Whilst this view remains largely speculative, particularly in the pig, the results of the previous chapter demonstrated that higher doses of phytase can effectively improve MYO availability and absorption in grower pigs. The plasma MYO results from the current study suggest that this is also true in pigs at the early finishing stage of production. Increasing doses of phytase continued to increase portal plasma MYO concentration in a linear manner up the highest dose tested (11,000 FTU/kg), with numerical increases apparent at the standard phytase doses. This shows that the additional MYO generated in response to phytase is being absorbed from the GIT. Phytase also increased MYO levels in the peripheral circulation; however, the magnitude of the increase was lower than that of the portal blood. Furthermore, increases in peripheral MYO were not apparent with the standard phytase doses. These results may indicate a possible role of the liver in storing or metabolising MYO; glucose (Burtle and Lovell, 1989), phosphatidylinositides (Prpic et al., 1982) and inositol phosphates (Shears and Hughes, 1990) can all be synthesised from MYO in the liver.

As hypothesised, increasing the phytase dose improved MYO bioavailability. However, this did not translate into improved growth performance. As theorised above, it is possible that older pigs, such as those used in this experiment, do not have an exogenous requirement for MYO. Alternatively, it is plausible that the experimental period was too short for any beneficial effects of MYO to be observed. More research into the effects of MYO on pig performance is necessary to validate the view that it is involved in the super-dosing growth response.

4.5.4 The effect of phytase on amino acid digestibility

There is much ambiguity surrounding the effects of phytase on protein digestibility in the pig. This is further compounded by the lack of any clear understanding of how phytate reduces protein availability. The current view is that binary phytate-protein complexes form *de novo* at regions of low pH, thereby rendering the bound protein unavailable for gastric proteolysis (Selle et al., 2012). Moreover, it is also thought that phytate can stimulate increased endogenous AA secretions (Cowieson et al., 2004) and compromise Na-dependent AA transport systems (Woyengo et al., 2012).

In this study, phytase had no effect on the apparent ileal digestibility of any of the AA measured. As previously discussed, the effects of phytase on AA digestibility are variable and inconsistent. Some studies have found no effect of phytase on AA digestibility (Traylor et al., 2001; Cervantes et al., 2004; Nitrayova et al., 2006; Woyengo et al., 2009b; Morales et al., 2012), whereas others have found that phytase improves the digestibility of some AA (Zeng et al., 2011; Guggenbuhl et al., 2012b; Adedokun et al., 2015; and Velayudhan et al., 2015). The inconsistencies in the AA phytase response may be linked to a number of factors, including protein source and solubility, phytate concentration, accessibility of protein to phytate, pH of media, choice of phytase enzyme, mineral concentration and protein-phytate-mineral interactions (Kemme et al., 1999; Liao et al., 2005b). In addition, the inherent protein digestibility of the diet will likely influence the phytase the response. Evidently, in this experiment, dietary phytate was not limiting AA digestibility in the finisher pigs.

From the literature it is clear that when phytase does improve AA digestibility the effects are marginal (Adeola and Sands, 2003). However, as protein is the second most expensive nutrient in the pig diet, even a small improvement in protein digestibility may significantly reduce feed costs. Therefore, further research into the factors influencing the phytase AA response could prove worthy, but until such factors have been identified, applying phytase AA matrix values to diets does not seem justified.

4.5.5 The effect of phytase on mineral availability

An alternative objective of this study was to determine the effect of increasing doses of phytase on mineral availability in the finisher pig. There is currently a lack of available information on the effects of high phytase doses on mineral availability in

pigs at this stage of production, particularly for minerals other than Ca and P. It has been suggested that while the effects of phytase on AA and DE are likely maximised at reasonably low doses, the effect on mineral digestibility continues in a linear fashion with incremental increases in dose (Cowieson et al., 2006).

4.5.5.1 Bone mineralisation

Phytase mediated increases in bone ash content are one of the most consistent effects of exogenous phytase supplementation (Harper et al., 1997; Brana et al., 2006; Zeng et al., 2014). The ash content of pigs fed the NC diet is in agreement with the value reported in the previous grower study. In this study, phytase quadratically increased the ash, Ca, P and Mg content of the third proximal phalanx. The quadratic increase in bone mineral retention in response to phytase is in line with the increase in phytate hydrolysis and can therefore be attributed to a reduction in complex phytate-mineral interactions occurring in the GIT, allowing for increased mineral utilisation.

Both Harper et al. (1997) and Brana et al. (2006) showed that microbial phytase can increase finisher pig bone ash content when added to a low P corn-SBM diet. This study demonstrated that phytase has the same effect when added to low P wheat-barley based diets, but also that increasing doses of phytase above industry standards (up to 11,000 FTU/kg) continues to increase bone mineral deposition in finishers, as has been demonstrated in weaners (Veum et al., 2006; Zeng et al., 2015) and growers (Brana et al., 2006; and previously reported study). This response appeared to plateau at 2,420 FTU/kg, which suggests that Ca, Mg and P were no longer limiting maximal osteoid mineralisation in finishers fed this treatment. Improvements in bone mineralisation associated with super-doses of phytase may be of more practical significance for the breeding herd, particularly for gilts or second-parity sows, where lameness is one of the primary causes of premature sow culling (Willgert et al., 2014).

4.5.5.2 Apparent ileal mineral digestibility and plasma mineral concentration

As expected apparent P ileal digestibility improved with increasing phytase dose; P AID is negatively correlated with P excretion, therefore, phytase is likely having a beneficial environmental impact by reducing finisher pig P excretion. The portal and peripheral plasma P data are in agreement with the AID P data and show quadratic increases in response to phytase supplementation. This finding differs to that of the previous experiment, in which phytase had no effect on blood P levels in grower pigs

and can most likely be attributed to differences in dietary P levels relative to requirement. Murry et al. (1997) noted in weaners that phytase increased serum P when added to low P diet, but its effect was diminished when added to P adequate diets. It is probable that in the previous experiment, dietary P levels were at or above requirement at the time of sample collection, whereas in the current study, P levels were below the requirement for finisher pigs of this weight. The observed effect on P digestibility and plasma P with increasing levels of phytase is in concurrence with many other studies (Simons et al., 1990; Lei et al., 1993; Zeng et al., 2015) and can be attributed to improved phytate hydrolysis and PP bioavailability, as supported by the ileal $InsP_x$ data.

Based on the plasma and digestibility data, phytase had no effect on Ca or Mg availability. This is in contrast to the bone data, which showed that phytase increased the retention of these minerals. Thus, these data imply that bone mineral concentration is a more sensitive measure of Ca and Mg availability than AID or plasma concentration. This is unsurprising given that circulating levels of these minerals are under strict homeostatic regulation (Li et al., 1998; de Baaij et al., 2012), and AID measurements are compounded by basal and specific endogenous mineral secretions (Stein et al., 2007).

Studies have shown that phytase can also improve the availability of some of the cationic trace-minerals. Jolliff and Mahan (2012) found that 1,000 FTU of phytase/kg increased Mn, S, Se and Zn levels in the bone and in the liver, Mn and Zn in the heart, and Mn in the kidney of weaner pigs. Likewise, Zeng et al. (2015) found that phytase increased the concentration of Zn and Mn in the bone; Zn in the heart, kidney and liver; Cu in the liver; and Mn in the kidney of weaner pigs fed a low Ca and P corn-SBM diet. In this finisher study, phytase linearly improved Cu AID to the highest tested dose; however, it is clear that this effect is less apparent when supplemented with a standard phytase dose. This is in agreement with the recent conclusions of Bikker et al. (2012), who conducted a meta-analysis and found that standard phytase doses (500 to 1,570 FTU/kg) generally have no effect on Cu digestibility in growing pigs. In addition, Zeng et al. (2015) found that phytase at 500 and 1,000 FTU/kg had no effect on weaner liver Cu concentration, whereas a super-dose of 20,000 FTU/kg significantly increased liver Cu concentration. These findings, together with the results of the current study, suggest that super-doses of phytase are necessary to

improve Cu availability in pigs. The ileal $InsP_{2-6}$ data from this experiment show that standard doses hydrolyse $InsP_6$ to mainly $InsP_4$ and $InsP_3$, whereas the super-doses of phytase begin to target these intermediate esters, thus facilitating complete phytate hydrolysis. Therefore, it is possible that the intermediate esters of phytate are still capable of binding significant quantities of Cu, and in order to improve Cu availability, phytate must be hydrolysed to $InsP_2$ or below. The *in vitro* work of Persson et al. (1998) supports this proposition as they demonstrated that $InsP_3$ is capable of chelating with up to 3 Cu^{2+} atoms at pH 5-6.

Fe and Zn digestibility were not influenced by phytase in this experiment, which is consistent with the findings of Rutherfurd et al. (2014a) in grower pigs. Studies have demonstrated that phytase can improve the bioavailability of these trace-minerals when they are provided at sub-optimal dietary concentrations. For instance, Stahl et al. (1999) reported improvements in blood haemoglobin concentration in anaemic weaner pigs when fed a low-Fe diet supplemented with 1,200 U/kg of phytase activity. In addition, Adeola et al. (1995) found that phytase improved apparent faecal Zn digestibility in weaners offered a low Zn diet, but had no effect when offered a Zn adequate diet. It is likely that dietary phytase had no effect on Fe and Zn digestibility in this study as both of these minerals were provided in excess of requirement (BSAS, 2003).

Recently, efforts to determine the effect of phytase on trace mineral availability in the pig have been small in comparison to that of P, protein and energy, and this is largely due to the potentially greater feed costs savings associated with these nutrients. However, although the economic value for trace mineral displacement with phytase may not be as great as that for P, protein or energy, reducing the dietary input of trace minerals would certainly have significant ecological value. This is particularly true for the environmentally damaging minerals Cu and Zn. Optimising mineral availability and reducing inorganic mineral supplementation are both ways in which levels of these pollutants in the excreta can be minimised (Mosenthin and Broz, 2010). Therefore, future research to clearly define the phytase effect on trace mineral availability seems justified. Efforts should be made to accurately determine phytase matrix values for the cationic trace minerals, allowing nutritionists to make the appropriate dietary modifications necessary to prevent over or under supply. This would maximise both

the economic and ecological value of phytase enzymes and likely contribute to improving pig production sustainability.

4.5.6 The effect of phytase on jejunal nutrient transporter gene expression

It has been reported that exogenous phytase can increase mineral, protein and energy digestibility in the pig; however, the underlying mechanism for the uptake of these nutrients from the intestinal lumen is unclear. Nutrients are absorbed from the lumen by way of active or passive mechanisms. Active nutrient uptake involves the absorption of nutrients from the lumen into enterocytes via carrier proteins (nutrient transporters) situated on the apical membrane. Therefore, factors influencing the expression of these nutrient transporters in the GIT largely influence the nutritional status of the animal. Studies have demonstrated that factors such as age (Nosworthy et al., 2012), gender (Mott et al., 2008), species (Gilbert et al., 2008) and presence of substrate in the intestinal lumen (Bar et al., 1990; Schiller et al., 1997; Ihara et al., 2000) can influence intestinal nutrient transporter expression. Therefore, in the present study, it was hypothesised that dietary phytase would modulate nutrient transporter gene expression in the jejunum by altering nutrient availability in the GIT.

NaPi-IIb is an intestinal, high affinity, Na^+-dependent phosphate cotransporter responsible for the active absorption of P across the brush border membrane of enterocytes (Hilfiker et al., 1998). Although phytase increased P digestibility in this experiment, it had no effect on jejunal NaPi-IIb gene expression. Vigors et al. (2014) recently reported that addition of 1,000 FTU of phytase/kg numerically increased ($P<0.1$) jejunal NaPi-IIb expression in grower pigs fed a low P diet. The lack of effect on NaPi-IIb gene expression the current experiment may be due to the dominance of passive (paracellular) P absorption. It is generally thought that the primary mode of intestinal P absorption occurs via the paracellular route when P is provided at or above requirement; however, under periods of P deprivation, the active transcellular route becomes dominant (Stein et al., 2008; Sabbagh et al., 2011). As all phytase treatments were formulated to contain adequate P, this may explain why there were no differences between the different tested phytase doses on NaPi-IIb gene expression.

Calbindin D9k is a small, cytosolic, vitamin-D sensitive calcium binding protein that is present in mammalian enterocytes, where it serves to transport absorbed Ca from the apical membrane to the basolateral membrane (Walters, 1989). Calbindin D9k

expression is upregulated in response to an increase in blood 1,25-dihydroxyvitamin D-3 levels during times of Ca deprivation. Armbrecht et al. (2003) and Li et al. (2012) demonstrated, in rats and broilers respectively, that low dietary Ca levels stimulate an increase in intestinal calbindin D9k gene expression. In this study, phytase supplementation prevented the up-regulation of calbindin D9k gene expression observed in those fed the non-supplemented low Ca NC treatment, which suggests improved Ca availability. This, taken together with the bone data, suggests that phytase is improving dietary Ca bioavailability.

Under normal physiological conditions, proteins are effectively degraded by protease enzymes secreted in the GIT to smaller peptides and AA. The primary hydrolysis products of protein digestion are di- and tri-peptides and these can be effectively transported from the gut lumen into the enterocyte by the H^+-dependent peptide transporter PepT1 (Botka et al., 2000). PepT1 is responsible for the majority of AA uptake from the lumen owing to its high capacity for peptide transport (Daniel, 2004). In pigs, PepT1 is expressed throughout the small intestine, with greatest expression in the jejunum (Chen et al., 1999). In this study, jejunal PepT1 gene expression was not influenced by phytase, which is in agreement with the apparent ileal AA digestibility data. Vigors et al. (2014) also found that phytase had no effect on jejunal PepT-1 gene expression; however, they did report a significant increase in PepT-1 expression at the ileum. Wang et al. (2012) found that feeding finisher pigs a lysine deficient diet reduced PepT-1 expression in the duodenum and ileum, but had no effect on the expression in the jejunum. Therefore, it is possible that jejunal PepT-1 expression is less sensitive to dietary changes in AA supply than duodenal or ileal PepT-1 expression.

SGLT1 and CD36 are intestinal transporters responsible for the uptake of glucose and long chained fatty acids respectively (Nassir et al., 2007; Wright et al., 2011). The expression of both of these transporters has been found to be correlated to substrate availability, thus providing a measure of luminal energy availability (Dyer et al., 1997; Chen et al., 2001). Finishers fed the NC diet tended to have a reduced expression of CD36 in comparison to those fed the PC diet. This can most likely be attributed to the lower ME content of the diet, which was met largely through a reduction in fat supplementation. Although not significant, there were numerical increases in CD36 gene expression in response to phytase which would suggest an increase in luminal fat

availability. It is speculated that phytate can restrict lipid digestibility through the formation of lipophytins within the intestines (Ravindran et al., 2000). Therefore, by degrading phytate in the proximal regions of the tract, phytase has the capacity to improve lipid availability (Selle et al., 2003). If indeed phytase is improving fat digestibility in the present experiment, this would help to explain how phytase supplementation is fully restorative of finisher performance when offered a low ME diet. Phytase had no effect on jejunal SGLT1 gene expression which is in agreement with the findings of Vigors et al. (2014) and suggests that phytase does not improve glucose availability at the jejunum.

The mechanism for MYO absorption across the brush border membrane in the pig is unclear. There are currently three known secondary active MYO transporters; one H^+-dependent (HMIT) transporter and two Na^+-dependent transporters (SMIT1 and SMIT2). HMIT is thought to be primarily expressed in neuronal tissue (Di Daniel et al., 2009), while SMIT1 is a basolaterally expressed protein present in the brain and renal medulla (Kwon et al., 1992). SMIT2 was discovered comparatively recently and has been detected in brain, kidney and intestinal tissue (Lahjouji et al., 2007). As demonstrated in rats, SMIT2 is thought to be responsible for intestinal MYO uptake (Aouameur et al., 2007). However, prior to this experiment, no published studies have looked at intestinal MYO transporters in the pig. A central discovery of the present study is that SMIT2 is indeed expressed in the pig intestine. Although the portal MYO data clearly demonstrate that phytase treatment increased intestinal MYO absorption, jejunal SMIT2 gene expression was not influenced by phytase. It is difficult to explain this lack of effect considering the dearth of available literature on SMIT2. It may be that maximal MYO absorption occurs at a different point in the small intestine such as the duodenum or ileum, or perhaps at a different point of the jejunum. It is also possible that an alternative as of yet unknown transporter is involved in MYO uptake that is more dominant than SMIT2. As with many other proteins, it could also be that SMIT2 is post-transcriptionally regulated, and thus mRNA abundance measurements are not a sensitive indicator of protein expression.

4.5.7 Conclusions

Microbial phytase supplementation effectively improved the growth performance of finisher pigs fed a low Ca, P and energy wheat-barley based diet. This effect can be attributed to improved phytate hydrolysis and the associated improvements in Ca and P

retention. In addition, although not measured in the current study, it may be that phytase is also improving lipid availability in the intestine (as indicated by a numerical increase in jejunal CD36 gene expression). Increasing the phytase dose from 751 to 11,000 FTU/kg had no effect on finisher pig growth performance.

Phytase effectively increased phytate hydrolysis at the terminal ileum in a dose-dependent manner up to the highest tested dose. The ileal inositol phosphate data show that at standard doses, phytase preferentially targets the $InsP_6$ and $InsP_5$ esters, resulting in an accumulation of $InsP_4$ and $InsP_3$ in the intestine. As the dose increases, phytase begins to hydrolyse the $InsP_4$ and $InsP_3$ esters, resulting in more complete phytate hydrolysis. Ileal $InsP_2$ concentration was unchanged irrespective of treatment, suggesting that the rate of formation of this phytate ester is equal to the rate of its hydrolysis/absorption. The more extensive phytate hydrolysis associated with the higher doses of phytase led to an increase in ileal MYO concentration and an increase in portal and peripheral plasma MYO levels.

Increasing doses of phytase increased Ca, P and Mg content in the bone in a quadratic manner, with the concentration of these minerals in the bone seemingly maximised at 2,420 FTU/kg. Phytase had no effect on apparent ileal AA digestibility, which may explain the lack of favourable growth response to high doses of phytase, as commonly seen in other studies. Phytase linearly increased apparent ileal Cu digestibility but had no effect on any of the other minerals measured.

Finishers responded to an inadequate Ca supply by up-regulating jejunal calbindin D9k gene expression. Phytase effectively prevented this up-regulation and maintained calbindin D9k gene expression at a level similar to the positive control, which suggests improved Ca availability. Jejunal NaPi-IIb, PepT1, SGLT1 and CD36 gene expression were not significantly affected by phytase. In addition, although the plasma data clearly show that MYO is being absorbed from the gut in response to phytase, SMIT2 gene expression in the jejunum was not altered. This suggests that jejunal SMIT2 mRNA concentration is not a sensitive measure of MYO absorption in finisher pigs.

These data show that although higher doses of phytase above current industry standards increase phytate degradation and MYO bioavailability, this does not translate into improved growth performance for pigs fed a wheat-barley based diet at the early finishing stage of production. It is possible that the experimental period was too short

for performance benefits to be observed, therefore, more research is needed before it can be concluded that super-dosing phytase has no beneficial effect on the performance of finisher pigs fed wheat-barley diets.

Chapter 5
The Effect of Phytase, Time and Freezing Temperature on Gastric Inositol Phosphate and *Myo*-inositol Concentration Following Sample Collection

5.1 Abstract

Phytate ($InsP_6$) and its degradation products are frequently measured in the digesta of monogastrics as a means of determining phytase efficacy. However, there is no standardised method for the collection and processing of digesta in such studies. This experiment set out to determine the effect of time and freezing temperature during sample processing on gastric inositol phosphate ($InsP_{2-6}$) concentration in pigs fed diets containing different levels of phytase. A total of 40 pigs were fed one of four pelleted wheat-barley diets *ad libitum* for 28 d. The diets comprised a nutritionally adequate diet (PC) with no added phytase, a similar diet but with Ca and P reduced by 1.6 and 1.24 g/kg respectively (NC), and this NC diet supplemented with 500 (STD) or 2,000 FTU/kg (SD). At the end of the experiment, pigs were euthanised and the total stomach digesta was collected and mixed thoroughly. Two subsamples of the digesta were frozen immediately; one at -78.5 °C and one at -26 °C. The remaining digesta was left to sit at room temperature (20 °C) for 5 min before a further two subsamples were collected and frozen as above. This process was repeated every five minutes for a total of 15 minutes from the time of mixing. Stomach digesta were analysed for total phytate, $InsP_{2-5}$ and MYO content.

There was no difference in gastric $InsP_6$ concentrations between the NC and PC treatments ($P>0.05$) or the STD and SD treatments ($P>0.05$); however, $InsP_6$ concentration was 82.4% lower in the diets with added phytase than in the control diets ($P<0.001$). Phytate was continuously degraded over time following sampling (linear, $P<0.001$), irrespective of dietary treatment or freezing temperature. Moreover, the concentration of $InsP_6$ ($P<0.001$) in the gastric digesta was influenced by a freezing temperature x diet interaction ($P<0.05$), with digesta frozen at -26 °C recovering less analysed phytate than that frozen at -78.5 °C. However, this difference was greater when pigs were fed the PC or NC diets. The total measured phytate hydrolysis products ($InsP_{2-5}$ + MYO) were also influenced by a freezing temperature x diet interaction ($P<0.01$); freezing at -78.5 °C recovered more hydrolysis products than

freezing at -26 °C in diets with added phytase, but there were no differences between freezing methods when fed non-supplemented diets. These results suggest gastric digesta should be frozen on dry ice as quickly as possible following sample collection, in order to minimise post-collection phytate degradation and prevent changes in the gastric inositol phosphate composition.

