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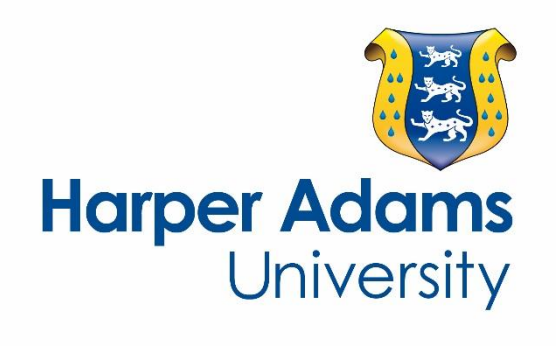

Strategies and efficacy of phytase supplementation and its interaction with pharmaceutical zinc oxide in newly weaned pigs

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Abstract

Feeding the World sustainably in the 21st century and beyond is perhaps one of the biggest challenges mankind has faced. Plant based pig feeds contain significant quantities of phosphorus, essential for animal health and performance, but which is locked-up in phytic acid and associated phytate salts (only around 30 % digestible). Since the 1990's, phytase enzymes have been added to non-ruminant feeds to enhance phytate digestibility. Recent evidence suggests that feeding super doses of phytase increases growth performance beyond that attributable to phosphorus release alone. In the UK, therapeutic levels of ZnO are often prescribed to prevent and treat post-weaning diarrhoea in newly weaned pigs, though some evidence suggests zinc may reduce the phosphorus releasing efficacy of phytase. Improving the efficiency of pig production, reducing environmental pollution and maximising the use of non-renewable resources may help contribute to a sustainable and secure global food chain. The objective of this study was to evaluate the optimal strategy for using phytase in weaner pig diets, in conjunction with pharmaceutical ZnO. Four live pig experiments were conducted to quantify the growth response to increasing dietary phosphorus, identify 'extra phosphoric' effects associated with phytase super dosing and to evaluate this response in ZnO medicated feed. Resulting recommendations are to super dose phytase (2000 - 8000 FTU/kg) for two weeks post-weaning; reducing to 1000 FTU/kg in the third week post-weaning to facilitate bone mineralisation. In this study, seemingly healthy pigs fed pharmaceutical ZnO at high levels (3100 mg/kg feed) showed reduced growth performance, possibly due to interactions between zinc and phosphorus. Further research into reducing levels of pharmaceutical ZnO in feed and the use of ZnO nanoparticles could help overcome these risks. There may also be justification for a higher inclusion of dietary digestible phosphorus in ZnO medicated diets post-weaning, especially in low phosphorus diets.

Declaration

I declare that this thesis has been composed entirely by the author and is a record of work undertaken following an original line of research. All help given by others has been acknowledged and no part or whole of this thesis has been previously submitted for an academic award by the author. This thesis contains no material that has been previously published, except where indicated or referenced and all quotations have been distinguished by quotation marks.

Stephen Mansbridge

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Published work

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Mansbridge, S.C., Mackenzie, A.M., Pirgozliev, V., Walk, C.L., Bedford, M.R., Wellock, I. and Stewart, A.H. 2015. The effects of dietary digestible phosphorous, phytase and zinc oxide on the growth performance of weaner pigs. *Advances in Animal Biosciences,* 6 (2), p. 234. (ISSN 2040-4700).

Mansbridge, S.C., Mackenzie, A.M., Pirgozliev, V., Skinner, L.S., Evans, N., Walk, C.L. and Stewart, A.H. 2014. Selection of reference genes suitable for normalisation of IFN-gamma and TNF mRNA expression by real-time SYBR Green polymerase chain reaction in porcine duodenum tissue. *Proceedings of the 2nd Annual International Conference on Veterinary Science Research*, 24th November, Singapore. pp. 28 – 31.

Mansbridge, S.C., Atkins, N and Mackenzie, A.M. 2014. Blood collection methods: Vacutainer® selection for serum or plasma mineral analysis (pigs). *Advances in Animal Biosciences*, 5 (1), p. 142.