5.2 Introduction

Microbial phytase enzymes effectively hydrolyse ingested phytate in the gastric regions of the tract, improving PP availability and reducing P excretion (Simons et al., 1990; Harper et al., 1997). The current commonplace inclusion of microbial phytases as a feed additive to pig diets globally is testament to persistent and successful phytase research spanning over two decades. Early phytase research focussed on producing, characterising and applying phytase enzymes mainly of fungal origin (principally *A. niger*). Efforts to develop more efficacious phytase enzymes have been constant since their commercial introduction in 1991. Whilst the search for superior phytase enzymes under physiologically relevant conditions continues unabated, more recently, attention has focussed on the extra-phosphoric effects of these enzymes and their potential to improve protein and energy availability, and animal growth performance.

However, despite a great deal of research, the phytase response is often inconsistent and thus the situation surrounding potential protein, energy and growth performance effects remains ambiguous. As discussed in Section 1.7, there are many well documented enzymatic, dietary and animal related factors that are known to influence the efficacy of phytase *in vivo*. Variations in such factors between studies have likely contributed to the inconsistent phytase responses reported, and as a result compounded efforts to discern the true effects of phytase supplementation. Furthermore, although less well documented, it seems reasonable to assume that the widespread study of phytase as a feed additive, and lack of inter-laboratory standardised methodology within the scientific community has played a major role in generating the inconsistencies seen in the literature.

Over the last few years, superior analytical methodologies for the accurate quantification of phytate in digesta and feedstuffs have been developed. This, in turn, has seen a rise in the number of studies measuring phytate ($InsP_6$) and its degradation

products in the digesta of monogastrics as a means of determining phytase efficacy. However, at present, there is no standardised method for the collection and processing of the digesta in such studies. Therefore, this short study set out to determine the effects of time and freezing temperature on phytate ($InsP_6$) and lower inositol phosphate ($InsP_{2-5}$) degradation occurring in gastric digesta during sample collection and processing. Digesta were obtained from pigs fed diets containing differing levels of phytase to determine if the response to different inclusion rates was influenced by processing method. As the stomach is the primary site of exogenous phytase activity (Kemme et al., 1998; Rapp et al., 2001), and it is clear that an early and rapid breakdown of phytate is necessary to alleviate the anti-nutritional effects of phytate (Adeola and Cowieson, 2011; Dersjant-Li et al., 2015), the extent of phytate hydrolysis occurring in the stomach is key to determining the magnitude of the phytase response.

5.2.1 Hypothesis

- Phytate in the gastric digesta will continue to be degraded over time following sample collection, thus altering the gastric InsP composition
- Phytate will be broken down faster in the digesta of pigs receiving the super-dose than the standard phytase dose treatment.
- Less post-sample collection phytate degradation will occur in samples frozen on dry ice (-78.5 °C) than in those frozen at -26 °C.

5.3 Methods

5.3.1 Animals, management and dietary treatments

Gastric digesta samples for this study were obtained from pigs in the previous experiment (Chapter 4), therefore, readers are referred to Sections 4.3.1 and 4.3.2 for a detailed description of animal housing, management and the dietary treatments. In brief, 288 finisher pigs (initial BW $\pm$ SE = 36.7 $\pm$ 0.3 kg) were housed into mixed sex pens of four and fed one of six wheat-barley based dietary treatments (n = 12) for 28 d. The dietary treatments included a nutritionally adequate (BSAS, 2003) positive control (PC) with no added phytase, a low Ca, P and ME negative control (NC) with no added phytase, and the NC supplemented with phytase at 500, 1,000, 2,000, or 8,000 FTU/kg; however, for this particular experiment, only samples from pigs fed the PC,

NC, NC+500 FTU/kg (STD) or the NC+2,000 FTU/kg (SD) treatments were used. The diet composition, including inositol phosphate content and calculated nutrient levels, are presented in Table 4.1. All diets were pelleted through a 3 mm die at a temperature of 62 ($\pm$ 2) °C and offered *ad libitum*.

5.3.2 Gastric digesta collection

This experiment set out to determine the effect of freezing temperature (-26 °C vs -78.5 °C) and time taken to freeze the sample (immediately, 5, 10 or 15 min) on gastric phytate degradation. At the end of the 28 d experiment, 10 pigs from each treatment were euthanised (as described in Section 4.3.2) and gastric digesta were collected from pigs fed the PC, NC, STD or SD treatments. To do this, an incision was made into the stomach and the total contents were poured into a large glass beaker. The stomach digesta were mixed thoroughly and the pH recorded using a handheld pH meter with a glass electrode (HI-99161, Hanna Instruments Ltd, Bedfordshire, UK). Two representative subsamples of the digesta were decanted into separate 30 ml polypropylene screw topped tubes and frozen immediately; one on dry ice (-78.5 °C) and one in the freezer (-26 °C). Thereafter, the remaining digesta was left to sit at room temperature (20 °C) for 5 minutes before a further 2 subsamples were collected and frozen as above. This process was repeated every 5 minutes for a total of 15 minutes from the time of mixing to give four time points: 0, 5, 10 and 15 min. It should be noted that the mixing of the stomach digesta occurred at approximately 4 min following the confirmation of death. All gastric digesta samples (including those frozen at -78.5 °C) were stored at -26 °C pending freeze-drying and subsequent inositol phosphate and MYO analyses. All samples were freeze-dried within 10 d of collection.

5.3.3 Gastric digesta analyses

Gastric digesta samples were freeze dried and ground to pass a 1 mm sieve prior to all subsequent analyses. The concentration of $InsP_{2-6}$ in gastric digesta was analysed by HPIC according to the method described in Section 2.5.11. MYO was quantified using HPLC according the method described in Section 2.5.10. TiO_2 was analysed in duplicate according to the method detailed in Section 2.5.4.

5.3.4 Calculations and statistical analysis

Gastric $InsP_6$ degradability was calculated according to the digestibility calculation outlined in Section 2.6.

All data were analysed as a 4 x 2 x 4 factorial using a three-way mixed ANOVA with the individual pig serving as the experimental unit for all analyses (SPSS Statistics, Version 22). The data were first tested for normality and sphericity using the Levene's test and Mauchly's Sphericity test respectively. Non-normal data were log transformed (log10 (x+1)) prior to statistical analysis. Data violating the assumption of sphericity were corrected for using the Greenhouse-Geisser correction. The statistical model included the effects of diet, freezing temperature, time and all associated interactions, with both time and freezing temperature included as repeated factors. Non-significant interactions were removed from the model one at a time, starting with the least significant. No three-way interactions were observed for any of the parameters measured. Polynomial contrasts were used to determine linear or quadratic effects of time. Differences were classed as significant if $P<0.05$ or as a trend if $P<0.10$.

5.4 Results

Experimental diets contained moderate amounts of phytate (7.1 – 7.9 g $InsP_6$/kg DM), which were in line with other wheat-barley based pig diets (Blaabjerg et al. 2010; Blaabjerg et al., 2011). The analysed phytase activity in the diets were as follows: PC = 85 FTU/kg, NC = <50 FTU/kg, STD = 751 FTU/kg and SD = 2,420 FTU/kg. For a detailed breakdown of the analysed nutrient composition of the experimental diets see Section 4.4 (Table 4.3).

The average pH of the gastric digesta from pigs fed the PC, NC, STD and SD diets were 4.09, 3.95, 3.56 and 3.68 respectively (SEM = 0.28, $P = 0.516$).

5.4.1 Gastric phytate hydrolysis

Figure 5.1 displays the effects of diet, time and freezing temperature on gastric $InsP_6$ degradation after sample collection. There was a significant diet by freezing temperature interaction ($P<0.05$) as less $InsP_6$ was degraded in digesta frozen on dry ice (-78.5 °C) than at -26 °C; however, the difference between the two freezing temperatures was greater in treatments devoid of supplementary phytase. This interaction can be more clearly observed in Figure A.1 (see appendix). Gastric $InsP_6$ degradation was significantly higher in the diets with added phytase than in the PC or NC diets ($P<0.001$); however, there was no difference between the two phytase test diets ($P = 0.967$) or the two control diets ($P = 0.948$).

As time did not interact with either dietary treatment or freezing temperature, its main effects were analysed independently and are presented in Figure A.2 (see appendix). $InsP_6$ degradation increased in a linear fashion over time ($P<0.001$). $InsP_6$ degradation was 1.7% higher in digesta frozen after 5 min than that frozen immediately (66.0 vs 64.3 %, $P<0.05$)

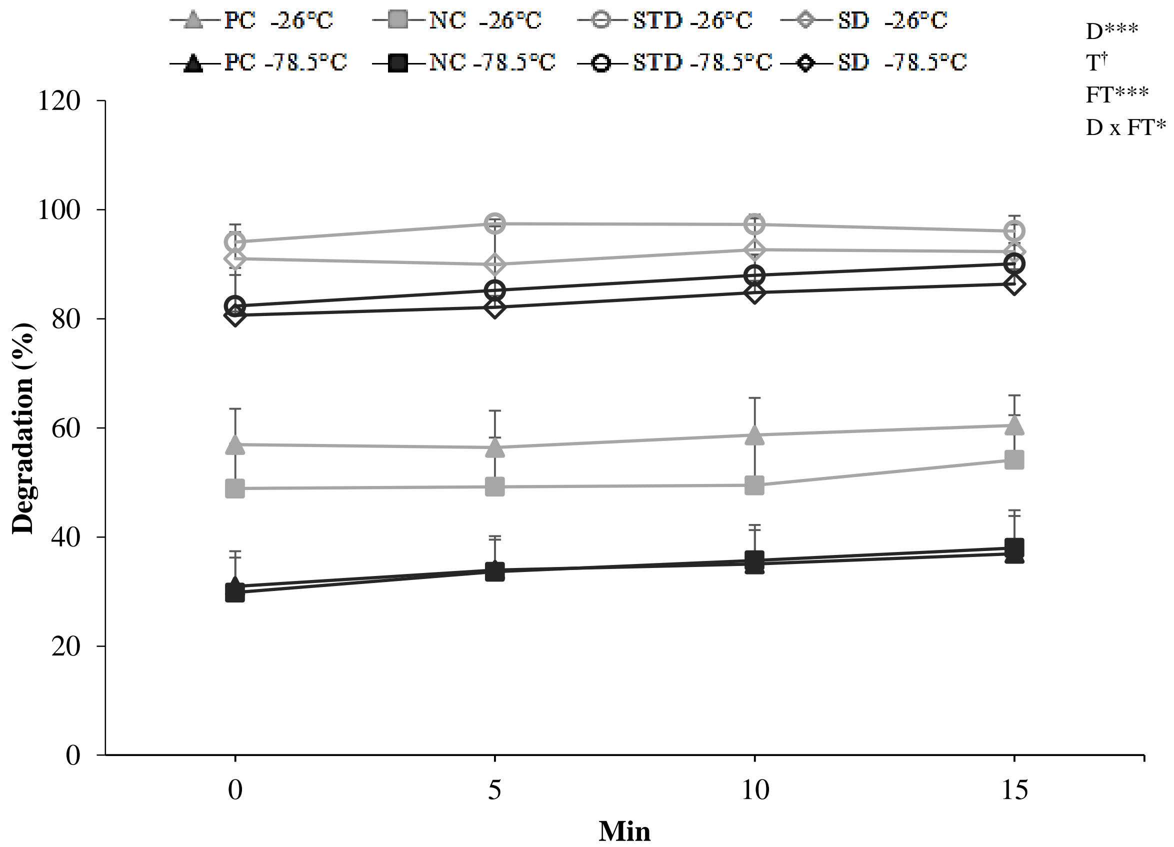

Figure 5.1 Effect of diet (D), freezing temperature (FT) and time (T) on gastric $InsP_6$ degradation (%) following sample collection

Values are means of 10 observations ± SEM. Statistical significance is shown: *P<0.05, **P<0.01, ***P<0.001, † linear effect (P<0.001). No D x T, FT x T or D x FT x T interactions were observed (P>0.05).

5.4.2 Gastric inositol phosphate and MYO concentration

The effect of diet, freezing method and time on gastric concentrations of phytate and its hydrolysis products ($InsP_2$ through $InsP_5$ and MYO) is presented in Table 5.1. In line with the hydrolysis data, gastric $InsP_6$ concentration was influenced by time (P<0.001) and a significant diet by freezing temperature interaction (P<0.05). The concentration of $InsP_6$ in the gastric digesta decreased by 13.4% from 0 to 15 min at a constant rate of approximately 32 nmol /mg TiO_2/min (P<0.001). As expected, the diet by freezing temperature interaction was the same as it was for the $InsP_6$ hydrolysis data, with less $InsP_6$ being measured in samples frozen at -26 °C than those frozen at -78.5 °C, and the effect being greater in the PC and NC than in the STD and SD

treatments. The concentration of lower inositol phosphates and MYO in the gastric digesta was influenced by a freezing temperature x diet interaction (*P*<0.01), but not by time (*P*>0.05). The data show that freezing temperature had no influence on the total concentration of lower phytate esters and MYO in the PC and NC diets; however, in diets with supplementary phytase, freezing at -78.5 °C produced greater gastric $InsP_{2-5}$ and MYO concentrations than freezing at -26 °C.

The effect of time on the concentrations of phytate hydrolysis products in the gastric digesta is presented in Figure A.3 (appendix). Leaving the gastric digesta to sit at room temperature for up to 15 min following sampling had no effect on $InsP_5$, $InsP_4$, $InsP_2$ or MYO concentrations (*P*>0.05). $InsP_3$ concentration increased in a linear manner (*P*<0.05) over time in the PC, NC and STD diets, but remained fairly constant in the SD diet, resulting in a tendency for a time x diet interaction (*P* = 0.06).

The effects of freezing temperature and diet on the concentration of the individual phytate hydrolysis products in the gastric digesta, including MYO, are presented in Figure 5.2. Within freezing temperature, the gastric inositol phosphate and MYO composition of pigs fed the PC and NC diets was almost identical. Adding phytase to the NC at either 500 or 2,000 FTU/kg significantly reduced $InsP_5$ content (*P*<0.001), though there were no differences between the two phytase doses (*P*>0.05). Digesta frozen at -26 °C measured 30.5% less $InsP_5$ than that frozen at -78.5 °C (402 vs. 280 nmol/mg TiO_2; *P*<0.001). $InsP_4$, $InsP_3$ and $InsP_2$ concentration were all influenced by a significant freezing temperature x diet interaction (*P*<0.001, *P*<0.001 and *P*<0.01 respectively). In the PC and NC diets, digesta frozen at -26 °C tended to have higher levels of $InsP_4$ than that frozen at -78.5 °C (*P*<0.1), whereas in the STD diet, freezing the digesta at -26 °C had the reverse effect and resulted in lower levels of measured $InsP_4$ (*P*<0.05). The freezing temperature x diet interaction for gastric $InsP_3$ was similar to that described for $InsP_4$. Within freezing temperature, increasing the phytase dose from a standard dose (500 FTU/kg) to a super-dose (2,000 FTU/kg) reduced the amount of $InsP_4$ and $InsP_3$ present in the stomach digesta (*P*<0.01). Levels of measured $InsP_2$ were similar irrespective of freezing temperature for the PC, NC and STD diets; however, in the SD diet, $InsP_2$ concentration was higher when frozen at -78.5 °C than at -26 °C. The concentration of gastric MYO was not influenced by treatment (*P*>0.05).

Table 5.1. Effect of diet, freezing temperature and time on gastric $InsP_6$ and $\sum InsP_{2-5}$ + MYO (nmol/mg TiO_2) concentration following sampling[a]

FT	-26 °C				-78.5 °C						
Diet	PC	NC	STD	SD	PC	NC	STD	SD	Average	Significance[b]	SEM
$InsP_6$										D^{***}, FT^{***}, $T^{\dagger}$, D x FT*	125
0 min	835	1098	110	166	1338	1489	327	356	*715*		
5 min	846	1091	47	185	1280	1425	274	329	*685*		
10 min	801	1085	50	135	1259	1380	223	280	*651*		
15 min	766	985	72	142	1222	1331	183	250	*619*		
Average	*812*	*1064*	*70*	*157*	*1275*	*1406*	*252*	*304*			
FT average		*526*				*809*					
$\sum InsP_{2-5}$ + MYO										D^{*}, FT^{**}, D x FT**	192
0 min	1942	1822	1360	940	1857	1856	1859	1386	*1426*		
5 min	1927	1755	1370	996	1876	1750	1844	1355	*1415*		
10 min	2019	1837	1368	806	1948	1786	1887	1328	*1425*		
15 min	1958	1905	1422	1013	2002	1910	1904	1327	*1471*		
Average	*1962*	*1830*	*1380*	*939*	*1921*	*1826*	*1874*	*1349*			
FT average		*1528*				*1742*					

[a] Values represent the mean of 10 observations.

[b] Significance level: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, † linear effect $P<0.001$. D = diet, FT = freezing temperature, T = time, and D x FT = diet by freezing temperature interaction. No D x T, FT x T or 3 way interactions were observed ($P>0.05$).

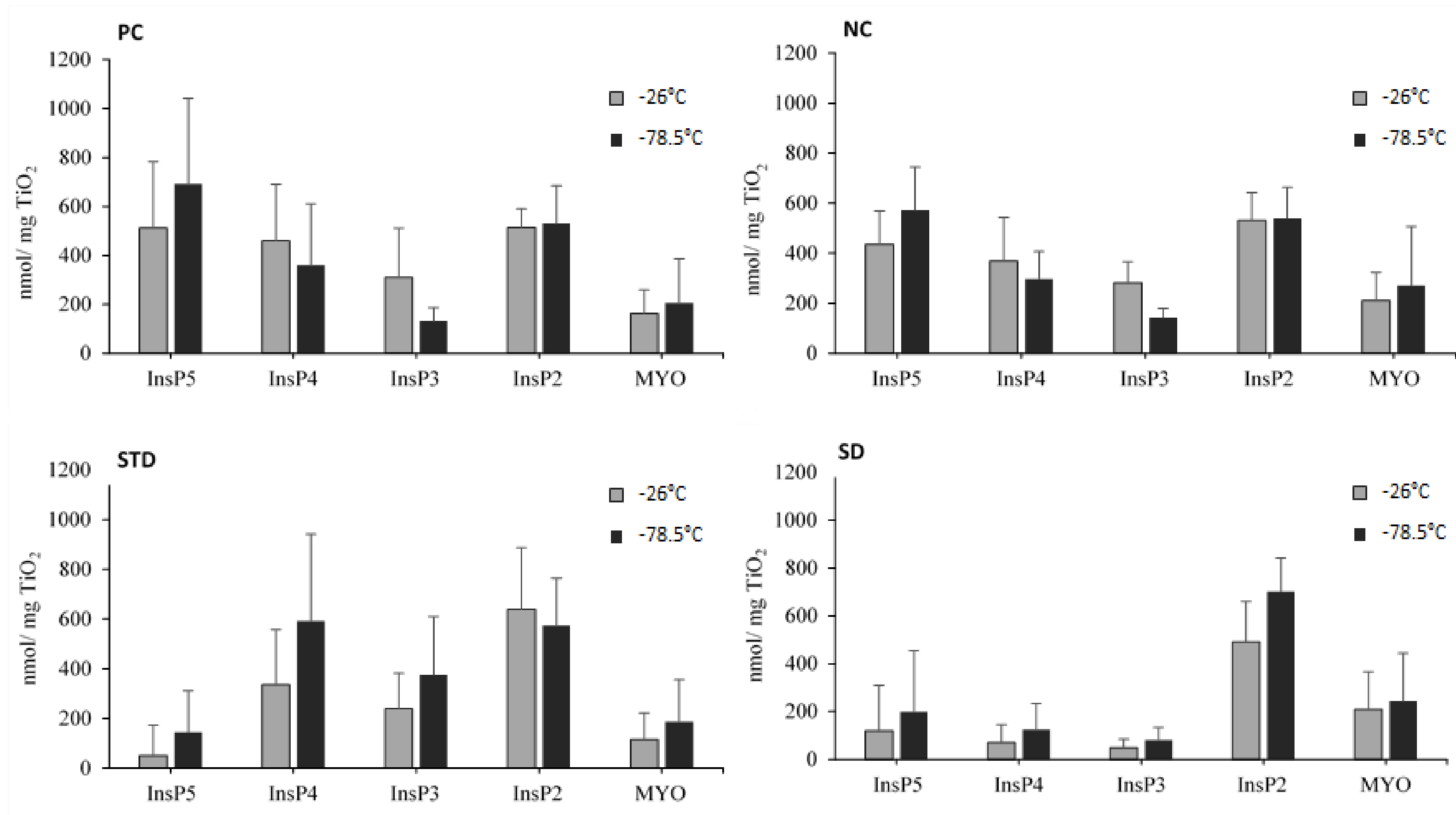

Figure 5.2 Interactive effects of freezing temperature and diet on gastric inositol pentakisphosphate (InsP5), inositol tetrakisphosphate (InsP4), inositol trisphosphate (InsP3), inositol bisphosphate (InsP2) and myo-inositol (MYO) concentration (nmol/mg TiO_2).

Values are means of 10 observations + SD.

5.5 Discussion

The present study demonstrates that the inositol phosphate composition of pig gastric digesta is influenced by both freezing temperature and time taken to freeze the sample following collection. It should be noted that although gastric phytate hydrolysis was measured in the present study, these data do not provide an accurate measure of post-prandial gastric phytate hydrolysis, as time between feed intake and sampling was not controlled. Nevertheless, gastric phytate hydrolysis provides a suitable means of assessing the influence of time and freezing temperature on phytate degradation and has thus been included in this thesis.

Phytase induced phytate hydrolysis is a time-dependent process which in the pig is often limited by the retention time of the digesta in the stomach (Blaajberg et al., 2011). Thus, it was unsurprising to learn that this enzyme catalysed reaction continues in the gastric digesta after sample collection from pigs fed diets with added phytase. Perhaps a more surprising discovery was that phytate continues to be hydrolysed in the gastric digesta after sampling from pigs fed diets with no added phytase. This is contrary to the results of Kemme et al. (2006), who found almost no phytate was degraded in the stomach of pigs when fed a low phytase diet. Both wheat and barley are known to possess high levels of phytase activity (Eeckhout and de Paepe, 1994; Viveros et al., 2000); although, this is generally lost during the pelleting process. In this study, dietary phytase analysis revealed that the inactivation of the intrinsic phytase activity during the pelleting process was more effective in the NC than in the PC (<50 vs 85 FTU/kg) treatment. It is possible that intrinsic phytase activity was responsible for the phytate hydrolysis occurring in the gastric digesta of pigs fed the non-phytase supplemented diets. However, this seems unlikely, particularly in the NC diet for which the analysed phytase activity was below the limit of detection (< 50 FTU/kg). The possibility of an additional source of microbial phytase cannot be excluded. Several studies have shown that some species of lactic acid bacteria reside within the stomach of pigs (Cranwell et al., 1976; Tannock, 1992; Hojberg et al., 2003; Chow and Lee, 2006); however, the ability of these to produce extracellular phytase is a contentious issue (Reale et al., 2007). Some have found that certain species of lactic acid bacteria are capable of producing extracellular phytase (Sreeramulu et al., 1996), whilst others have not (Fredrikson et al., 2002; Palacios et

al., 2005). Nevertheless, if indeed microbes residing in the stomach of the pig are capable of producing phytase, their quantitative importance for *in vivo* phytate hydrolysis is untested and would be worthy of further study.

Another key finding of the current study is that freezing temperature has a significant influence on gastric phytate hydrolysis; samples frozen at -26 °C recovered less analysed phytate than those frozen at -78.5 °C. This suggests that phytate hydrolysis continues during the freezing process, and digesta must be frozen rapidly in order to terminate the enzyme catalysed reaction and prevent possible erroneous calculation of *in vivo* phytate hydrolysis. There was a significant diet by freezing temperature interaction on gastric phytate hydrolysis, as freezing at -26 °C recovered proportionally less phytate than that frozen at -78.5 °C in diets devoid of added phytase than in diets with added phytase. This interaction may reflect the fact that digesta samples used in this study likely reflect digesta at different stages of digestion. As a result, some of the pigs receiving a diet with added phytase, most likely at the later stages of gastric digestion, had completely degraded (i.e. no phytate detected) or were close to completely degrading the ingested phytate. Logically, the choice of freezing temperature will have little to no effect on phytate hydrolysis in these samples. Gastric phytate hydrolysis was much lower in pigs fed diets devoid of added phytase, and thus the potential scope for continued phytate hydrolysis during processing in such samples is much greater. It would be interesting to see if this interaction would stand if time from feeding to sampling had been controlled and all pigs were at an early stage in gastric digestion.

The gastric lower inositol phosphate and MYO profiles in diets without added phytase were almost identical. This suggests that the phytate in these diets is likely being degraded by the same mechanism, through similar phytases with similar specificities and reaction kinetics. Supplementing the NC diet with a standard dose of phytase changed the inositol phosphate composition of the digesta, with reductions in $InsP_5$ and concurrent increases in $InsP_4$ and $InsP_3$ content. Adding a super-dose of phytase to the diet effectively diminished the build of $InsP_4$ and $InsP_3$, resulting in more complete phytate hydrolysis. These findings are consistent with the ileal inositol phosphate data from the previous chapter and confirms that $InsP_6$ and $InsP_5$ are the primary substrates for standard doses of this *E. coli* phytase, whereas $InsP_4$ and $InsP_3$ phytate esters are also targeted upon the addition of a super-dose.

Interestingly, the more complete phytate hydrolysis associated with the standard phytase dose frozen at -26 °C and the super-dosing diets was not met with clear changes in $InsP_2$ or MYO concentration. Furthermore, the total sum of measured phytate hydrolysis products in the digesta of these pigs was lower than that of the controls, which likely indicates $InsP_1$ formation. Unfortunately, it was not possible to measure $InsP_1$ using the inositol phosphate quantitation methodology used in the present study. $InsP_1$ is considered to be a transient compound that is present in trace amounts in ileal digesta, as it is rapidly dephosphorylated by endogenous phosphatases or possibly absorbed directly by the small intestine (Adeola and Cowieson, 2011). Thus, it is frequently dismissed as having minor quantitative importance in ileal digesta; however, this study would suggest that this is not the case in gastric digesta. Moreover, these results indicate that this particular *E.* coli phytase is unable to completely dephosphorylate phytate to MYO, which is in agreement with the current view that most microbial phytases are not capable of completely dephosphorylating phytate. This is thought to be due to the axial arrangement of the phosphate group positioned at C2 on the inositol nucleus (Wyss et al., 1999). However, the MYO data presented in the previous chapter clearly demonstrate that supplementing this phytase increased MYO levels in the ileal digesta of the pigs. Thus, it seems probable that the phytase degraded ingested phytate to lower, more soluble, inositol phosphate esters ($InsP_2$ and $InsP_1$) in the gastric phase, which were then available to endogenous luminal and mucosal phosphatases in the intestine for further dephosphorylation to MYO.

It can be concluded that significant phytate hydrolysis occurs in the gastric digesta during collection and processing. In the current experiment, delaying the freezing of gastric digesta by just 5 min resulted in significant $InsP_6$ hydrolysis. Therefore, digesta should be processed as quickly as possible and frozen on dry ice in order to minimise post-collection phytate degradation and changes in the gastric inositol phosphate profile. At present, many papers fail to provide an adequate level of detail in their methodology with regards to the sampling and processing of digesta prior to inositol phosphate quantitation, making it difficult for one to draw firm conclusions and draw cross-study comparisons. Clearly, differences in sample processing methodologies can have a large impact on the digesta inositol phosphate composition and it seems highly likely that such differences have contributed to the variable

outcomes surrounding the effect of phytase in the literature. This highlights the necessity for the implementation of standardised methodology in phytate degradation studies.

Chapter 6
Effect of Phytase and Iron Supplementation on Weaner Pig Growth Performance, Haematological Status, Mineral Availability and Inositol Phosphate Degradation

6.1 Abstract

This experiment set out to determine the effect of a super-dose of phytase on the Fe status of weaner pigs fed a low-Fe wheat-SBM based diet. A total of 234 mixed sex pigs were weaned at 28 ($\pm$ 4 d) d of age and blocked according to weight, sex and litter of origin. Dietary treatments were arranged as a 3 x 2 factorial and included a low-Fe basal wheat-SBM diet supplemented with three levels of microbial phytase (0, 500 or 2,500 FTU/kg) and two levels of Fe (50 [L-Fe] or 300 [H-Fe] mg/kg as $FeSO_4$). Each treatment was fed to 8 replicate pens (comprising 4 or 5 mixed sex pigs) for 20 d post-weaning. At the end of the study one pig from each pen (n = 48) was euthanised for the collection of whole blood, portal and peripheral plasma, ileal digesta, liver, M3 bone and duodenal mucosa.

Weaner pigs fed the L-Fe diet had a lower ADFI than those fed the H-Fe diet (P<0.01). ADG was influenced by a significant Fe x phytase treatment interaction (P<0.05); phytase increased ADG; however, the magnitude of the response was greater when added to the L-Fe diet. Supplementing the diet with 2,500 FTU/kg phytase improved weaner pig FCR (P<0.01) over those fed the non-supplemented phytase diet (1.23 vs 1.38), irrespective of dietary Fe concentration.

Weaners fed the L-Fe diet tended (P<0.1) to have a lower haemoglobin concentration than those fed the H-Fe treatment (10.0 vs 11.0 g/dl); however, neither of these treatments resulted in Fe deficiency anaemia. Supplemental phytase had no effect on weaner haemoglobin concentration (P>0.05). Pigs fed the L-Fe treatment were Fe deficient as demonstrated by the depleted Fe liver (P<0.001) and bone (P<0.05) concentrations, reduced plasma Fe (P<0.05) and ferritin (P<0.05), increased plasma transferrin (P<0.05), and increased duodenal DMT1 (P<0.05) and TFRC (P<0.001) gene expression. Phytase had no effect on any of the indices of Fe status (P>0.05).

Ileal phytate degradation was influenced by an Fe x phytase interaction (P<0.05) similar to that observed for ADG. In diets without added phytase, weaners receiving

the H-Fe diet degraded significantly more phytate by the terminal ileum than those on the L-Fe treatment. Whereas, in diets with added phytase, the dietary Fe concentration seemingly had no effect on ileal phytate degradation. Both doses of phytase reduced the amount of phytate in the digesta at the terminal ileum (P<0.001); however, the standard dose resulted in a small increase in $InsP_4$ and $InsP_3$ concentrations, whereas the super-dose prevented this build up (P<0.01), resulting in more complete phytate degradation. As a result, there was an increase in ileal MYO concentration (P<0.001) and a corresponding increase in portal (P<0.05) and peripheral plasma (P<0.01) MYO concentration in response to the 2,500 FTU/kg treatment.

Phytase increased bone (P<0.001) and plasma Mg (P<0.01) concentrations. Super-dosing further increased bone Mg content and also increased plasma Zn (P<0.01), bone Ca (P<0.05) and P (P<0.01) concentrations. In conclusion, feeding weaner pigs a wheat-SBM based diet containing 100 mg Fe/kg was not sufficient to maintain adequate Fe reserves. Adding a super-dose of phytase (2,500 FTU/kg) to this diet effectively improved weaner pig growth performance to a level comparable with those offered the high-Fe diet (320 mg/kg), but was not sufficient to prevent a decline in pig Fe status.

6.2 Introduction

Iron (Fe) is an essential micronutrient required by almost all forms of life for regular growth and metabolism. This trace element has a diverse array of biological functions in the body which include oxygen transfer, electron transport and DNA synthesis (Beard, 2001). Under physiological conditions Fe typically exists under one of two oxidation states: ferrous (Fe^{2+}) or ferric (Fe^{3+}). The two oxidation states are readily inter-changeable which makes Fe an appropriate cofactor for a number of enzymes involved in reduction and oxidation, such as the heme-enzymes, NADH-cytochrome reductase and xanthine oxidase.

In the body, unbound Fe is extremely cytotoxic as it readily reacts with cellular oxygen to form free radicals via the Fenton reaction (Jenkins and Kramer, 1988). Therefore, over 90% of total body Fe is incorporated into proteins with the remainder commonly associated with small organic molecules (Pond et al., 2005). Fe serves as a vital ligand for the binding of oxygen to hemeproteins, therefore, one of its primary biological

functions is to transport oxygen around the body. Accordingly, between 60 and 80% of the total body Fe is present in erythrocytes as haemoglobin (Hb) and up to 20% in muscle as myoglobin (Pond et al., 2005). Much of the remaining body Fe is stored as ferritin in the liver, kidney and spleen (Pond et al., 2005). Ferritin is an Fe storage protein that can hold up to 4,500 atoms of Fe in the ferric state (Kim et al., 2003). Fe stored within this complex is readily available for Hb synthesis, and can be transported in the blood plasma bound to the glycoprotein transferrin. Due to poor solubility, the body has a very limited capacity to excrete Fe and thus Fe homeostasis is predominantly regulated at the level of intestinal absorption (Hallberg and Hulthen, 2000). Iron is mainly absorbed in the duodenum via divalent metal transporter 1 (DMT1; Andrews, 1999). Expression of this transport protein is known to be negatively regulated by hepcidin, a peptide hormone produced in the liver in response to high ceullular Fe stores (Atanasiu et al., 2007).

Fe deficiency, as characterised by microcytic hypochromic anaemia, is the most common micronutritional deficiency in mammals (Lipinski et al., 2010). Piglets have low-Fe reserves at birth (~50 mg; Venn et al., 1947). In addition, sow milk is a poor source of Fe. Therefore, unless an external source of Fe is available, neonatal pigs are at particular risk of developing Fe deficiency anaemia. For many years pigs have been selectively bred for faster growth rates, as a result, it is not uncommon for a modern pig to increase its BW 4 to 5-fold in the first 3 weeks of life. This rapid growth rate and the corresponding increase in blood volume predispose the suckling pig to Fe deficiency anaemia. Suckling pigs require around 15 mg of Fe per day, of which only 7% is provided by the sow's milk (Venn et al., 1947). Increasing the sow's Fe intake during late gestation has been shown to have no effect on piglet Fe reserves at birth or on the concentration of Fe in the sow's milk (Venn et al., 1947; Veum et al., 1965; Ducsay et al., 1984). Therefore, it is common practice for pigs reared indoors to receive an intramuscular injection of 150 to 200 mg Fe, usually in the form of Fe-dextran or gleptoferron, at around 3 days of age to maintain normal Hb concentrations through to weaning.

As with neonatal pigs, weaner pigs also have a high growth rate and thus a rapid increase in blood volume. According to the NRC (2012) pigs at weaning require 80 mg Fe/kg DM; however, this value was set based on the work of Pickett et al. (1960), which was conducted over half a century ago. Decades of selection for faster growing

pigs have likely resulted in changes in the pigs' post-weaning Fe requirement. A more recent study using a comparatively modern pig breed suggested that typical weaner diets should be supplemented with at least 100 mg of an available Fe source/kg in order to maintain adequate Fe stores and prevent the onset of Fe deficiency anaemia (Rincker et al., 2004). The minimum level of Fe supplementation to weaned pigs of 10 to 30 kg BW according to the BSAS (2003) is 120 mg/kg air dry feed. As the animal ages the requirement for Fe declines due a reduction in the rate of blood volume expansion and an increase in feed intake (NRC, 2012).

Pig diets typically contain sufficient Fe to satisfy the requirement the animal, particularly in the latter stages of production. However, there is limited information available on the bioavailability of Fe from the different feedstuffs. Moreover, Fe digestibility is known to be influenced by a multitude of factors including: animal Fe status, dietary mineral concentration (notably Fe, Zn, and Ca; (Hallberg et al., 1992; Camara et al., 2007)), duodenal pH and the presence of iron absorption enhancers (such as ascorbate and citrate; Teucher et al., 2004) or inhibitors (such as polyphenols and tannins; Brune et al., 1989). Therefore, pig diets are frequently supplemented with a highly available inorganic source of Fe such as ferrous sulphate ($FeSO_4$) or ferric chloride ($FeCl_3$). Such sources of Fe are inexpensive and have been shown to be an effective means of preventing Fe deficiency anaemia (Ammerman and Miller, 1972), and therefore, are commonly added to pig diets in excess of requirement.

The primary determinant of Fe availability in grain based diets is phytate concentration (Bohn et al., 2008). As with other cationic ions, phytate has a high capacity to chelate Fe, and in doing so form an insoluble complex that is refractory to digestion and absorption (Tang et al., 2006). Several studies have shown that phytate is a contributing factor in the aetiology of Fe deficiency in humans (Reddy et al., 1996; Hurrell et al., 2003; Koreissi-Dembele et al., 2013). Phytate is a ubiquitous component of pig diets and is typically present in concentrations of between 7 to 10 g/kg. Although phytase is commonly added to pig diets at a rate of 500 FTU/kg to breakdown phytate, the results of the previous studies have demonstrated that at this dose a large portion of the phytate remains intact. In addition, most of the degraded phytate is broken down to $InsP_4$ and $InsP_3$. This is pertinent as the recent in vitro study of Yu et al. (2012) demonstrated that the lower phytate esters $InsP_5$, $InsP_4$ and $InsP_3$ still have 73, 35 and 30% of the binding potential to Fe of $InsP_6$. In their study,

degradation to $InsP_2$ or $InsP_1$ was necessary for alleviating the inhibitory effect of phytate on Fe availability. Based on these data, it seems likely that at standard doses of phytase the potent Fe inhibitor, phytate, is being converted into another Fe inhibitor in $InsP_4$ or $InsP_3$.

The results of Chapters 4 and 5 have shown that at super-dosing levels phytase targets the $InsP_4$ or $InsP_3$ phytate esters, thus preventing the build-up of these esters seen with standard phytase doses. Therefore, it seems plausible that super-doses of phytase may provide an effective means of improving the bioavailability of phytate-bound Fe and thus a useful tool in the prevention of Fe deficiency induced anaemia in young pigs.

6.2.1 Study Aims

This study set out to determine if a phytase super-dose could be used to improve the performance and Fe status of weaner pigs fed a low-Fe diet. In addition, the effects of both Fe and phytase supplementation on ileal $InsP_{2-6}$ and MYO concentration, plasma MYO, mineral availability and intestinal nutrient transporter gene expression were assessed.

6.2.2 Hypotheses

- Super-dosing phytase will improve the performance of weaner pigs fed the low-Fe diet to a level comparable with those receiving the high-Fe diet.
- Super-dosing phytase will improve the haematological status of pigs fed the low-Fe diet to a level comparable with those fed the high-Fe diets.
- Dietary Fe concentration will have no effect on ileal $InsP_{2-6}$ or MYO concentration. The super-dose of phytase will reduce ileal $InsP_{2-6}$ concentration and increase MYO concentrations. Similarly the standard dose will decrease $InsP_{5-6}$ and increase both MYO concentrations and $InsP_{3-4}$ concentrations.
- Dietary Fe level will have no effect on plasma MYO concentration. Phytase will increase plasma MYO concentration, with the super-dose having a greater effect than the standard dose.
- Phytase will improve weaner tissue mineral concentration, with the super-dose having a greater effect than the standard dose. Weaners fed the high-Fe diet will have higher Fe tissue concentrations than those fed the low-Fe diet.

- Reducing the dietary Fe concentration will increase duodenal DMT1, TFRC and ZIP14 gene expression. Adding a super-dose of phytase to this diet will prevent the increase in DMT1, TFRC and ZIP14 gene expression.
- Phytase will reduce duodenal NaPi-IIb and calbindin D9k and increase SMIT2 gene expression, whereas dietary Fe concentration will have no effect.

6.3 Materials and methods

6.3.1 Experimental design and dietary treatments

Weaner pigs were offered one of six wheat-SBM based diets throughout this 20 d feeding experiment. Dietary treatments had a 2 x 3 factorial arrangement, with two levels of supplemental Fe (50 [L-Fe] or 300 mg/kg [H-Fe]) and 3 levels of supplemental phytase (0, 500 and 2,500 FTU/kg). Fe was added to the basal diet (analysed intrinsic Fe content of 39 mg/kg) in the form of the highly available ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$). The test levels of Fe were selected as a low and high concentration based on the BSAS Nutrient Requirement Standards (2003), which suggest that weaned pigs of 10 to 30 kg require 120 mg of supplemental Fe/kg. Diets were formulated to meet or exceed the nutritional requirements for all nutrients other than Fe (BSAS, 2003). To achieve this, a low-Fe (10 g/kg as fed) vitamin and mineral premix was used, which provided Fe at approximately 16% the rate of standard commercial premixes. TiO_2 was added to all diets at a rate of 5 g/kg as an inert dietary marker.

The basal diet was prepared as a single batch at Target Feeds Ltd (Shropshire, UK) with the vitamin and mineral premix added a rate to provide 50 mg of supplementary Fe/kg (as $FeSO_4 \cdot 7H_2O$). This mix was split into two and ferrous sulphate was added to one lot at 250 mg/kg to create the H-Fe (300 mg of supplemental Fe/kg) test diet. The two lots were each split into three and phytase was added as appropriate to create the six test diets. All diets were pelleted (3 mm) at a temperature of 62 $\pm$ 2 °C (as described in Section 2.2.2). The ingredient composition and the calculated nutrient levels of the test diets are presented in Table 6.1.

Table 6.1. Composition and nutrient specifications of experimental diets (%, as-fed basis)

Supplemental Fe (mg/kg)	50			300[1]		
Phytase (FTU/kg)	0	500	2500	0	500	2500
Ingredient						
Wheat	36.41	36.40	36.36	36.28	36.27	36.23
Soybean meal	22.41	22.41	22.41	22.41	22.41	22.41
Micronised barley	15.00	15.00	15.00	15.00	15.00	15.00
Whey powder	6.94	6.94	6.94	6.94	6.94	6.94
Fishmeal	5.50	5.50	5.50	5.50	5.50	5.50
Micronised wheat	5.00	5.00	5.00	5.00	5.00	5.00
Full fat soyabean	3.00	3.00	3.00	3.00	3.00	3.00
Soya oil	2.49	2.49	2.49	2.49	2.49	2.49
Vitamin-mineral premix[2]	1.25	1.25	1.25	1.25	1.25	1.25
Lignobond DD®[3]	0.63	0.63	0.63	0.63	0.63	0.63
TiO_2	0.50	0.50	0.50	0.50	0.50	0.50
Benzoic Acid	0.50	0.50	0.50	0.50	0.50	0.50
Dicalcium phosphate	0.39	0.39	0.39	0.39	0.39	0.39
Salt	0.20	0.20	0.20	0.20	0.20	0.20
L-lysine HCL	0.155	0.155	0.155	0.155	0.155	0.155
DL-Methionine	0.053	0.053	0.053	0.053	0.053	0.053
L-Threonine	0.047	0.047	0.047	0.047	0.047	0.047
$FeSO_4 \cdot 7H_2O$	-	-	-	0.125	0.125	0.125
Quantum Blue[4]	-	0.008	0.040	-	0.008	0.040
Pan-Tek® Robust[5]	0.015	0.015	0.015	0.015	0.015	0.015
Sucram®[5]	0.01	0.01	0.01	0.01	0.01	0.01
Nutrient content						
Net energy (MJ/kg)	10.2	10.2	10.2	10.2	10.2	10.2
Crude protein	21.9	21.9	21.9	21.9	21.9	21.9
Crude fibre	2.52	2.52	2.52	2.52	2.52	2.52
SID lysine	1.20	1.20	1.20	1.20	1.20	1.20
Calcium	0.72	0.72	0.72	0.72	0.72	0.72
Total P	0.66	0.66	0.66	0.66	0.66	0.66
Available P	0.40	0.40	0.40	0.40	0.40	0.40
Ca:P	1.09	1.09	1.09	1.09	1.09	1.09

[1] Fe was added to the basal diet as $FeSO_4$ at the expense of wheat to create the high-Fe test diet.