Undergraduate Honours Research Projects

The following undergraduate research projects have been supported through various elements of this thesis and some data is reproduced or re-analysed in/from these publications:

Morris, K. 2016. *The effect of phytase on bone characteristics of weaned pigs, fed a phosphorus adequate diet: being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree in Bio-Veterinary Science*. Newport: Harper Adams University.

Evans, N.C. 2015. *The effect of dietary phytase and zinc supplementation on cytokine expression in the small intestine of weaner pigs: being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree in Bio-Veterinary Science*. Newport: Harper Adams University.

Newby, T.A. 2015. *The effect of increasing phytase doses on the growth, mineral digestibility and physiology of the weaner pig: being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree in Bio-Veterinary Science*. Newport: Harper Adams University.

Slater, E.R. 2015. *The effect of varying phytase levels on the growth performance and intestinal morphology of weaned pigs (3-7 weeks of age): being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree in Agriculture and Animal Science*. Newport: Harper Adams University.

Bosley, M.J. 2014. *The effect of phytase on the strength of bones in weaner pigs: being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree in Agriculture and Animal Science*. Newport: Harper Adams University.

Dooley, N.M. 2014. The effect of phosphorus, zinc and phytase on the immune system of weaner pigs: *being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree in Bio-Veterinary Science*. Newport: Harper Adams University.

Wilson, A. 2014. *The effects of phytase and zinc on the immune system of pigs: being an Honours Research Project submitted in partial fulfilment of the requirements for the BSc (Honours) Degree in Bio-Veterinary Science*. Newport: Harper Adams University.

List of Common Abbreviations

AMGP	Anti-Microbial Growth Promoter
ANF	Anti-Nutritional Factor
ANOVA	Analysis of Variance
ATTD	Apparent Total Tract Digestibility
BPPhy	β-propeller phytase
CAT	Clot Activating Tube
CC	Chemokine
CCK	Cholecystokinin
CV	Coefficient of Variation
DF	Daily Feed Disappearance
DFI	Daily Feed Intake
dgP	Digestible Phosphorus
DLWG	Daily Live Weight Gain
FCR	Feed Conversion Ratio
FTU	Phytase Units
GALT	Gut-Associated Lymphoid Tissue
GI	Gastro-Intestinal
GLP-1	Glucagon-like peptide-1
GOI	Gene of Interest
HAPhy	Histidine Acid Phytases
Hb	Hemoglobin
Hct	Hematocrit
HPLC	High Performance Liquid Chromatography
IAP	Intestinal Alkaline Phosphatase
ICP – MS	Inductively Coupled Plasma – Mass Spectrometer
Ig	Immunoglobulins
IL	Interleukin
iIELs	intestinal intraepithelial lymphocytes
INF	Interferon
IP	Inositol Phosphate
IP_6	Inositol Hexaphosphate
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LPS	Lipopolysaccharide
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin per Cell
MCV	Mean Corpuscular Volume
MHC	Major Histocompatibility Complex

MPV	Mean Platelet Volume
NC	Negative Control
NDV	Newcastle Disease Virus
NIR	Near Inferred
NLR	Nucleotide oligomerisation domain-like receptors
NMR	Nuclear Magnetic Resonance
NRQ	Normalised Relative Quantity
nPP	Non Phytate Phosphorus
NPT2b	Type 2 sodium-dependent phosphorous co-transporter
P	Phosphorus
p	p-value (α-level of significance)
P_i	Inorganic Phosphorus
PA	Phytic Acid
PAPhy	Purple Acid Phosphatases
PC	Positive Control
Pct	Procalcitonin
PDW	Platelet Distribution Width
PepT1	Peptide transporter 1
PP	Phytate Phosphorus
PPE	Potential Physical Energy
PRR	Pattern Recognition Receptors
PTH	Parathyroid Hormone
PTPhy	Protein Tyrosine Phytase
PYY	Peptide YY
QC	Quality Control
RBC	Red Blood Cells
RDW	Red Blood Cell Distribution Width
RG	Reference Gene
RLRs	RIG-I-like receptors
RT-qPCR	Reverse Transcription – quantitative Polymerase Chain Reaction
SD	Standard Deviation
SEM	Standard Error of the Mean
SED	Standard Error of the Difference of Means
SGLT-1	Sodium-dependent glucose co-transporter member 1
SIg	Secretory Immunoglobulin
STTD	Standardised Total Tract Digestibility
TGF	Transforming Growth Factor
Th	T-helper cell
THR	Thrombocytopenia