[2] Vitamin and trace mineral premix provided per kg of diet: 12,500 IU vitamin A, 2,000 IU vitamin D_3, 60 IU vitamin E, 4 mg vitamin K, 4.2 mg thiamine (B_1), 5.6 mg riboflavin (B_2), 5 mg pyridoxine (B_6), 50 µg cyanocobalamin (B_{12}), 20 mg pantothenic acid, 40 mg nicotinic acid, 150 µg biotin, 1 mg folic acid, 50 mg Fe as $FeSO_4$, 160 mg Cu, 2.2 mg I, 62 mg Mn, 0.3 mg Se, 100 mg Zn

[3] Lignobond DD® is a pellet binder (100% lignosulphonate)

[4] Phytase was added to the basal diet at the expense of wheat to create the 500 and 2,500 FTU/kg test diets respectively.

[5] Pan-Tek® Robust is a flavouring palatant.

[5] Sucram ® is an artificial sweetener.

6.3.2 Animals and management

Two hundred and thirty-four pigs were weaned onto trial at 28 $\pm$ 4 d of age (mean initial BW $\pm$ SE 7.6 $\pm$ 0.16 kg). Treatments were replicated eight times, with each replicate pen consisting of 5 or 4 mixed sex pigs (7 reps of 5 pigs/pen and 1 rep of 4 pigs/pen). Within replicate, pens were balanced for weight, gender and litter of origin. Pigs were housed in a weaner-grower facility which consisted of 8 identical rooms each comprising 16 fully slatted floored pens (135 x 155 cm); 8 either side of a central passageway. The initial room temperature was 29 $\pm$ 2 °C and this was gradually reduced to 22 $\pm$ 2 °C over the course of the 20 d experiment. Each pen was equipped with a multi-space trough feeder, two nipple drinkers and a ball on chain. Feed and water were provided *ad libitum* throughout the study. The mean analysed Fe concentration of the drinking water throughout the experiment was 63 µg/l, and thus Fe contribution from water intake was considered negligible.

Prior to the commencement of the experiment pigs received an intra-muscular injection of 200 mg of Fe as gleptoferron within the first three days of life, as is standard Spen Farm practise (see Section 2.2.1). However, to ensure low body Fe reserves at the commencement of the experiment, precautions were taken to ensure pigs had minimal access to exogenous Fe sources in the farrowing house; pigs had no access to creep feed, sow faeces was cleared twice daily, and pigs were separated from the sow as she was fed to prevent access to sow feed.

Pigs were weighed individually at the beginning of the experiment and then again on d 7, 14 and 20 for the determination of pen ADG. Health and faecal scores were recorded daily, as described in Section 2.2.3. Troughs were weighed daily for the determination of pen feed intake and FCR. Both ADFI and FCR were adjusted for pigs taken off trial as described in Section 3.3.2. Samples of the drinking water were collected weekly throughout the experiment. At the end of the 20 d experiment, 48 mixed sex pigs (1 per pen) were slaughtered via captive bolt penetration (death confirmed by pithing) after sedation with an intra-muscular injection of azaperone (4 mg/kg BW; Stresnil, Elanco, Hampshire, UK). Dissections took place over two consecutive days.

6.3.3 Sample collection

Following the confirmation of death, portal and peripheral blood were collected into heparinised Vacutainers as described in Section 2.4.1. Two subsamples of the heparinised peripheral whole blood were collected prior to plasma separation; one for immediate haematocrit (Hct) analysis and the other frozen at -20 °C for subsequent Hb analysis. The two Vacutainers were centrifuged according to the procedure outlined in Section 2.4.1 for the collection of portal and peripheral plasma. Digesta were collected from the terminal ileum and frozen at -20 °C (Section 2.4.2). Mucosal scrapings from duodenal tissue (60 cm distal to the pyloric sphincter) were collected into Trizol and snap frozen in liquid nitrogen, as described in Section 2.4.4. The whole liver was removed from the carcass and weighed to the nearest 0.1 g. A 3 x 3 cm sample from the left lobe of the liver was collected, rinsed with ice cold PBS to remove blood contamination, wrapped in tin foil and snap frozen in liquid nitrogen. The M3 bone was collected from the right foot as described in Section 2.4.3.

6.3.4 Laboratory analysis

Feed samples were sent to ESC for phytase and phytate analysis (Section 2.5.3), and to Sciantec Analytical Services Ltd (Cawood, UK) for crude protein, crude fibre, and total fat analysis. Feed and digesta were analysed for DM (Section 2.5.2), minerals (Ca, P, Mg, Na, K, Mn, Cu, Fe and Zn; Section 2.5.7 and 2.5.8), $InsP_{2-6}$ (Section 2.5.11), MYO (Section 2.5.10), and TiO_2 (Section 2.5.4) in triplicate and duplicate respectively. The total Fe content of the drinking water was measured by ICP-OES (Section 2.5.8).

Both portal and peripheral plasma were analysed for minerals (Ca, P, Mg, Na, K, Fe, Cu and Zn) including total Fe by ICP-OES (Section 2.5.7.3), and for MYO by HPLC (Section 2.5.9.2). Commercially available Sandwich ELISA assay kits were used for the quantitation of peripheral plasma transferrin (#E-EL-P1384; Elabscience Biotechnology Co. Ltd., China) and ferritin (#SEA518Po; Cloud-Clone Corp. Business Co. Ltd., China) according to manufacturer's instructions. Plasma was first diluted 1:10 and 1:400,000 with DI water prior to ferritin and transferrin analyses respectively. All plasma transferrin and ferritin analyses were performed in duplicate.

Whole blood were analysed for Hct within 30 min of collection using the microhaematocrit method. In brief, a heparinised microcapillary was filled

(approximately ¾ full) with blood, sealed with clay, and spun at 12,000 rpm in a microhaematocrit (IEC Model MB, Damon, USA) for 5 min. The haematocrit was measured using a Microhaematocrit Tube Reader (Hawksley, W. Sussex, UK). Hb content of the heparinised whole blood were analysed colourimetrically using Drabkin's Reagent (#D5941; Sigma Chemical Co.) according the manufacturer's instructions. The Hb standard used was Haemoglobin Porcine (#H4131, Sigma Chemical Co.) and the samples were read at 540 nm using a Jenway 6300 spectrophotometer (Bibby Scientific Ltd., Staffordshire, UK).

Liver DM was determined after drying at 100 °C for 24 h in a convection oven, as described in Section 2.5.2. The organic content of the liver sample was removed following combustion in a muffle furnace at 600 °C for 16 h. The Fe, Cu, Zn and Mn content of the resulting ash was determined by ICP-OES (Section 2.5.8) after dissolution in 10 ml of 5M HCl and filtration through a Whatman 541 filter paper.

Bone measurements, fat free dry weight and ash, P, Ca, Mg, Zn and Fe content were determined according to the methods described in Sections 2.4.3 and 2.5.6.

The relative gene expression of selected nutrient transporters (Table 6.2) in the duodenal mucosa was determined by qPCR. Total RNA extraction, cDNA synthesis, and qPCR were performed as described in Section 2.5.12. The stability of three commonly used reference genes (*ACTB, HMBS* and *HPRT*) was assessed using the geNorm function of qbasePLUS (Biogazelle). The most stable combination of reference genes tested was all three (M = 0.42, CV = 0.15), therefore, the geometric mean of these three reference genes was used as the normalisation factor for all genes of interest (GOI). All primers were purchased from Bio-Rad Ltd as predesigned PrimePCR™ Assays, with the exception of *DMT1* and *SMIT2*, for which primers were designed using the NCBI Primer-Blast tool (Ye et al. 2012) and purchased as a custom PrimePCR™ Assay. Primer efficiency was calculated for all PrimePCR™ Assays from a standard curve generated from a serially diluted (1:10) pooled cDNA mix as described in Section 2.5.12.3.

Table 6.2. Selected genes for nutrient transporter gene expression analysis

Gene name	Symbol	Function	Unique Assay ID	Amplicon size (bp)	Efficiency (%)
Reference genes					
Beta-actin	*ACTB*	Actin synthesis	qSscCED0016579	110	97
Hydroxymethylbilane synthase	*HMBS*	Porphyrin metabolism	qSscCID001281	116	104
Hypoxanthine phosphoribosyltransferase 1	*HPRT1*	Purine synthesis	qSscCID0002342	100	110
Nutrient transporter GOI					
Na-dependent phosphate transporter 2b	*NaPi-IIb*	Phosphate transporter	qSscCID0011723	108	98
Calbindin D9k	*S100G*	Calcium binding protein D9k	qSscCED0015930	113	100
Divalent metal transporter 1	*DMT1*	Iron importer	N/A[1]	195	104
Transferrin receptor	*TFRC*	Cellular iron uptake	qSscCED0019652	93	100
Zrt- and Irt-like protein 14	*ZIP14*	Iron and zinc transporter	qSscCED0017928	119	104
Na/myo-inositol transporter 2	*SMIT2*	MYO transporter	N/A[2]	88	97

[1] *DMT1* primer sequence: forward (5'to 3') AAGGTTCCGCGAATTATCCT, reverse (5' to 3') TAGCTTCCGCAAGCCATACT.
[2] *SMIT2* primer sequence: forward (5'to 3') GTTTACTCGCCATGACCCCA, reverse (5' to 3') TGGTGTCCCGTTCTGAGAGA.

6.3.5 Calculations and statistical analysis

Apparent ileal nutrient digestibility was calculated using the analysed nutrient values (Table 6.3) according to the digestibility calculation described in Section 2.6.

Blood mean corpuscular haemoglobin concentration (MCHC) was calculated according to the following calculation: MCHC (g Hb/dl) = [Hb (g/dl) × 100]/ Hct (%).

Data were analysed as a two-way ANOVA using the GLM procedure of SPSS Statistics (version 22.0, SPSS Inc., Chicago IL, US). The pen mean served as the experimental unit for all growth performance analyses, whereas the individual pig served as the experimental unit for all other analyses. Data were first tested for homogeneity of variance and normality using the Levene's test and the Kolmogorov-Smirnov test respectively. Non-normal data or data displaying heteroscedasticity were log10 transformed prior to analysis. The statistical model included the effects of block (rep), Fe, Phytase and Fe x phytase interactions. Non-significant interactions were removed from the model and the main effects were analysed individually. Slaughter BW was added to the model as a covariate for all bone and liver analyses. A Pearson's Product-Moment Correlation test was conducted to test for associations between plasma MYO and weaner pig ADG. Differences were classed as significant if $P<0.05$ or as a trend if $P<0.10$. Significantly different means were separated using the Tukey's post-hoc test. Data are expressed as least-square means along with the pooled SEM.

6.4 Results

Table 6.3. Analysed phytase, nutrient and inositol phosphate composition of the experimental diets (as-fed basis)

Fe	L-Fe[a]			H-Fe[b]		
Phytase (FTU/kg)	0	500	2500	0	500	2500
Item						
DM (%)	88.60	88.80	88.70	88.80	88.60	88.80
Total fat (%)	5.16	5.03	5.18	5.05	5.18	4.88
Crude protein (%)	21.30	23.00	23.10	22.00	22.10	22.90
Crude fibre (%)	2.10	1.90	2.20	2.00	2.00	2.20
Ash (%)	5.50	5.80	5.80	6.00	6.10	5.80
Ca (%)	0.78	0.77	0.78	0.77	0.77	0.76
Total P (%)	0.59	0.59	0.60	0.59	0.61	0.61
Available P (%)	0.37	0.36	0.37	0.36	0.37	0.38
Phytate P (%)	0.22	0.24	0.23	0.23	0.24	0.23
Ca:P	1.32	1.30	1.30	1.30	1.27	1.25
Na (%)	0.18	0.23	0.27	0.20	0.24	0.26
K (%)	0.97	0.93	0.91	0.90	0.95	0.94
Mg (%)	0.17	0.16	0.16	0.16	0.16	0.17
Fe (mg/kg)	107	105	102	315	329	342
Mn (mg/kg)	82	84	90	86	93	97
Cu (mg/kg)	139	135	159	136	165	145
Zn (mg/kg)	169	156	167	136	146	161
Phytase (FTU/kg)	147	592	2230	116	543	2810
Inositol phosphate (nmol/g)						
$InsP_6$	9162	11053	9335	9833	10896	10547
$InsP_5$	1740	1491	1357	1822	1202	1724
$InsP_4$	218	92	296	173	261	192
$InsP_3$	251	175	223	279	202	218
$InsP_2$	ND[1]	ND	ND	ND	ND	ND
MYO	538	596	663	630	655	674

[1]ND = not detected.
[a] L-Fe = low iron (formulated 50 mg supplemental Fe/kg wet feed).
[b] H-Fe = high iron (formulated 300 mg supplemental Fe/kg wet feed).

6.4.1 Dietary analysis

The analysed nutrient values for the experimental diets are presented in Table 6.3. Measured phytase activity levels were close to expected and within an acceptable range when sampling and assay error were considered. Diets supplemented with 50 mg Fe/kg contained around 100 mg Fe/kg as fed, and were thus approximately 20 mg/kg below the recommended level as set by BSAS (2003). The H-Fe treatments

(supplemented with 300 mg Fe/kg) contained approximately 320 mg/kg Fe. All three low and high-Fe treatments were within an acceptable range of each other. Fat, crude-protein, crude-fibre, Ca, P and phytate content were similar between diets.

6.4.2 Pig health

Table 6.4. Effect of Fe and phytase treatment on average faecal scores

Dietary Fe	PHY (FTU/kg)	d 0-7	d 7-14	d 14-20	Overall
L-Fe	0	2.87	2.38	2.56	2.61
L-Fe	500	2.84	2.45	2.63	2.64
L-Fe	2500	3.04	2.41	2.63	2.69
H-Fe	0	2.93	2.34	2.56	2.61
H-Fe	500	3.00	2.41	2.58	2.67
H-Fe	2500	2.82	2.43	2.33	2.54
	SEM	0.10	0.09	0.09	0.05
Main effects					
Fe					
	100	2.92	2.41	2.61	2.65
	320	2.92	2.39	2.49	2.61
	SEM	0.06	0.05	0.05	0.03
PHY	0	2.90	2.36	2.56	2.61
	500	2.92	2.43	2.61	2.65
	2500	2.93	2.42	2.48	2.62
	SEM	0.07	0.06	0.06	0.04
P-value					
Fe		1.000	0.198	0.133	0.370
PHY		0.967	0.335	0.379	0.678
Fe x PHY		0.162	0.730	0.224	0.176

In the immediate post-weaning period general pig health was poor with many pigs displaying signs of post-weaning enteric disorder (PWED). The prevalence of post-weaning diarrhoea was high (diarrheic pools found on floor of 68% of the pens on trial; not treatment related, $P>0.05$). Consequently, on d 8 a three day course of amoxicillin antibiotic batch medication was initiated (Citramox; 40mg/kg BW), which was added to the drinking water. Following this course of treatment, pig health appeared to quickly pick up. One pig died during the experiment (cause unknown) and 4 pigs were taken off trial due to weight loss; two from the L-Fe + 2,500 FTU/kg

treatment, 1 from the H-Fe + 500 FTU/kg treatment and 1 from the H-Fe + 0 FTU/kg treatment. Table 6.4 displays the average fecal scores for days 1 to 7, 7 to 14, 14 to 20 and 1 to 20 for each of the dietary treatments. Although the average faecal scores were relatively high throughout the 20 d trial (mean $\pm$ SE = 2.6 $\pm$ 0.02), these were not influenced by dietary treatment (P>0.05).

6.4.3 Growth performance

The effect of Fe and phytase on weaner pig growth performance is presented in Table 6.4. Weaner pigs receiving the L-Fe diet had a lower ADFI throughout the experiment compared to those receiving the H-Fe diet (P<0.01). This increase in feed intake was most apparent in the final five days of the study in which pigs receiving the high Fe diet ate 52 g more a day (P<0.01) than those fed the low Fe treatment. Phytase supplementation at 2,500 FTU/kg tended to increase pig ADFI (P = 0.055), whereas supplementation at 500 FTU/kg had no effect.

Weaner pig ADG in the first 7 d of the trial was very low; however, those receiving the high-Fe diet had a significantly higher ADG than those fed the low-Fe diet (12 vs 28 g; P<0.05). Moreover, although adding phytase at 500 FTU/kg had no benefit on initial (d 1 to 7) weaner ADG, adding 2,500 FTU/kg significantly improved it by 21 g (P<0.05). In diets with no added phytase, overall pig growth rate was higher when fed the high-Fe treatment (P<0.05). Weaners fed the L-Fe diet with no supplementary phytase had the lowest ADG throughout the experiment and those fed the H-Fe diet with 2,500 FTU/kg of phytase had the highest (numerically). Adding a super-dose of phytase to the L-Fe diet improved weaner ADG to a level not dissimilar from the fastest growing pigs, whereas adding a standard phytase dose had a marginal but non-significant effect. The 2,500 FTU/kg dose of phytase appeared to numerically improve weaner pig performance irrespective of dietary Fe level; however, the standard phytase dose (500 FTU/kg) only influenced pig growth when supplemented to the L-Fe diet, thus resulting in a significant Fe x phytase interaction for ADG (P<0.05) and final BW (P<0.05). Furthermore, the varying ADG response to the phytase super-dose treatment is likely to have contributed to this interaction.

Table 6.5. Effect of Fe and phytase treatment on weaner pig growth performance[1]

	Fe (mg/kg)			PHY (FTU/kg)				100 mg Fe/kg			320 mg/ Fe/kg				*P*-value		
	100	320	SEM	0	500	2500	SEM	0	500	2500	0	500	2500	SEM	Fe	PHY	Fe x PHY
Start BW (kg)	7.5	7.6	0.03	7.6	7.6	7.5	0.03	7.5	7.6	7.5	7.6	7.6	7.6	0.05	0.116	0.466	0.965
Final BW (kg)	12.0	12.6	0.10	11.9	12.2	12.8	0.13	11.4^{a}	12.0^{ab}	12.7^{bc}	12.5^{bc}	12.5^{bc}	12.9^{c}	0.18	<0.001	<0.001	<0.05
ADFI (g)																	
d 0-7	125	140	4.1	129	129	139	5.0	121	121	132	136	136	147	5.8	<0.05	0.240	0.699
d 7-14	293	306	7.4	295	293	311	9.0	288	286	305	302	299	318	10.4	0.209	0.292	0.640
d 14-20	520	572	9.8	534	538	568	11.8	486	513	562	582	562	573	16.9	<0.01	0.100	0.052
Overall	294	318	5.3	300	299	319	6.5	288	287	308	312	311	331	7.6	<0.01	0.055	0.213
ADG (g)																	
d 0-7	12	28	5.42	13^{a}	13^{a}	34^{b}	6.6	5	5	26	20	20	42	7.6	<0.05	<0.05	0.225
d 7-14	275	289	10.3	258^{a}	278^{ab}	311^{b}	12.3	251	271	304	265	284	318	14.2	0.339	<0.05	0.300
d 14-20	402	468	13.1	409	433	463	15.9	376	400	430	442	466	496	18.9	<0.01	0.065	0.484
Overall	220	252	5.5	218^{a}	233^{a}	259^{b}	6.6	189^{a}	219^{ab}	253^{bc}	247^{bc}	246^{bc}	264^{c}	9.4	<0.001	<0.001	<0.05
FCR																	
d 0-7	7.22	8.79	4.51	5.91	4.89	13.22	5.52	5.13	4.11	12.43	6.70	5.68	14.00	6.38	0.807	0.515	0.220
d 7-14	1.11	1.09	0.03	1.17^{a}	1.10^{ab}	1.02^{b}	0.04	1.20	1.13	1.00	1.14	1.08	1.04	0.05	0.617	<0.05	0.609
d 14-20	1.32	1.25	0.03	1.32	1.29	1.24	0.04	1.36	1.32	1.28	1.29	1.25	1.21	0.05	0.174	0.419	0.778
Overall	1.34	1.28	0.03	1.38^{a}	1.32^{ab}	1.23^{b}	0.03	1.41	1.35	1.26	1.35	1.29	1.19	0.04	0.115	<0.01	0.250

[1]Data are means of 8 replicate pens of 5 or 4 mixed sex pigs.

Super-dosing phytase numerically improved weaner pig ADG by 7% when supplemented to the H-Fe diet; however, its effect was much greater when added to the L-Fe diet, with a significant 34% improvement in ADG (P<0.05).

There was no significant Fe x phytase interaction for pig FCR throughout this experiment (P>0.05). Supplementing the diet with a super-dose of phytase improved weaner pig FCR irrespective of Fe level (P<0.01). Those receiving the 500 FTU/kg treatment had an FCR not dissimilar from the 0 or 2,500 FTU/kg phytase treatments. This observed improvement in FCR in response to the 2,500 FTU/kg was most apparent throughout the second week of the study.

6.4.4 Ileal phytate hydrolysis and inositol phosphate and MYO concentration

Phytate degradation occurring within the gastrointestinal tract by the terminal ileum was influenced by an Fe x phytase interaction (P<0.05; Figure 6.1). Dietary Fe concentration had no effect on ileal $InsP_6$ degradation in pigs fed diets with added phytase. However, in diets devoid of supplementary phytase, Fe influenced $InsP_6$ degradation with those receiving the H-Fe diet degrading 34% more phytate than those fed the L-Fe diet (P<0.05).

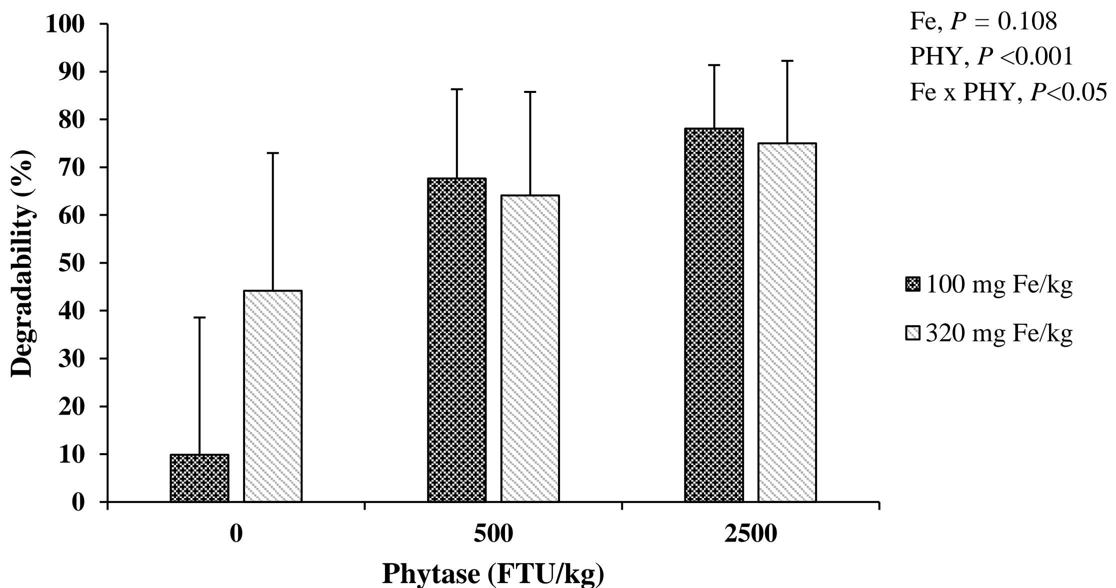

Figure 6.1. Effect of Fe and phytase on ileal InsP6 degradability (%)

Bars represent the mean of 8 replicate pigs ± standard deviation.

The effect of Fe and phytase on ileal inositol phosphate ($InsP_{2-6}$) and MYO concentration is presented in Table 6.5. Supplementary phytase reduced the concentration of $InsP_6$ in the digesta at the terminal ileum (P<0.001); however, there was no significant difference between the standard and super-dose treatments (P>0.05). As with the degradability data, there was a tendency (P = 0.059) for an Fe x phytase interaction on ileal $InsP_6$ concentration. Dietary Fe concentration had no influence on ileal $InsP_6$ concentration in diets with added phytase; however, in diets with no added phytase, the phytate concentration was higher when the dietary Fe level was low. This interaction was also true for ileal $InsP_5$ concentration (P<0.01).

Dietary Fe concentration had no effect on ileal $InsP_{2-4}$ or MYO concentration. Supplementing the diet with 500 FTU/kg of phytase resulted in a numerical increase in ileal $InsP_4$, whereas adding 2,500 FTU/kg significantly reduced ileal $InsP_4$ concentration (P<0.01). Similarly, 500 FTU/kg numerically increased $InsP_3$ whereas 2,500 FTU/kg decreased it; as a result, the difference between the standard and high phytase dose was significantly different (P<0.01). Phytase had no effect on ileal $InsP_2$ concentration (P>0.05), but super-dosing phytase did increase ileal MYO concentration from 266 to 673 nmol/mg TiO_2 (P<0.001).

Dietary Fe concentration had a significant effect on the proportion of $InsP_4$ in the ileal digesta of the weaner pigs (P<0.01). In pigs receiving the L-Fe diet $InsP_4$ accounted for 25% of the sum of phytate hydrolysis products. This increased to 31% upon increasing the dietary Fe concentration from ~100 to 320 mg/kg. $InsP_5$ and $InsP_4$ were the predominant phytate hydrolysis products in diets with no added phytase. Adding 500 FTU/kg to the diet resulted in a decrease in the proportion of $InsP_5$: $\sum InsP_{2-5}$ (P<0.001), a numerical increase in $InsP_4$:$\sum InsP_{2-5}$, and an increase in $InsP_3$: $\sum InsP_{2-5}$ (P<0.001). Adding 2,500 FTU/kg also reduced the proportion of $InsP_5$, but, in contrast to the standard dose, it reduced the proportion of $InsP_3$ and $InsP_4$ in the ileal digesta. As a result, there was a significant rise in $InsP_2$:$\sum InsP_{2-5}$ (P<0.001) with the 2,500 FTU/kg treatment.

Table 6.6. The effect of Fe and phytase on ileal inositol phosphate ($InsP_{2-6}$) and MYO concentration (nmol/mg TiO_2)

Dietary Fe	PHY (FTU/kg)	$InsP_6$	$InsP_5$	$InsP_4$	$InsP_3$	$InsP_2$	MYO	$InsP_5$: $\sum InsP_{2-5}$	$InsP_4$: $\sum InsP_{2-5}$	$InsP_3$: $\sum InsP_{2-5}$	$InsP_2$: $\sum InsP_{2-5}$
L-Fe	0	1584	312^{a}	240	131	218	232	0.37	0.25	0.14	0.24
L-Fe	500	720	130^{bcd}	319	234	332	426	0.15	0.31	0.22	0.32
L-Fe	2500	380	49^{d}	100	93	313	853	0.10	0.18	0.16	0.56
H-Fe	0	1060	212^{b}	302	162	232	312	0.25	0.32	0.17	0.26
H-Fe	500	774	144^{bc}	304	177	219	271	0.17	0.36	0.22	0.24
H-Fe	2500	495	64^{cd}	162	101	252	495	0.10	0.25	0.17	0.48
	SEM	156	21	44	30	39	107	0.03	0.03	0.02	0.03
Main effects											
Fe	L-Fe	895	164	220	153	291	503	0.21	0.25	0.17	0.37
	H-Fe	777	140	256	147	234	355	0.17	0.31	0.19	0.33
	SEM	83	12	25	17	25	74	0.02	0.02	0.01	0.02
PHY	0	1322^{a}	262^{a}	271^{a}	147^{ab}	225	266^{a}	0.31^{a}	0.29^{a}	0.16^{a}	0.25^{a}
	500	747^{b}	137^{b}	311^{a}	206^{a}	275	348^{a}	0.16^{b}	0.34^{a}	0.22^{b}	0.28^{a}
	2500	437^{b}	56^{c}	131^{b}	97^{b}	287	673^{b}	0.10^{b}	0.21^{b}	0.17^{a}	0.52^{b}
	SEM	102	14	31	21	31	92	0.02	0.02	0.01	0.02
P-value											
Fe		0.302	0.146	0.328	0.804	0.082	0.518	0.201	<0.01	0.260	0.050
PHY		<0.001	<0.001	<0.01	<0.01	0.244	<0.001	<0.001	<0.001	<0.001	<0.001
Fe x PHY		0.059	<0.01	0.618	0.419	0.274	0.135	0.062	0.896	0.568	0.189

[a-c] Means within column that do not share a common superscript are significantly different ($P<0.05$).

6.4.5 Plasma MYO

Table 6.7. Effect of Fe and phytase on portal and peripheral plasma MYO concentration (nmol/ml)

		Blood	
Dietary Fe	PHY (FTU/kg)	Portal	Peripheral
L-Fe	0	25.8	48.8
L-Fe	500	32.7	54.0
L-Fe	2500	38.9	67.2
H-Fe	0	24.6	42.5
H-Fe	500	31.5	56.2
H-Fe	2500	37.7	70.9
	SEM	3.5	6.8
Main effects			
Fe	L-Fe	32.5	56.7
	H-Fe	31.3	56.5
	SEM	2.4	3.7
PHY	0	25.2^{a}	45.6^{a}
	500	32.1^{ab}	55.1^{ab}
	2500	38.3^{b}	69.1^{b}
	SEM	3.0	4.6
P-value			
Sampling site[1]		<0.001	
Fe x sampling site		0.884	
PHY x sampling site		0.229	
Fe x PHY x sampling site		0.754	
Fe		0.727	0.975
PHY		<0.05	<0.01
Fe x PHY		0.584	0.553

[1] Paired portal vs peripheral analysis.
[a-c] Means within column that do not share a common superscript are significantly different (*P*<0.05).

Dietary Fe concentration had no influence on MYO levels in the portal or peripheral plasma in weaner pigs (*P*>0.05) at the end of the experiment (Table 6.6). Supplementing the diet with 2,500 FTU/kg significantly increased MYO concentration in both the portal (*P*<0.01) and peripheral (*P*<0.01) plasma. MYO levels were not influenced by an Fe x PHY interaction (*P*>0.05), and were approximately 1.7-1.9 fold higher in the peripheral plasma than the portal plasma (*P*<0.001). This is in contrast to the previous grower and finisher experiments in which MYO levels were higher in the portal than in the peripheral blood.

There was a moderate positive correlation between portal plasma MYO concentration and weaner pig ADG (r = 0.365, *P* = 0.012; see Figure A.4 in appendix). Peripheral MYO however, was not significantly correlated with pig growth rate (r = 0.211, *P* = 0.160; data not presented).

6.4.6 Bone characteristics

As demonstrated in Table 6.7, metatarsal size measurements were not influenced by Fe or phytase treatment (*P*>0.05), although there was a tendency for an Fe x phytase interaction with the 2,500 FTU/kg dose increasing midshaft width at the widest point when supplemented to a H-Fe diet (*P* = 0.057). Bone ash weight increased in response to the 2,500 FTU/kg treatment (1.05 vs 1.17 g; *P*<0.01). P (*P*<0.01), Ca (*P*<0.05) and Mg (*P*<0.001) concentrations in the bone were all influenced by phytase treatment. Supplementing the weaner diets with 2,500 FTU/kg significantly increased P, Ca and Mg content. Whereas, supplementing the diets with 500 FTU/kg had no effect on bone P, increased bone Ca to a level not significantly different from the 0 or 2,500 FTU/kg treatments, and significantly increased bone Mg, but not to the same extent as the 2,500 FTU/kg treatment.

Bone Fe content was influenced by dietary Fe concentration (*P*<0.05); weaner pigs fed the H-Fe treatment had 7.7 mg/kg more Fe in the bone than those fed the L-Fe treatment. There were numerical increases in bone Fe content in response to the 2,500 FTU/kg treatment; however, this effect was not significant. Bone Zn content was not influenced by Fe or phytase treatment.

6.4.7 Effect of Fe and phytase on liver mineral concentrations

Weaner pigs receiving the H-Fe diet tended to have heavier livers (*P*=0.056) than those fed the L-Fe diet (Table 6.8). This difference was significant when expressed as a percentage of BW (*P*<0.05). Liver Fe concentration was 67% higher in the H-Fe pigs compared with the L-Fe pigs (*P*<0.001). There was a significant Fe x phytase interaction for liver Cu and Mn concentrations (*P*<0.05). Increasing levels of phytase increased liver Cu in the L-Fe fed pigs, but had the reverse effect when added to the H-Fe diet. Likewise, phytase increased liver Mn content when added to the L-Fe diet; however, here, the level of phytase had no effect on liver Mn when added to the H-Fe diet. Liver Zn content was not influenced by Fe or phytase treatment (*P*>0.05).

Table 6.8. Effect of Fe and phytase treatment on weaner pig metatarsal bone traits

Dietary Fe	PHY (FTU/kg)	FFDW (g)*	Width widest (mm)*	Width narrowest (mm)*	Bone length (mm)*	Ash wt (g)*	Bone ash (% ffdw)	P (% ffdw)	Ca (% ffdw)	Mg (% ffdw)	Fe (mg/kg ffdw)	Zn (mg/kg ffdw)
L-Fe	0	2.95	14.89	9.14	51.10	1.04	36.5	6.48	13.94	0.281	74.6	100.6
L-Fe	500	2.99	15.10	9.19	52.10	1.05	36.8	6.61	14.19	0.296	76.3	96.7
L-Fe	2500	2.95	14.84	8.89	51.63	1.16	38.3	6.92	14.67	0.330	81.7	103.6
H-Fe	0	2.94	14.81	9.14	51.40	1.07	36.3	6.50	13.98	0.283	82.3	103.5
H-Fe	500	2.97	14.66	9.19	50.84	1.07	36.7	6.63	14.22	0.298	84.0	99.6
H-Fe	2500	2.94	15.43	8.89	51.00	1.19	38.1	6.94	14.70	0.332	89.4	106.4
	SEM	0.06	0.21	0.15	0.57	0.03	0.60	0.10	0.23	0.330	3.35	2.67
Main effects												
Fe	L-Fe	2.96	14.94	9.07	51.61	1.08	37.2	6.67	14.27	0.302	77.5	100.3
	H-Fe	2.95	14.97	9.08	51.08	1.11	37.0	6.69	14.30	0.304	85.2	103.2
	SEM	0.04	0.12	0.10	0.33	0.02	0.42	0.07	0.16	0.004	2.31	1.91
PHY	0	2.94	14.85	9.14	51.25	1.05[a]	36.4[a]	6.49[a]	13.96[a]	0.282[a]	78.4	102.1
	500	2.98	14.88	9.19	51.47	1.06[a]	36.7[ab]	6.62[a]	14.21[ab]	0.297[b]	80.1	98.2
	2500	2.94	15.13	8.89	51.31	1.17[b]	38.2[b]	6.93[b]	14.69[b]	0.331[c]	85.6	105.0
	SEM	0.05	0.15	0.13	0.40	0.02	0.52	0.09	0.20	0.005	2.86	2.37
P-value												
Fe		0.847	0.901	0.990	0.257	0.310	0.781	0.854	0.878	0.731	<0.05	0.285
PHY		0.831	0.371	0.201	0.926	<0.01	<0.05	<0.01	<0.05	<0.001	0.189	0.121
Fe x PHY		0.216	0.057	0.928	0.386	0.377	0.940	0.958	0.960	0.926	0.405	0.918

[a-c] Means within column that do not share a common superscript are significantly different (*P*<0.05).
*Slaughter weight covariate significant: fat-free dry weight (*P*<0.001); width widest (*P*<0.001); width narrowest (*P*<0.001); bone length (*P*<0.001); ash weight (*P*<0.001). The relationship between the covariate and the independent variables was positive in each case.

Table 6.9. Effect of Fe and phytase on liver mineral concentrations

					Mineral content, mg/kg DM				Mineral content, mg/liver			
Dietary Fe	PHY (FTU/kg)	Liver wt[1*] (g)	Liver wt[*] (% of BW)	Liver DM (%)	Fe	Cu	Zn	Mn	Fe	Cu	Zn[*]	Mn[*]
L-Fe	0	439	3.61	26.0	156.9	56.4^{ab}	164.6	11.25^{a}	17.09	6.47^{ab}	19.97	1.41
L-Fe	500	437	3.60	25.9	198.8	67.1^{ab}	165.4	13.50^{ab}	21.11	7.35^{ab}	20.40	1.55
L-Fe	2500	426	3.43	24.8	176.9	85.3^{a}	177.0	16.13^{c}	17.79	8.96^{a}	18.10	1.74
H-Fe	0	466	3.80	25.4	277.8	79.4^{ab}	188.7	12.86^{ab}	32.67	9.15^{a}	20.18	1.34
H-Fe	500	465	3.77	25.2	319.7	56.1^{ab}	186.4	13.38^{ab}	36.69	6.73^{ab}	20.61	1.48
H-Fe	2500	445	3.72	24.9	297.7	40.6^{b}	156.6	14.57^{bc}	33.37	4.47^{b}	18.30	1.68
	SEM	16.0	0.13	0.44	23.98	10.4	17.42	0.58	2.73	1.18	1.62	0.06
Main effects												
Fe	L-Fe	434	3.55	25.6	177.5	69.6	169.0	13.63	18.66	7.59	19.49	1.57
	H-Fe	459	3.76	25.2	298.4	58.7	177.2	13.60	34.24	6.79	19.70	1.50
	SEM	9.2	0.07	0.25	17.48	6.3	10.5	0.35	1.92	0.67	1.13	0.04
PHY	0	452	3.71	25.7	217.4	67.9	176.7	12.05	24.88	7.81	20.08	1.37^{a}
	500	451	3.68	25.6	259.3	61.6	175.9	13.44	28.90	7.04	20.51	1.52^{a}
	2500	436	3.58	24.9	237.3	62.9	166.8	15.35	25.58	6.72	18.20	1.71^{b}
	SEM	11.4	0.09	0.31	21.17	7.6	12.8	0.43	2.40	0.83	1.39	0.05
P-value												
Fe		0.056	<0.05	0.246	<0.001	0.217	0.550	0.961	<0.001	0.400	0.901	0.307
PHY		0.506	0.541	0.135	0.372	0.824	0.845	<0.001	0.435	0.623	0.460	<0.001
Fe x PHY		0.950	0.890	0.371	0.900	<0.05	0.333	<0.05	0.713	<0.05	0.614	0.159

[1]Expressed on a wet tissue basis.

[*]Slaughter weight covariate significant: liver weight (g; $P<0.001$); liver weight (% of BW; $P<0.01$); Zn (mg/liver; $P<0.05$); Mn (mg/liver; $P<0.05$). The relationship between the covariate and the independent variables was positive in each case, with the exception of liver weight (% of BW) which was negative.

6.4.8 Haematological parameters

Table 6.10. Effect of Fe and phytase on haematological status and plasma transferrin and ferritin concentration

Dietary Fe	PHY (FTU/kg)	Transferrin (mg/ml)	Ferritin (ng/ml)	Haematocrit (%)	Haemoglobin (g/dL)	MCHC[1] (g/dL)
L-Fe	0	46.53	57.04	25.11	9.66	37.90
L-Fe	500	45.93	67.26	26.45	9.98	38.84
L-Fe	2500	42.60	74.15	27.64	10.38	38.81
H-Fe	0	39.55	78.70	29.25	10.68	36.65
H-Fe	500	38.95	81.76	30.58	11.00	37.58
H-Fe	2500	35.62	81.98	31.77	11.40	37.55
	SEM	3.29	8.93	0.93	0.55	2.01
Main effects						
Fe	L-Fe	45.02	66.15	26.40	10.0	37.90
	H-Fe	38.04	80.81	30.53	11.0	37.26
	SEM	2.22	4.81	0.64	0.38	1.40
PHY	0	43.04	67.87	27.18	10.17	37.28
	500	42.44	74.51	28.51	10.49	38.21
	2500	39.11	78.06	29.70	10.89	38.18
	SEM	2.82	6.32	0.80	0.47	1.75
P-value						
Fe		<0.05	<0.05	<0.001	0.051	0.653
PHY		0.554	0.395	0.085	0.495	0.962
Fe x PHY		0.939	0.465	0.777	0.879	0.806

[1] MCHC = Mean corpuscular haemoglobin concentration.

Plasma transferrin concentration was higher in weaners fed the L-Fe than the H-Fe diet ($P<0.05$). Increasing the dietary Fe concentration from ~100 to 320 mg/kg resulted in a 22% increase in plasma ferritin concentration.

Weaners fed the H-Fe treatment had a higher Hct ($P<0.001$) and tended to have a higher Hb concentration ($P=0.051$) than those fed the L-Fe treatment. Phytase had no influence on plasma transferrin, ferritin, Hb concentration or MCHC, but did tend to increase Hct ($P=0.085$).

6.4.9 Plasma minerals

The effect of Fe and phytase treatment on plasma mineral concentration is presented in Table 6.10. Increasing the level of supplemental Fe added to the diet significantly increased Fe levels in both the portal ($P<0.01$) and peripheral ($P<0.05$) plasma, irrespective of phytase treatment. Phytase tended to increase peripheral P concentration ($P=0.075$), with the 2,500 FTU/kg dose having a greater effect than the 500 FTU/kg dose. Plasma Ca levels in the peripheral circulation were not influenced by phytase treatment, but were significantly lower in weaners fed the L-Fe than the H-Fe treatment ($P<0.05$). The level of dietary Fe had no effect on portal Mg concentration in diets with added phytase; however, in diets devoid of added phytase, increasing the dietary Fe content reduced the amount of Mg in the portal plasma. As a result, there was a significant Fe x phytase interaction ($P<0.01$) for portal plasma Mg. Peripheral plasma Mg, however, was not influenced by this interaction, but did increase in response to both the 500 and 2,500 FTU/kg treatments ($P<0.01$).

Cu concentration in the portal plasma was affected by an Fe x phytase treatment interaction ($P<0.01$). Supplementing the diet with 500 FTU/kg had no effect on Cu concentration; however, supplementing the diet with 2,500 FTU/kg increased Cu concentration in L-Fe diet, but had the reverse effect in the H-Fe diet and numerically decreased portal Cu. Zn levels in the peripheral plasma increased in response to the phytase super-dose treatment ($P<0.01$). The portal Zn data show a similar response although this was only a trend ($P=0.068$). Neither Na nor K concentrations in portal or peripheral plasma were influenced by either of the dietary treatments ($P>0.05$). With the exception of Zn, the concentration of all the minerals measured was higher in the portal than the peripheral plasma ($P<0.01$).

Table 6.11. Effect of Fe and phytase on portal and peripheral plasma mineral concentration (µg/ml)

		P		Ca		Mg		Na		K		Fe		Cu		Zn	
Dietary Fe	PHY (FTU/kg)	Port	Peri	Port	Peri	Port	Peri	Port	Peri	Port	Peri	Port	Peri	Port	Peri	Port	Peri
L-Fe	0	110.5	92.2	117.5	92.4	31.0^{a}	17.3	3625	3094	305	187	1.11	0.97	1.42^{ab}	1.24	0.59	0.57
L-Fe	500	112.8	99.4	116.2	93.6	23.7^{bc}	18.6	3584	3114	319	202	1.17	1.05	1.40^{a}	1.28	0.52	0.50
L-Fe	2500	122.1	107.8	117.4	91.9	27.0^{ab}	18.9	3581	3074	310	192	1.30	1.27	1.59^{b}	1.26	0.61	0.61
H-Fe	0	108.6	100.5	114.1	95.2	21.5^{c}	17.6	3561	3120	293	203	1.45	1.30	1.55^{ab}	1.29	0.63	0.59
H-Fe	500	110.9	102.4	112.8	96.4	23.7^{bc}	19.1	3520	3083	307	182	1.51	1.36	1.55^{ab}	1.34	0.57	0.53
H-Fe	2500	120.1	107.3	114.0	94.7	25.2^{bc}	20.0	3517	3077	298	182	1.64	1.45	1.44^{ab}	1.31	0.66	0.72
	SEM	4.9	5.1	2.2	1.4	1.58	0.53	51	39	20.1	9.5	0.12	0.15	0.07	0.06	0.03	0.04
Main effects																	
Fe	L-Fe	115.1	99.8	117.1	92.6	27.23	18.27	3597	3094	311.1	194.6	1.19	1.10	1.47	1.26	0.57	0.56
	H-Fe	113.2	103.4	113.6	95.4	23.47	18.89	3533	3094	299.6	188.8	1.53	1.37	1.51	1.31	0.62	0.62
	SEM	3.3	2.7	1.6	1.0	0.88	0.30	36	21	14.2	5.0	0.08	0.09	0.04	0.04	0.02	0.02
PHY	0	109.6	96.3	115.8	93.8	26.22	17.44^{a}	3593	3107	299.3	195.5	1.28	1.14	1.49	1.26	0.61	0.58^{a}
	500	111.8	100.9	114.5	95.0	23.71	18.85^{b}	3552	3098	312.7	191.8	1.34	1.21	1.48	1.31	0.54	0.52^{a}
	2500	121.1	107.5	115.7	93.3	26.11	19.44^{b}	3549	3076	303.9	186.7	1.47	1.36	1.52	1.29	0.63	0.67^{b}
	SEM	4.2	3.4	1.9	1.2	1.08	0.38	44	26	17.4	6.3	0.11	0.11	0.05	0.05	0.03	0.03
P-value																	
Sampling site		<0.001		<0.001		<0.001		<0.001		<0.001		<0.01		<0.001		0.852	
Fe		0.682	0.344	0.130	0.049	<0.01	0.127	0.219	0.983	0.569	0.459	<0.01	<0.05	0.443	0.383	0.132	0.059
PHY		0.115	0.075	0.872	0.576	0.174	<0.01	0.735	0.652	0.859	0.760	0.414	0.290	0.822	0.810	0.068	<0.01
Fe x PHY		0.298	0.664	0.483	0.745	<0.01	0.726	0.441	0.740	0.188	0.118	0.938	0.885	<0.01	0.242	0.236	0.342

Table 6.11 continued

	P	Ca	Mg	Na	K	Fe	Cu	Zn
P-value	Port Peri	Port Peri	Port Peri	Port Peri	Port Peri	Port Peri	Port Peri	Port Peri
Fe x sampling site	0.354	0.056	<0.01	0.409	0.221	0.213	0.994	0.574
PHY x sampling site	0.952	0.831	0.165	0.963	0.620	0.789	0.687	0.245
Fe x PHY x sampling site	0.127	0.295	<0.01	0.598	0.251	0.689	0.370	<0.10

6.4.10 Apparent ileal DM and mineral digestibility

Apparent ileal DM digestibility was not influenced by either Fe or phytase treatment (*P*>0.05; Table 6.11). Weaners offered the H-Fe diet had a higher Fe AID then those on the L-Fe treatment (*P*<0.001). Phytase supplementation at 2,500 FTU/kg improved P digestibility by 12.6% compared to the non-phytase supplemented diet (*P*<0.05). Supplementing the diet with 500 FTU/kg resulted in a slight increase in apparent P digestibility, to a level not dissimilar from the 0 or 2,500 FTU/kg diets. There was a trend for increasing doses of phytase to improve Na digestibility (*P*=0.083). Apparent ileal Ca, Mg, K, Cu and Zn digestibility was not affected by either Fe or phytase treatments (*P*>0.05).

Table 6.12. Effect of Fe and phytase on apparent ileal DM and mineral digestibility (%)

Dietary Fe	PHY (FTU/kg)	DM	Ca	P	Mg	Na	K	Fe	Cu	Zn
L-Fe	0	65.1	54.9	46.7	-21.5	-314	94.6	-28.03	-26.9	-39.2
L-Fe	500	64.5	58.9	54.7	-15.1	-256	94.2	-33.92	-43.2	-55.6
L-Fe	2500	67.3	65.2	59.3	-34.9	-196	94.1	-44.51	-49.0	-26.7
H-Fe	0	65.1	50.9	47.6	-12.6	-289	94.7	-2.06	-46.4	-22.9
H-Fe	500	64.4	55.0	55.6	-6.2	-232	94.3	-7.95	-30.3	-30.3
H-Fe	2500	66.0	61.2	60.2	-26.0	-171	94.2	-18.54	-44.0	-25.9
	SEM	2.7	4.5	3.9	9.6	45.2	0.7	7.45	9.5	11.9
Main effects										
Fe	L-Fe	65.6	59.7	53.6	-23.8	-255	94.3	-35.49	-39.7	-40.5
	H-Fe	65.2	55.7	54.5	-15.0	-231	94.4	-9.51	-40.2	-26.4
	SEM	1.4	3.2	2.8	6.9	31.0	0.5	5.24	5.5	6.4
PHY	0	65.1	52.9	47.2[a]	-17.1	-301	94.7	-15.04	-36.6	-31.1
	500	64.4	56.9	55.2[ab]	-10.7	-244	94.2	-20.94	-36.7	-42.9
	2500	66.7	63.2	59.8[b]	-30.4	-184	94.2	-31.52	-46.5	-26.3
	SEM	1.7	3.9	3.3	8.0	37.6	0.5	6.28	6.5	7.9
P-value										
Fe		0.820	0.363	0.810	0.347	0.555	0.843	<0.001	0.948	0.115
PHY		0.621	0.163	<0.05	0.218	0.083	0.737	0.172	0.500	0.276
Fe x PHY		0.951	0.431	0.250	0.654	0.811	0.369	0.861	0.199	0.513

6.4.11 Nutrient transporter gene expression

The expression of selected genes involved in nutrient absorption and Fe metabolism was assayed by qPCR (Table 6.12). Duodenal DMT1 (2.3-fold) and TFRC (1.9-fold) gene expression was higher in L-Fe than H-Fe fed pigs ($P<0.01$). Changes in dietary Fe or phytase concentration had no influence on NaPi-IIb, calbindin D9k, ZIP14 or SMIT2 gene expression in the duodenal mucosa ($P>0.05$).

Table 6.13. Effect of Fe and phytase on the normalised relative abundance of duodenal nutrient transporter mRNA[1]

Dietary Fe	PHY (FTU/kg)	NaPi-IIb	Calbindin D9k	DMT1	TFRC	ZIP14	SMIT2
L-Fe	0	2.80	1.30	1.89	1.56	0.99	1.27
L-Fe	500	2.99	1.16	1.86	1.41	1.27	1.39
L-Fe	2500	2.86	0.96	1.88	1.48	1.17	1.03
H-Fe	0	2.86	0.98	0.84	0.87	0.98	1.39
H-Fe	500	3.05	1.21	0.81	0.73	1.26	1.52
H-Fe	2500	2.91	1.14	0.83	0.80	1.15	1.15
	SEM	0.08	0.14	0.27	0.13	0.20	0.24
Main effects							
Fe	L-Fe	2.88	1.14	1.88	1.48	1.14	1.23
	H-Fe	2.94	1.11	0.83	0.80	1.13	1.35
	SEM	0.06	0.08	0.19	0.09	0.14	0.17
PHY	0	2.83	1.14	1.37	1.22	0.98	1.33
	500	3.02	1.19	1.33	1.07	1.26	1.46
	2500	2.89	1.05	1.36	1.14	1.16	1.09
	SEM	0.07	0.10	0.23	0.12	0.17	0.20
P-value							
Fe		0.498	0.473	<0.01	<0.001	0.461	0.852
PHY		0.316	0.323	0.773	0.502	0.701	0.445
Fe x PHY		0.948	0.214	0.533	0.697	0.943	0.888

[1]Means are relative expression of target gene normalization to geometric mean of selected housekeeping genes.

6.5 Discussion

Before the results are discussed in detail it should be stressed that for the first 9 days of the experiment the pigs were immunologically challenged due to an outbreak of post-weaning enteric disease. While the reason for this outbreak is unclear, it is possible that both the absence of supplementary creep feed throughout lactation and the absence of zinc oxide in the weaner diets made these pigs particularly susceptible to infection. As a result of the post-weaning disease, faecal scores were relatively high and the initial growth performance of these pigs was comparatively low for this genotype (in comparison to in house data).

Therefore, in order to treat the post-weaning diarrhoea, all pigs received a 3 d course of amoxicillin from d 8 to 10. While this treatment was applied to all pigs on trial, the possibility of potentially synergistic effects between the antibiotic and the dietary treatments cannot be excluded. Rosen (2001) reviewed the enzyme and antibiotic literature and found that while both may be added to monogastric diets to improve animal performance, the presence of one may mask or reduce the effect of the other. Moreover, as the control animals were initially performing sub-optimally, the potential scope for improvements through the use of feed additives is greater than it would be in pigs performing closer to their optimum. It is well established that the health status of an animal can influence both growth performance and nutrient requirement (Bedford, 2016), thus all treatment effects in the current experiment should be considered in light of this information.

6.5.1 Iron deficiency

The physiological changes that follow a period of suboptimal Fe supply have been well characterised and can be split into 4 stages: depletion, deficiency, dysfunction and disease (Suttle, 1999). Depletion is the first stage of Fe deprivation and is commonly marked by a reduction of body Fe stores (primarily as ferritin and hemosiderin) in organs such as the liver and spleen. These changes are often associated with a reduction in plasma ferritin concentration. The duration and extent of Fe deprivation, together with the initial Fe stores, govern the severity of this phase. Following Fe depletion comes deficiency. This stage is characterised by decrements in plasma Fe concentration, haemoglobin, MCHC and haematocrit, and elevated levels of

circulating transferrin. Depending on the severity of Fe deprivation iron deficiency anaemia may develop at this stage; a Hb concentration of <7 g/dl suggests anaemia, 9 g/dl borderline anaemia, whereas a Hb concentration of 10-11 g/dl is considered normal (Zimmerman, 1980). Dysfunction is the third stage of Fe deficiency, in which the growth of the animal is negatively affected due to the impairment of metabolic processes involving Fe dependent enzymes such as cytochrome c oxidase and succinate dehydrogenase. The final stage, disease, is characterised by poor growth, an increase in respiration rate, listlessness, pallor, and in severe cases, death.

In the present experiment, the L-Fe diets contained 102-107 mg Fe/kg, which according to the NRC (2012), is enough to satisfy the requirement of the pig during the initial post-weaning period. However, pigs receiving this treatment had lower liver and bone Fe reserves, lower Hct, plasma Fe and ferritin concentrations, elevated transferrin, and greater duodenal DMT1 and TFRC gene expression than those receiving the high-Fe (315-342 mg Fe/kg) treatment, all of which are indicative of Fe deficiency. Therefore, these findings concur with the suggestion of Jolliff and Mahan (2011) in that the post-weaning Fe requirement of the modern pig is well in excess of the 80 mg/kg proposed by the NRC (1998). Based on the aforementioned stages of Fe deprivation, it is likely that the weaner pigs receiving the low-Fe treatment in the current study were in the second stage, and were therefore, non-anaemic Fe deficient. It is possible that the higher Fe requirement of pigs in the current study than that suggested by the NRC (1998) is due to sub-optimal pig health status.

6.5.2 The effect of dietary Fe and phytase on weaner pig growth performance

Growth performance is generally considered an insensitive response criterion to animal Fe status as growth is usually only depressed in cases of severe anaemia (Amine et al., 1972). All pigs received an intra-muscular injection of 200 mg of Fe within the first three days post-partum, therefore, it is unlikely that pigs in the current experiment were anaemic. Moreover, no pigs displayed visual signs of anaemia such as laboured breathing or pallor. Nonetheless, pigs offered the low-Fe diet had a lower ADFI throughout the post-weaning period than those fed the high-Fe diet, with most of the differences in intake appearing in the latter stages of the study. Using pigs of similar genetics to that used in the present experiment [(Large White x Landrace) x PIC], Jolliff and Mahan (2011) also found that young pigs are indeed responsive to

increasing dietary Fe concentration in terms of growth performance, particularly in the latter post-weaning period. In their study, pigs were weaned at 17 d of age and fed a diet (intrinsic Fe content = 200 mg/kg) supplemented with 0, 80 or 160 mg/kg of Fe as ferrous sulphate for 35 d. The authors reported linear improvements in ADFI from d 21 to 35 and overall ADG in response to increasing levels of dietary Fe.

The growth rate of the pigs in the first week of the experiment was low for all pigs, and while this can largely be attributed to the poor health status, the low ADG was exacerbated by a low-Fe diet. This effect can be attributed to a reduction in feed intake. Interestingly, super-dosing phytase improved the initial performance of the pigs during this immuno-challenging time (prior to antibiotic treatment on d 8), without a corresponding increase in ADFI. These data suggest, that despite having no beneficial effect on faecal scores, super-doses of phytase may be used as a nutritional tool to mitigate the post-weaning growth check commonly experienced by pigs in modern production systems.

In terms of overall post-weaning performance, the results presented herein support the original hypothesis and demonstrate that 2,500 FTU/kg can effectively replace ~220 mg/kg of supplementary Fe in the post-weaning period with no adverse effects on growth performance. Weaner ADG was influenced by a dietary Fe by phytase treatment interaction. In diets without added phytase, pigs receiving the low-Fe treatment had a much reduced ADG. Adding a standard dose of phytase to this diet marginally improved ADG, whereas the super-dose substantially improved weaner pig ADG, to a level not dissimilar from the fastest growing treatment (H-Fe+2,500 FTU/kg). However, when supplemented to the high-Fe diet, phytase had no measurable effect on weaner pig ADG at the standard dose, and its effect was much reduced at the super-dosing level. Few studies have investigated the effect of phytase supplementation on Fe availability in the pig, therefore, this study is the first to show that the effect of phytase is dependent on the dietary Fe concentration. This interaction may be attributed to the level of phytate degradation occurring within the GIT, as ileal phytate degradation appeared to closely reflect pig performance and also displayed a similar Fe by phytase treatment interaction (discussed below in Section 6.5.3). In diets without added phytase, weaners receiving the high-Fe diet degraded substantially more ingested phytate than those fed the low-Fe diet. Consequently, as with performance, supplementary phytase had a greater effect on phytate degradation when added to the

low-Fe diet. This is likely the result of greater substrate availability for phytase in pigs offered the low-Fe treatment.

As discussed in Section 1.5, phytate is a potent anti-nutritional factor that reduces the nutritional (protein, energy and mineral) value of the diet, interferes with gastric proteolysis and stimulates endogenous AA secretions and losses (Woyengo and Nyachoti, 2013; Dersjant-Li et al., 2015). Studies have demonstrated that increasing the dietary phytate content can have a negative effect on weaner pig growth rate and FCR (Woyengo et al., 2012). Therefore, the improvements in performance in response to added phytase can likely be attributed to amelioration of the anti-nutritional effects of phytate, with the super-dose degrading more phytate and thus having a greater ameliorative effect. The interaction likely stems from the fact that in diets with no phytase, pigs fed the high-Fe diet degraded significantly more phytate than those fed the low-Fe diet.

Super-dosing phytase improved post-weaning FCR irrespective of the dietary Fe concentration. Several others have reported a similar FCR response to super-doses of phytase in weaner pigs (Kies et al., 2006; Veum et al., 2006; Zeng et al., 2014; Zeng et al., 2015), and this is generally attributed to improvements in cationic mineral, AA, carbohydrate digestibility, reduced endogenous secretions and losses, and the associated improvement in dietary DE. More recently, it has been proposed that the additional MYO generated with such high phytase doses, as exemplified in this study, may be involved in this performance response (Cowieson et al., 2013a).

6.5.3 Ileal inositol phosphate and MYO concentration

At physiologically relevant pH ranges (~2-7), *myo*-inositol hexa- to tri-phosphates readily chelates with Fe to form unavailable Fe-phytate complexes, principally monoferric phytate (Bretti et al., 2012; Yu et al., 2012). As a result, small degrees of phytate degradation that result in the formation of $InsP_{3-5}$, such as those achieved with standard doses of phytase, are not expected to yield significant improvements in Fe bioavailability. Therefore, in the current study it was hypothesised that super-dosing phytase would improve dietary Fe bioavailability by effectively degrading phytate, including the $InsP_{3-5}$ esters, in the proximal regions of the GIT.

Ileal phytate degradation was influenced by an Fe by phytase treatment interaction, suggesting that the effect of phytase was dependent on the dietary Fe concentration.

However, this may be somewhat misleading as the results show that dietary Fe concentration had no measurable effect on phytate hydrolysis in diets with supplementary phytase. It is in fact the diets with no added phytase that respond differently to the differing levels of dietary Fe; phytate degradation was substantially higher in weaners receiving the high-Fe treatment. The reason for this finding is unclear; however, in heat treated diets devoid of added phytase, phytate hydrolysis occurring prior to the terminal ileum is thought to originate primarily from the animals' endogenous phytase enzymes at the brush border membrane (Selle and Ravindran, 2008). It is possible that Fe is a necessary cofactor for mucosal phytase in pigs. Purple acid phosphatase enzymes (PAPs) are a group of metallophosphatases that contain up to two Fe atoms in their catalytic core (Schenk et al., 2013). PAPs have been detected in the spleen, lung tissue and uterine fluid; however, their physiological relevance in mammals is largely unknown (Olczak et al., 2003). It is not known if PAPs are expressed in the intestinal tissue of the pig. If indeed PAP enzymes do contribute to intestinal phytate hydrolysis, it may be that in pigs fed the low-Fe diet the available Fe was prioritised for Hb synthesis, and as a result, the activity of Fe-dependent enzymes such as PAP was diminished. Maenz and Classen (1998) found that the addition of 25 mM $MgCl_2$ to a brush border membrane preparation increased the rate of chick brush border phytase activity 2-fold. This finding agrees with the proposal of Maenz (2001) who suggested that animal phytase enzymes likely require mineral cofactors for optimal catalytic activity. Thus, it is plausible that in the present experiment, Fe improved phytate hydrolysis in diets with no added microbial phytase by increasing mucosal phytase activity. This would also help to explain the widely varying phytate hydrolysis values reported in the literature for diets with no added phytase.

The situation with $InsP_5$ closely reflected that of phytate and therefore was also affected by the Fe by phytase treatment interaction. As with phytate hydrolysis, dietary Fe had no influence on $InsP_5$ concentration in diets containing added microbial phytase; however, in diets with no added phytase, more $InsP_5$ was degraded in the high-Fe diet. This suggests that Fe also improves the catalytic activity of mucosal phytase enzymes for $InsP_5$. In diets with added phytase, those receiving the super-dose had significantly less $InsP_5$ in the digesta than those receiving the standard dose. The pattern of phytate degradation is consistent with that observed in Chapter 4; the

standard dose of phytase increased $InsP_4$ and $InsP_3$ (numerically) concentrations in the digesta, whereas the super-dose reduced them to low levels, below that of the non-supplemented diet. Using the same enzyme as that used in the present study, Holloway et al. (2016) also found that standard doses of phytase increased ileal concentrations of $InsP_4$ and $InsP_3$ in pigs, whereas the super-dose decreased them.

The more extensive phytate degradation seen with the super-dose of phytase resulted in a ~2 fold increase in the amount of MYO in the digesta reaching the terminal ileum. This is in contrast to the marginal, non-significant increase observed in response to the standard phytase dose.

6.5.4 The effect of dietary Fe and phytase on plasma MYO concentration

The level of dietary Fe had no influence on weaner pig portal or peripheral plasma MYO concentration. As was previously demonstrated in grower (Chapter 3) and finisher (Chapter 4) pigs, super-dosing phytase also increased plasma MYO levels in both the portal and peripheral circulation in weaner pigs. This reaffirms that the additional MYO liberated in response to high doses of phytase is available for absorption and systemic distribution. However, in contrast to the grower and finisher experiments, super-dosing mediated performance benefits were observed in the current weaner experiment.

Many have speculated that MYO has a role in eliciting the favourable super-dosing growth response; however, evidence to support this view, particularly in the pig, is lacking. A central finding of the present study is the observed positive correlation between portal plasma MYO concentration blood and weaner pig ADG. As the amount of MYO absorbed from the gut is directly linked to the degree of phytate degradation, this relationship may be due to the alleviation of the anti-nutritional effects of phytate, the growth promoting properties of MYO, or a combination of both. Results presented by Walk et al. (2014) are also supportive of a relationship between MYO and growth performance, as they found a positive correlation between the MYO concentration in the gizzard of broilers, as generated by phytase, and bird ADG.

As previously mentioned, the nutritional significance of dietary MYO in pig nutrition is unclear. Yamashita et al. (2013) recently demonstrated that MYO has insulin-like properties, which in mammals can stimulate the mobilisation of GLUT4 from intracellular vesicles to the plasma membrane. GLUT4 is an insulin sensitive glucose

transporter found primarily in adipose and skeletal tissue (Pessin and Bell, 1992). The content of GLUT4 at the plasma membrane is normally increased in response to insulin in order to reduce levels of blood glucose. It has therefore been suggested that the growth promoting properties of MYO may stem from insulin-like effects on glucose uptake, gluconeogenesis and protein accretion (Cowieson et al., 2014).

Previous studies have shown that the concentration of MYO in the tissue of developing foetuses and juveniles is relatively high, and as the animal ages, the concentration begins to decrease (Lewin et al., 1978; Quirk and Bleasdale, 1983; Pereira et al., 1990). The results of this thesis may support this finding as the concentration of MYO in the peripheral blood appeared to decrease (relative to portal blood) as the animal aged. However, direct comparisons across the studies should be done with caution as different pigs from different experiments were used. In addition, the hydration status of the portal and peripheral blood was not tested. It was earlier hypothesised that younger animals may have a greater requirement for exogenous MYO than older animals (Section 4.5.3). Therefore, it is of particular interest to note that a favourable growth response to super-dosing phytase was observed in the present experiment with young pigs, but not in the two previously reported experiments involving older pigs. These findings are consistent with the current body of literature and imply that favourable super-dosing responses are more commonly observed in younger animals.

6.5.5 The effect of dietary Fe and phytase on mineral availability

Another objective of this study was to determine the effect of supplementary phytase on mineral availability (particularly Fe) in the weaner pig. As noted earlier, one of the most consistent effects of phytase supplementation is the improvement in bone mineralisation traits. In this experiment, the high phytase dose significantly increased the metatarsal bone ash content of pigs fed a nutritionally adequate diet. Several others have also demonstrated phytase-mediated increases in bone ash in animals fed nutritionally sufficient diets (Murry et al., 1997; Watson et al., 2006; Pagano et al., 2007). This effect can be explained as the Ca and P requirement for maximal bone mineralisation is higher than that for optimal growth in pigs (NRC, 1998). Therefore, bone mineral deposition continues upon the provision of increasing dietary Ca and P availability after the requirement for optimal growth has been met. Murry et al. (1997) reported that phytase significantly increased bone ash in weaners fed P deficient or P

adequate diets; however, the magnitude of the phytase response was smaller when added to the P adequate diet.

Super-dosing phytase in the current experiment increased weaner pig bone P, Ca and Mg content, which is consistent with the findings of Chapters 3 and 4 in growers and finishers respectively. This can be attributed to greater release of phytate bound minerals in the proximal regions of the GIT, prior to the site of absorption. A significant portion of body Fe is stored in the reticuloendothelial cells of bone marrow as hemosiderin or ferritin (Path, 1963). Bone Fe measurements are therefore considered a sensitive means of determining Fe status (Rocha et al., 2009). In this study, weaners fed the low-Fe diet had a lower bone Fe content than those fed the high-Fe diet. Adding phytase to the low-Fe diet, particularly at the super-dosing level, appeared to increase bone Fe concentration, although this effect was not significant. Few studies have assessed the effects of phytase on bone Fe; however, Zeng et al. (2015) also found that supplementing a low P corn-SBM diet (adequate Fe) with increasing doses of phytase (up to 20,000 FTU/kg) had no effect on weaner metacarpal bone Fe concentration. In contrast, Shelton et al. (2005) noted that 500 FTU/kg of phytase increased coccygeal bone Fe concentration in weaners when added to a corn-SBM diet. The positive phytase response on bone Fe observed in the study of Shelton et al. (2005) may have been due to the lower Fe content of the negative control diet.

The liver is the major site of Fe storage in the body. Pigs fed the high-Fe diet had heavier livers (relative to BW) than those fed the low-Fe diet. This effect was not anticipated, but may be attributed to the higher ADFI of these pigs, which likely resulted in an increase in liver metabolic activity. This effect was demonstrated in the study of Lange et al. (2003) as they found that an increase in feed intake increased viscera growth in grower pigs. Depletion of hepatic Fe reserves is one of the first biochemical changes experienced during iron deprivation, therefore, liver Fe measurements provide a more sensitive measure of Fe status than most commonly measured haematological parameters (i.e. Hb, Hct, plasma Fe), particularly at the early stages of Fe deficiency (Smith et al., 1984). The hepatic Fe concentration of pigs fed the high-Fe diet was similar to other reported values for young pigs fed Fe adequate diets (Hansen et al., 2009; Fang et al., 2013). Reducing the dietary Fe content (low-Fe diet) resulted in a 40% reduction in liver total Fe concentration, indicating that the liver Fe stores were mobilised to meet the demands of Fe requiring tissue in pigs offered

this treatment. This effect has been noted before in pigs, as Yu et al. (2000) reported that decreasing the level of supplemental Fe from 120 to 0 mg/kg resulted in a linear reduction in both total Fe and ferritin in the liver of weaner pigs. Supplementary phytase had no measurable effect on liver Fe stores in this study.

The absorption and metabolism of Fe, Cu, Zn and Mn are known to be closely linked to one another due to the possession of similar physiochemical properties and uptake pathways (Arredondo et al., 2006). In this experiment, phytase increased both Cu and Mn levels in the liver when added to the low-Fe diet. This is in agreement with the results of Zeng et al. (2015) who also found that phytase increased liver Cu and Mn content in weaner pigs. Interestingly, in this experiment, phytase had the reverse effect on liver Cu when added to the high-Fe diet, and reduced it. In addition, the effect of phytase on liver Mn was much muted when added to the high-Fe diet. These interactions can likely be attributed to competitive inhibition of uptake occurring within the GIT. Transition metal transporters such as DMT1 (principal Fe transporter) and ZIP14 (principal Zn transporter) appear to have broad substrate specificities (Hansen et al., 2009). For example, although DMT1 is best known for its role in Fe uptake, there is evidence to suggest that Cu, Mn, Zn, Cd and Pb can all pass through this carrier protein (Gunshin et al., 1997; Hansen et al., 2009). In the current experiment, duodenal DMT1 expression in pigs fed the low-Fe diet was high. Therefore, it is possible that phytase, particularly at the super-dosing level, improved Cu and Mn solubility within the GIT, and due to the high expression of DMT1 these minerals were readily absorbed from the intestinal lumen. Whereas, in the high-Fe treatment, however, DMT1 expression was comparatively low, thus the additional Mn released in response to the super-dose was not absorbed as efficiently. It is unclear why phytase had the reverse effect on Cu absorption when added to a high-Fe diet; however, it seems that through phytate degradation, phytase is improving the solubility of minerals which, in the presence of high levels of Fe, create unfavourable conditions for Cu absorption. Many studies have demonstrated antagonistic interactive effects between Fe, Cu and Zn, in which an imbalance in one can have adverse effects on the uptake of another (Elshobaki and Rummel, 1979; Blakeborough and Salter, 1987; Arredondo et al., 2006).

Levels of minerals in the plasma reflect a balance between those absorbed from the GIT, those being diverted to or from tissue, and those in circulation bound to plasma

proteins. In the present experiment, pigs on all treatments had plasma Fe levels that fell within the physiological 'normal' range (Underwood and Suttle, 1999). However, pigs receiving the low-Fe diets had lower circulating levels of Fe in both the portal and peripheral blood than those on the high-Fe diet. Adding phytase to the diets had no measurable effect on plasma Fe. Although it is apparent that plasma Fe is sensitive to changes in Fe status (Rincker et al., 2004; Zhang et al., 2013), there is uncertainty surrounding the suitability of this measure to diagnose Fe status due to the rapid turnover of Fe in blood plasma. It is thought that an Fe atom spends only ~120 minutes in the plasma before uptake into haemopoietic or storage tissue (Schreiber et al., 1989). Moreover, plasma Fe is sensitive to a range of other factors including chronic infection, tissue damage and inflammation (Smith et al., 1986).

Copper levels in the portal blood were influenced by a dietary Fe and phytase treatment interaction, similar to that described for the liver. As levels in portal flow are closely related to absorption from the GIT, this result supports the earlier proposed contention that this interaction is occurring within the GIT, and therefore, is most likely due to competitive inhibition of uptake. Plasma Zn increased in response to the super-dosing treatment whereas the standard dose had no effect. Han et al. (1994) found that although the inhibitory effect of phytate attenuated as the degree of phosphorylation decreased, $InsP_3$ was still capable of binding sufficient Zn. This may explain why improvements in Zn status were not observed with the standard phytase dose in the current experiment. Phytase exerted similar effects on plasma Mg, however, here a standard dose was sufficient to improve Mg status. Similar effects of phytase on circulating Zn and Mg in weaners have been reported by several others (Adeola et al., 1995; Zeng et al., 2014; Zeng et al., 2015).

Adeola et al. (1995) suggested that the performance benefits observed in response to phytase may in part be due to improved mineral bioavailability. In the current study, super-dosing improved Mg and Zn bioavailability (as demonstrated by the bone and plasma data respectively) in comparison to the standard phytase dose. Both Zn and Mg are essential cofactors for over 300 different enzyme systems and thus are involved in a variety of biological functions in the body. Mg is the fourth most common cationic mineral in the body and is essential for normal cellular respiration, DNA synthesis and protein synthesis (de Baaij et al., 2012). Zn is the second most abundant micro-mineral in the body and is required for regular cell division and differentiation,

nutrient oxidation, immunity and DNA transcription (Kelleher et al., 2011). Therefore, it is possible that the super-dosing derived performance benefits seen in the current experiment were the result of improved Zn and Mg bioavailability. Phytate is known to have a negative effect of Mg retention through stimulating Mg endogenous secretions (Brink et al., 1992). As phytase had no effect on Mg AID in the present experiment, it seems likely that phytase increased plasma Mg by reducing endogenous secretions into the GIT. This is also likely true for Zn.

6.5.6 The effect of dietary Fe and phytase on haematological status

Two proteins central for maintaining normal Fe homeostasis are ferritin and transferrin, the principal Fe storage and transport proteins respectively. Ferritin is found in most body tissue and its expression is directly proportional to Fe stores, making it a sensitive diagnostic measurement of total Fe status. Smith et al. (1984) found that circulating ferritin provided a better estimate of liver Fe concentration than most traditional haematological indicators of Fe status such as Hct, Hb, MCHC and plasma Fe. Transferrin is a liver derived protein found predominantly in the plasma, where its primary function is to transport non-heme Fe around the body (Gkouvatsos et al., 2012). In contrast to ferritin, transferrin expression is inversely proportional to Fe status (McKnight et al., 1980; Rincker et al., 2004). There is limited data available on plasma transferrin and ferritin levels in pigs, therefore, 'normal' reference values for these parameters have not been determined. However, the reported values in this study for transferrin are similar to those published by Rincker et al. (2004), and the ferritin values fall within the range published by Smith et al. (1984).

Feeding pigs the low-Fe diet resulted in an 18% reduction in plasma ferritin and proportional increase in plasma transferrin at d 20 post weaning. These data demonstrate that under times of Fe deficiency, pigs respond by increasing transferrin expression in order to mobilise Fe from storage to erythropoietic tissue. Adding a super-dose of phytase to the low-Fe diet appeared to increase serum ferritin (57.04 to 74.15 ng/ml) to levels comparable with those offered the high-Fe treatment, however, this effect was not significant. This may have been due to the high level of variation observed in plasma transferrin between pigs. The presently reported results are consistent with those of Rincker et al. (2004) who observed a linear decrease in plasma transferrin, from 44.66 to 39.08 g/l, in response to increasing supplemental Fe concentration (0 to 150 mg/kg).

Hb and Hct are two of the most commonly measured criteria for determining animal Fe status due to relative ease of sample collection and analysis. Both provide a measure of the amount of active Fe within the body (Crichton, 2006). In this study, offering weaners a low-Fe diet resulted in a significant decline in Hct (30.5 to 26.4%) and a small non-significant reduction in Hb concentration (11.0 to 10.0 g/dl). These data concur with those of Jolliff and Mahan (2014) and suggest that Hct is a more sensitive measure of Fe status than Hb concentration. The average Hb concentration of weaner pigs receiving the low and high-Fe treatments both fell within the adequate reference range published by Thorn (2010). Taken together with the other indices of Fe status, these data demonstrate that under periods of Fe deprivation, pigs draw upon their Fe reserves to maintain adequate Hb synthesis. The relatively long life-span of erythrocytes (~72 d) can delay the onset of iron deficient microcytic hypochromic anaemia in pigs receiving a marginally Fe deficient diet (Withrow and Bell, 1969). In this experiment, pigs were on trial for 20 d. It is possible that had the duration of the experiment been extended such that there was a full turnover of erythrocytes, pigs on the low-Fe diet may have developed anaemia due to inadequate Fe for erythropoiesis. Phytase supplementation at 2,500 FTU/kg tended to increase pig Hct concentration. It may be that super-doses of phytase have a restorative effect on Hct by increasing the bioavailability phytate-bound Fe through sufficient phytate hydrolysis. This was demonstrated in the work of Stahl et al. (1999) who showed that the addition of 1,200 FTU/kg of a fungal-derived phytase to a corn-SBM diet effectively improved the bioavailability of phytate-bound Fe for Hb and Hct repletion in anaemic weaner pigs. The authors proposed that phytase improves Fe availability through one of two ways: releasing phytate-bound Fe and/or preventing phytate from chelating with other sources of Fe within the GIT.

6.5.7 The effect of dietary Fe and phytase on duodenal nutrient transporter gene expression

Mammals have a very limited capacity to excrete Fe, therefore, homeostasis of this mineral is largely regulated at the level of intestinal absorption. Non-heme iron is absorbed primarily as Fe^{2+} in the duodenum via the apical proton-coupled metal cotransporter DMT1 (De Domenico et al., 2008). Hansen et al. (2009) recently demonstrated an inverse relationship between DMT1 expression and dietary Fe concentration in pigs. The DMT1 gene expression data presented in the current

experiment are consistent with those of Hansel et al. (2009) and others (Hansen et al., 2010; Fang et al., 2013; Espinoza et al., 2014) and indicate an upregulation in DMT1 expression in response to Fe deficiency, in an effort to maximise Fe uptake. Conversely, when Fe status is high, DMT1 expression is downregulated in the intestine to prevent cells from Fe overload and oxidative damage (Gunshin et al., 2001). DMT1 expression was not influenced by phytase treatment, which suggests that phytase is not influencing weaner pig Fe status.

Other genes encoding putative proteins involved in Fe homeostasis measured in this study include transferrin receptor (TFRC) and Zrt- and Irt-Like Protein 14 (ZIP14). TFRC has a central role in the cellular acquisition of circulating transferrin-bound Fe through receptor-mediated endocytosis. In the epithelial cells of the intestine, TFRC expression is limited to the basal and lateral surfaces where it is thought to provide the rapidly dividing crypt cells with Fe (Anderson et al., 1991). Data obtained from rats and cultured Caco-2 cells have shown that intestinal TFRC expression is negatively correlated with body Fe reserves, and thus serves as a reliable indicator of Fe status (Anderson et al., 1990; Nunez et al., 1996). However, *in vivo* work on the effects of dietary Fe concentration on duodenal TFRC expression are limited. In this study, it was demonstrated that reducing the dietary Fe concentration from ~320 to 100 mg/kg increased duodenal TFRC gene expression 1.9-fold. This increase indicates Fe deficiency and confirms that TFRC expression is a sensitive measure of Fe status in young pigs. As with DMT1, supplementary phytase had no influence on TFRC gene expression.

ZIP14 is a member of the mammalian ZIP metal-ion transporter family with known specificity for Zn and Fe (Liuzzi et al., 2006). This protein has recently been detected in the pig duodenal mucosa of young pigs; however, its contribution to dietary Fe absorption is unclear (Hansen et al., 2009). In this experiment ZIP14 gene expression in the duodenum was not influenced by dietary Fe concentration. This is contrary to the results of Hansen et al. (2009) who found that decreasing the dietary Fe content increased the relative expression of ZIP14 in the duodenum. In their experiment, the choice of treatments were more extreme; the low-Fe diet contained only 20 mg Fe/kg (80 mg/kg less than that of the current study) and the high-Fe diet contained 520 mg Fe/kg (200 mg/kg above that used in the current study). It is possible that ZIP14 plays a more important role intestinal Fe absorption during periods of severe Fe deprivation.

The increase in circulating Zn concentration observed in pigs receiving the super-dose treatment was not met with an increase in ZIP14 gene expression. This finding lends support to the above-mentioned suggestion that phytase is improving Zn availability via a reduction in endogenous secretions, rather than increasing intestinal absorption.

Phytase is known to influence P, Ca and MYO digestibility, therefore, this study set out determine the relative intestinal expression of NaPi-IIb, calbindin D9k and SMIT2 mRNA in response to phytase and Fe treatments. Despite increases in bone P content, NaPi-IIb expression was not influenced by phytase treatment. This finding is in agreement with those of Chapter 4 and can likely be attributed to the dominance of the paracellular route of P absorption in times of adequate P supply (Stein et al., 2008). As with P, the bone data also show an increase in Ca bioavailability in response to phytase treatment which was not met with a change in calbindin D9k expression. Although the duodenum is the main site of intestinal Ca absorption, it has been suggested that jejunal calbindin D9k measurements are more sensitive to changes in dietary Ca concentration (Pointillart et al., 1986). Moreover, similarly to P, the transcellular route of absorption is dominant under adequate or low Ca supply; however, the contribution of the passive paracellular route of absorption is thought to increase with Ca intake (Bronner, 1987). This may explain the lack of calbindin D9k response to phytase treatment.

In this study, the relative duodenal gene expression of the putative intestinal MYO transporter SMIT2 was not influenced by phytase treatment. This is in accordance with the data presented in Chapter 4, in which increasing doses of phytase had no influence on SMIT2 expression in the jejunum of finisher pigs. As previously discussed, it is difficult to explain why SMIT2 mRNA expression was not affected by phytase considering the limited amount of available data on this protein, particularly in the pig. While phytase had no influence on SMIT2 expression at the transcript level, it is possible that phytase affected the expression at the protein level; however, protein quantitation research would be necessary to confirm this.

6.5.8 Conclusions

In conclusion, feeding weaner pigs a wheat-SBM based diet containing 100 mg Fe/kg (20 mg/kg above the NRC requirement) was not sufficient to maintain adequate Fe reserves. Adding a super-dose of phytase (2,500 FTU/kg) effectively improved weaner pig growth performance to a level comparable with those offered the high-Fe diet (320 mg/kg), but was not sufficient to replenish the depleted Fe reserves. This performance benefit was met through an improvement in FCR, a small increase in ADFI and possibly through improved phytate-bound Fe bioavailability.

Weaners receiving the low-Fe treatment had reduced liver and bone Fe stores, a lower Hct and Hb (trend) concentration, reduced circulating ferritin and Fe, increased plasma transferrin, and increased mRNA expression of genes encoding proteins involved in Fe uptake (DMT1 and TFRC). These data all point towards Fe deficiency. However, the Hb content of pigs fed this diet was adequate (10 g/dl), therefore, these pigs were considered to be non-anaemic Fe deficient. The decrease in Fe stores and increase in plasma transferrin suggest that Fe was being mobilised from the storage pools in order to meet the Fe demands of the erythropoietic tissue for Hb production. Further, in order to maximise Fe intake, the pigs responded by up-regulating the expression of intestinal proteins involved in Fe absorption.

Supplementary phytase had no significant effect on any of the Fe status measurements recorded; however, phytase, particularly at the super-dosing level, resulted in numerical improvements in bone Fe, blood Hb and Hct, plasma Fe and ferritin, and reduced plasma transferrin. Although none of these responses were significant at the *P* equals 0.05 level, writing off the possibility that super-dosing phytase improved performance through improvements in Fe bioavailability does not seem fully justified and warrants further investigation.

Phytase effectively degraded phytate by the terminal ileum. In diets without added phytase, dietary Fe concentration affected phytate hydrolysis, with those on the high-Fe diet degrading significantly more than those fed the low-Fe diet. This effect may be due to the possible involvement of Fe as a cofactor for the animals' mucosal phytase enzymes. In agreement with Chapter 4, the inositol phosphate data show that standard doses of phytase are associated with a build-up of $InsP_4$ and $InsP_3$ esters in the ileal digesta. Adding a super-dose of phytase prevents this build up, resulting in more

complete phytate degradation and thus greater MYO generation. This increase in intestinal MYO availability was met with an increase in circulating levels of MYO. It is possible that an elevated MYO status was involved in the performance benefits observed in response to the phytase super-dose.

The effect of phytase on mineral availability is inconsistent and appears to be tissue dependent. Phytase increased P, Ca and Mg bioavailability as demonstrated with the bone data. In the plasma, both Zn and Mg content increased in response to phytase treatment, whereas Ca, Na, K, Cu, Fe content were not affected. A dietary Fe and phytase treatment interaction for portal plasma Cu and Mg and liver Cu and Mn was observed, demonstrating that the effect of phytase on the availability of these minerals is dependent on the level of Fe in the diet.

Chapter 7
General Discussion

Phytase enzymes are commonly added to pig diets at a rate of 500 to 750 FTU/kg to improve the bioavailability of phytate phosphorus and in doing so reduce the need for phosphate supplementation. However, over the last decade it has become increasingly apparent that phytate has inimical effects on mineral, AA and energy digestibility, endogenous secretions, nutrient absorption and endogenous enzyme activity (Selle and Ravindran, 2008; Woyengo and Nyachoti 2013). As a result, recent research efforts have focused on maximising phytate degradation through the use of super-doses (>1,500 FTU/kg) of phytase, in order to alleviate the anti-nutritional effects of phytate and improve animal performance.

Declining enzyme costs together with the development of more efficacious third generation microbial phytases have made super-dosing a feasible practise. Super-doses of phytase have been shown to improve monogastric growth performance beyond that of a high P positive control or a standard phytase dose diet (Cowieson et al. 2006; Karadas et al. 2010; Zeng et al., 2014). However, the mode of action for this response requires clarification. Furthermore, most of the research on super-dosing phytase has been conducted in poultry, and of that available in pigs, most has been conducted in weaners. This research set out to determine the effects of super-dosing phytase on the growing pig, with a view to providing more clarification on the underlying mechanism, particularly in relation to the possible involvement of MYO.

7.1 The effect of super-dosing phytase on pig growth performance

One of the primary objectives of this research was to determine the effects of super-dosing phytase on the growth performance of the growing pig. In Chapter 3, phytase improved the ADFI and ADG of grower pigs fed a low P diet to a level similar to those fed a high P control diet. However, increasing the phytase dose from 500 to 2,000 FTU/kg delivered no further improvements in performance. A similar response was observed in Chapter 4 with pigs at the early finishing stage of production. In this experiment, although increasing doses of phytase continued to degrade phytate in a linear fashion up the highest tested dose of 11,000 FTU/kg, there were no differences between any of the phytase doses tested in terms of finisher pig growth performance.

In contrast, in Chapter 6, super-dosing phytase (2,500 FTU/kg) significantly improved weaner pig growth performance in comparison to the non-phytase supplemented P adequate diet, and the same diet supplemented with a standard phytase dose.

These data support the current body of literature and suggest that the super-dosing response on pig growth performance is inconsistent. This is likely a reflection of the multitude of factors known to influence phytase induced phytate degradation *in vivo*, including: enzyme source, phytase activity (both supplemental and intrinsic), dietary ingredients, ambient mineral concentration (particularly Ca) and substrate concentration (Section 1.7). The implementation of standardised methodology, controlling for many of these variables, may help to reduce inter-experimental variation and thus help discern the true phytase response.

In addition, the findings of this research support the observation described in Chapter 4 (Section 4.5.1) in that the favourable growth responses to high phytase doses are more commonly observed in weaner pigs. This view was recently shared in a report by Goncalves et al. (2016). It is difficult to speculate why this may be without a clear understanding of the super-dosing mechanism; however, as discussed in Chapter 4, it is possible that younger pigs are more sensitive to the anti-nutritional effects of phytate and are thus more responsive to the extra-phosphoric effects of phytase supplementation. Another proposition advanced in this thesis is that younger pigs may have a relatively greater dietary requirement for MYO (discussed below in Section 7.2); this would help to explain why phytase-mediated performance benefits are more commonly observed in younger pigs.

7.2 The effect of super-dosing phytase on ileal phytate degradation and MYO formation

Part of performance benefits observed in response to super-doses of phytase are thought to stem from alleviation of the anti-nutritional effects of phytate through greater phytate degradation. While many studies have demonstrated improved mineral (Veum et al., 2006; Kies et al., 2006b; Zeng et al., 2016), AA (Adedokun et al., 2015; Zeng et al., 2016) and energy (Kiarie et al., 2015; Zeng et al., 2016) digestibility in response to high phytase dosing, very few have actually measured phytate degradation occurring at the ileal level. Of the studies that have measured phytate degradation, most have measured phytate P disappearance which provides little information on the

extent, and no information on the pattern, of phytate degradation. Such information would provide an insight into the mechanism of phytate degradation within the pig GIT and could provide instructive information to help shed light on the underlying super-dosing mechanism. Therefore, one of the key objectives of this study was determine the effect of super-dosing phytase on phytate degradation (with discrimination for the different InsP esters) and MYO generation in the GIT.

Across all experiments, supplementation of a standard phytase dose consistently reduced the amount of $InsP_6$ and $InsP_5$ in the digesta reaching the terminal ileum. This supports the current view that at standard doses, modern bacterial phytase enzymes have a selective preference for the higher molecular weight ($InsP_6$ and $InsP_5$) InsP esters (Greiner and Farouk, 2007). In Chapter 3, increasing the phytase dose from 500 to 2,000 FTU/kg delivered no further improvements in ileal phytate degradation in grower pigs. This finding was not expected but was likely the result of excessive dietary Ca levels (Ca:P ratio of 1.46 to 1.49), which is known to have an adverse effect of phytase efficacy (Qian et al., 1996). In both Chapters 4 (finisher) and 6 (weaners), the standard dose of phytase resulted in a small accumulation of $InsP_4$ and $InsP_3$, suggesting that conversion of $InsP_4$ to $InsP_3$ is the rate limiting step in complete phytate hydrolysis. However, by increasing the phytase dose to a super-dose, the build-up of these intermediate esters began to diminish. This is important as both $InsP_4$ and $InsP_3$ still exhibit anti-nutritional effects on protein and mineral availability, therefore, it has been suggested that degradation to at least $InsP_2$ is required in order to alleviate these adverse effects (Yu et al., 2012; Dersjant-Li, 2015). This may offer an explanation as to why super-doses are known to improve monogastric growth performance, while standard doses are not.

An alternative, as yet untested hypothesis for the super-dosing mode of action is that high doses of phytase are generating bio-active intermediates that promote growth (Bedford and Cowieson, 2012). Phytase catalyses phytate in a step-wise manner, resulting in the production of a series of lower InsP esters in the digesta. This thesis has shown that super-doses are associated with more complete phytate degradation and thus alter the composition of InsPs in the digesta. Intracellularly, inositol phosphates are important secondary messengers that predicate cell signalling biology. There are over 700 different enantiomers of the different inositol phosphates, therefore, it is possible that super-doses of phytase mediate the production of certain inositol

phosphates within the GIT that exert biological activity. There is currently no evidence to support this theory, however, future research in this area may warrant attention.

It has recently been suggested that MYO, the product of complete phytate dephosphorylation, is involved in the favourable super-dosing growth response (Cowieson et al., 2013a; Cowieson et al., 2015). However, as previously mentioned, evidence to support this view, particularly in the pig, is limited. Moreover, evidence to show that phytate is completely hydrolysed in the pig GIT is also lacking. The research presented in this thesis has demonstrated that increasing the phytase dose is indeed associated with an increase in MYO generation in the ileal digesta. Thus, these data are suggestive of complete phytate dephosphorylation. However, the gastric data from Chapter 5 suggests that phytate can be broken down to $InsP_1$, but not to MYO within the stomach. Therefore, it may be that exogenous phytase effectively degrades phytate to lower, more soluble esters, which are then available for complete dephosphorylation by the endogenous phytases. This finding supports the view that most microbial phytases are unable to completely dephosphorylate phytate because of an axially orientated Pi group on the C2 of the inositol ring, which is resistant to the action of most phytases (Wyss et al., 1999).

Another central finding of the research presented in this thesis is that super-doses of phytase increased levels of circulating MYO in weaners (Chapter 6), growers (Chapter 3) and finishers (Chapter 4). Increases were seen in both the portal and peripheral blood, which demonstrates that the MYO is effectively absorbed from the gut and available for systemic distribution. This study is the first to confirm that super-dosing improves the MYO status in pigs, and agrees with the findings of Cowieson et al. (2015) who demonstrated a similar effect in broilers. It is noteworthy that in all experiments, levels of circulating MYO in pigs receiving a standard dose of phytase did not differ from the controls. If indeed MYO is involved in the super-dosing growth response, this may offer an explanation as to why standard doses of phytase are not associated with performance benefits.

Interestingly, plasma MYO only correlated with pig performance in the weaner experiment (Chapter 6), which was also the only experiment to demonstrate super-dosing performance benefits. This observation supports the notion proposed in Chapter 4 (Section 4.5.3) that young animals may have a dietary requirement for

MYO. Further research to test this theory could prove beneficial, and may provide an explanation as to why favourable growth responses to super-doses are more commonly observed in weaner pigs than in older pigs.

7.3 The effect of super-dosing phytase on nutrient availability

It is well established that microbial phytase supplementation increases bone ash concentration in the pig, and that this is largely met through improvements in Ca and P bioavailability. This effect was consistent throughout this thesis for pigs at the weaner, grower and finisher stages of production. A less well documented effect of phytase is that on bone Mg. In each of the reported experiments, phytase consistently increased bone Mg concentration, in a dose-dependent manner. This finding is in accordance with the recent observation of Madrid et al (2013), who also found that phytase (at 500 FTU/kg) increased grower pig Mg metacarpal content. Mg in the bone is found primarily as part of the of apatite complex, providing strength and firmness to the bone (Castiglioni et al., 2013). As with Ca and P, Mg deficiency is also associated with osteoporosis (Belluci et al., 2011). These data demonstrate that super-doses of phytase are associated with increases in bone mineralisation. This finding may be of more practical significance in the breeding herd, where the premature culling of sows due to lameness is an issue.

It is well known that phytase improves both P and Ca bioavailability in the pig (Selle and Ravindran, 2008). Less well understood is the effect of phytase on the divalent trace elements. Some studies have suggested that phytase improves Zn, Fe, Cu, and Mn digestibility in pigs (Pallauf et al. 1992; Adeola et al. 1995; Kies et al., 2006b), while others have reported no effect (Valencia and Chavez, 2002; Rutherfurd et al., 2014a). In the currently presented work, the effect of super-dosing phytase on trace mineral availability was inconsistent. Madrid et al. (2013) found that Cu was the trace-mineral that was most influenced by phytase treatment in pigs. The data presented in this thesis support this finding, as there was evidence of improved Cu availability across all experiments. Super-dosing improved Cu AID in Chapters 3 and 4, and increased liver Cu concentration in Chapter 6, but only when added to the low-Fe diet. There were suggestions of improved Zn availability in response to super-dosing phytase in Chapters 3 and 6, with increases in AID and plasma Zn respectively. Fe digestibility was not influenced by phytase in Chapter 3, 4 or 6, although there were

indications of improved Fe bioavailability in Chapter 6, as super-dosing phytase improved the performance of weaners fed a low-Fe diet to a level comparable with pigs fed a high-Fe diet. In Chapter 6, super-dosing phytase increased liver Mn concentration; however, the size of the effect was diminished when supplementary phytase was added to a high-Fe diet.

It is difficult to draw any firm conclusions of the effects of super-dosing phytase on trace mineral availability based on the present work. However, in each experiment, there were clear indications that phytase was influencing at least one of the trace minerals under study. It is possible that as the dietary trace elements were provided in excess of requirement (except Fe in Chapter 6) the phytase response was diminished. To determine the true effect of phytase on mineral availability, the diets should be deficient in the mineral under investigation (Augspurger et al., 2003). It was, however, apparent from this thesis that the magnitude, and even the direction (in the case of Cu), of the phytase response on a trace-mineral availability was dependent on the concentration of other trace-minerals. This was most clearly demonstrated by the dietary Fe and phytase treatment interaction for liver Cu and Mn concentration observed in the Chapter 6, in which phytase was added to either a low or a high-Fe diet. This effect was attributed to antagonistic interactions occurring between elements with similar physiochemical properties, and demonstrates that an imbalance of one can result in the inhibition of uptake of another.

To prevent possible misuse of supplementary phytase and potential mineral imbalances, future work should be directed at identifying and quantifying the true mineral response to phytase so that accurate mineral ration dilutions can be made. Such matrix values will increase the economic and ecological value of phytase further. This is particularly relevant at present as there are growing concerns over heavy metal (notably Cu and Zn) emissions from intensive livestock operations. These concerns have seen legislation designed to curb Cu and Zn emissions introduced into the European Union (Regulation (EC) No 166/2006 of the European Parliament and of the Council). It may be that high-doses of phytase have a place in ensuring these regulations are met.

7.4 Conclusions

This research set out to determine the effects of super-dosing phytase on the growing pig, with a view to providing more clarification on the underlying mechanism, particularly in relation to the possible involvement of MYO. From this work, it can be concluded that the effect of super-dosing phytase on pig growth performance is inconsistent; super-dosing improved weaner pig performance, but had no influence on the performance of grower or finisher pigs. Super-doses of phytase consistently increased MYO generation in the gut, which was effectively absorbed by pigs at all stages of production, resulting in increased circulating MYO concentration. These results are suggestive of complete phytate dephosphorylation within the gut of pigs, and support the current view that MYO may be involved in the super-dosing response.

Based on the findings of the current work and those of others, it is clear that there are considerable opportunities to maximise the extra-phosphoric effects of phytase through the use of super-doses, particularly in the weaner pig. However, whether these opportunities remain in the latter stages of production requires clarification. Future work should continue to focus on identifying the mechanism by which super-doses of phytase improve pig growth performance. In addition, efforts should be made to identify the factors responsible for the inconsistencies observed in the literature. In the current study, plasma MYO correlated with performance in the weaner phase, but not in the grower or finisher stages. It would be interesting to test the proposition that younger animals have a greater relative exogenous requirement for MYO. Efforts should also focus on identifying the true effect of phytase on nutrient digestibility (AA, divalent minerals [particularly Cu and Zn] and energy), so that appropriate dietary dilutions can be made. With the decline in phytase costs together with the rising feed costs, it is likely that super-dosing phytase will become common practise. Research in these suggested areas should maximise the economic and environmental value of phytase, thus contributing to improving the sustainability of pig production.

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Appendix A

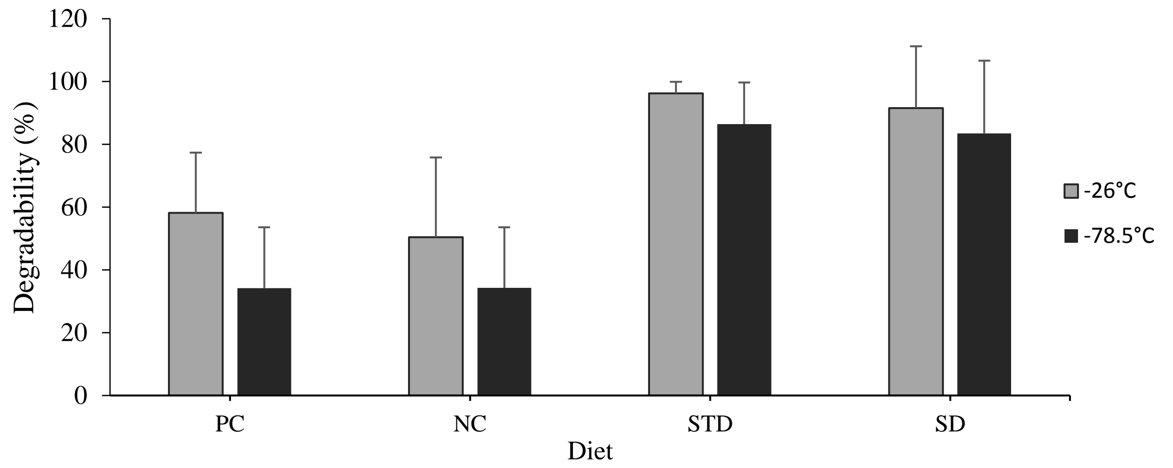

Figure A.1 Effect of diet and freezing temperature on gastric $InsP_6$ degradation (%) following sample collection

Values are means of 10 observations ± SD.

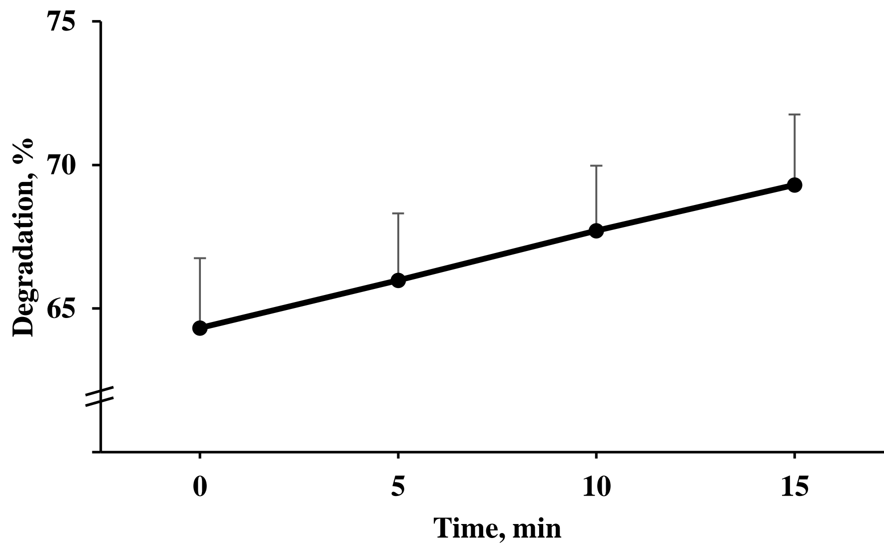

Figure A.2 Effect of time on gastric $InsP_6$ degradation (%) following sample collection.

Values are means of 72 observations ± SEM.

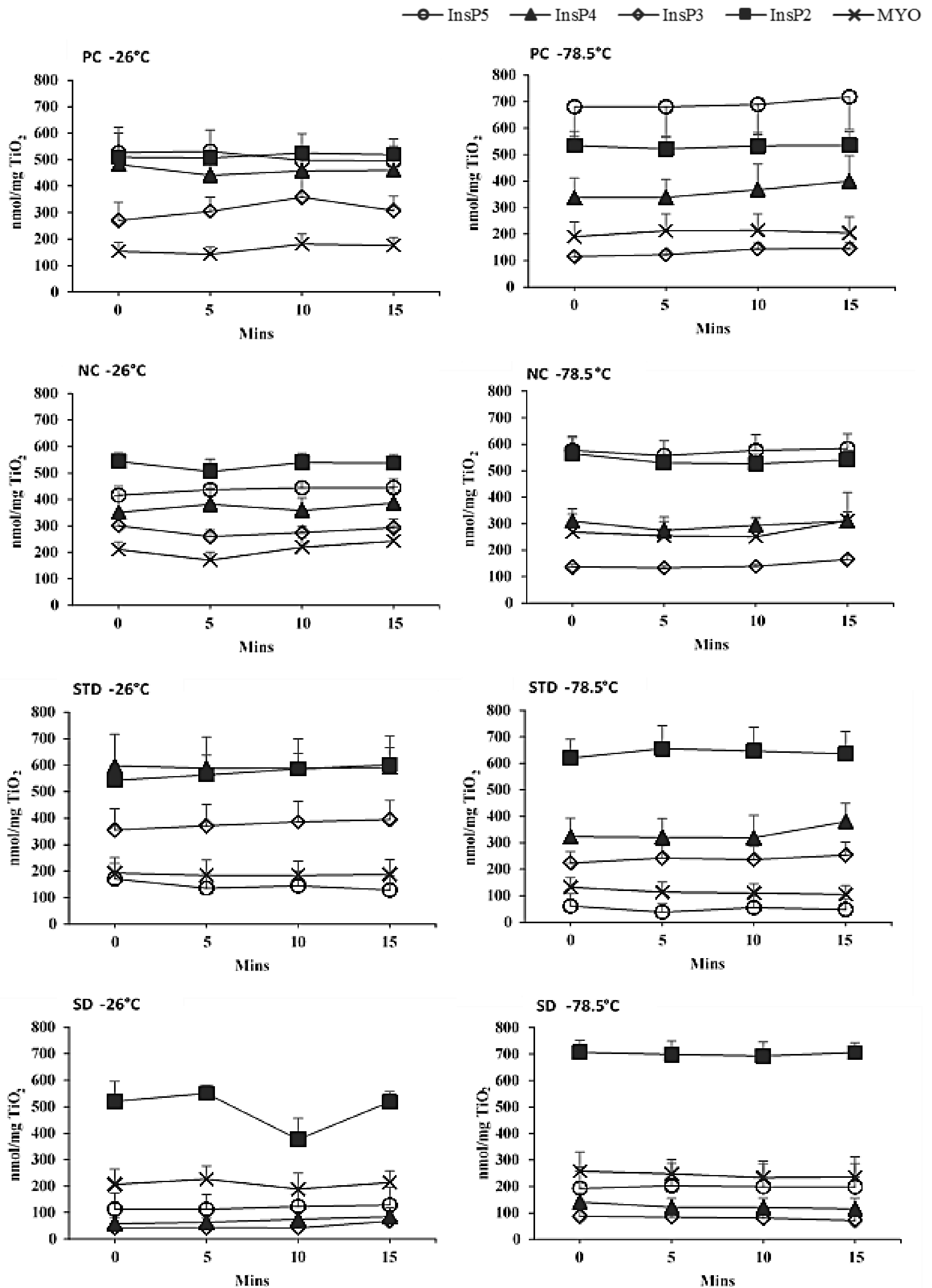

Figure A.3 Effect of time and freezing temperature on $InsP_{2-5}$ and MYO concentrations in the gastric digesta of pigs fed a nutritionally adequate diet (PC), a low Ca and P diet (NC), or the NC with 500 (STD) or 2,000 FTU/kg (SD).

Values are means of 10 observations $\pm$ SD. Time had no influence on $InsP_5$, $InsP_4$, $InsP_2$ or MYO concentration (P>0.05), but did influence $InsP_3$ concentration (linear, P<0.05).

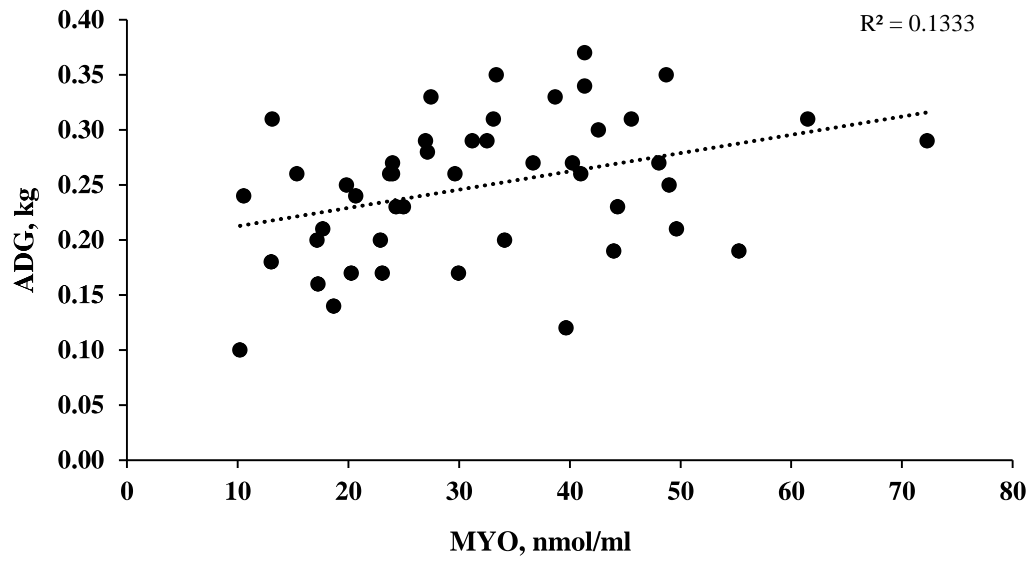

Figure A.4 Correlation between portal plasma MYO concentration and ADG in weaner pigs

Values represent data from individual pig (n = 48). Pearson product-moment correlation coefficient = 0.365; P-value = 0.012.