TiO_2	Titanium Dioxide
TLR	Toll-like receptors
TNF	Tumour Necrosis Factor
tP	Total Phosphorus
ZnO	Zinc Oxide
ZnO-np	Zinc Oxide – Nano Particles
$ZnSo_4$	Zinc Sulphate

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CHAPTER ONE

General introduction

1.0. The rising population

Feeding the World in the 21st century and beyond is perhaps one of the biggest challenges mankind has ever faced. The population of humans has exhibited unprecedented expansion in the last two hundred years, increasing from one billion in 1804 to six billion by 1999 (McQueen, 2000). Projected growth to the year 2100 by the United Nations (2011) suggests this trend will continue with an estimated increase to 10.1 billion. At the same time, there is a notable shift from those living in rural areas to urban environments (Figure 1). Those people living in urban areas tend to require more commercially prepared foods which are typically easier to transport but increase the requirements for grains, sugar and animal products, such as fats and proteins (McQueen, 2000).

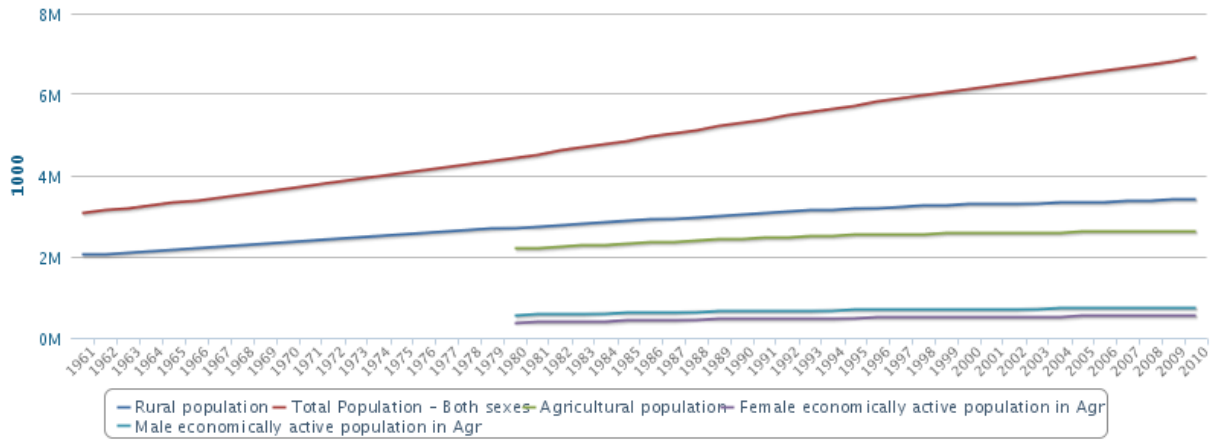

Figure 1: Global human population dynamics from 1961 – 2010 with trends indicated for rural and total populations, male and female economically active agricultural populations. (Source: FAOSTAT, 2012).

As the human population increases, so does the competition for natural resources such as nitrogen (N) and phosphorous (P), which are also attributed to both direct and indirect consequences on the global environment (Holdren and Ehlich, 1974). For example, N has been extracted from the air for industrial fertiliser and explosives since the invention of the Haber–Bosch process, in which, as Fritz Haber described in his 1920 Nobel Prize speech, “Gaseous nitrogen combines with gaseous hydrogen in simple quantitative proportions to produce gaseous ammonia” (Haber, 2002). It is this process, in combination with N mobilisation from processes like biomass burning, which contributes to huge amounts of N fixation in the terrestrial environment (Vitousek *et al.,* 1997). This N can lead to acidification and eutrophication of water courses (Vitousek *et al.,* 1997) and if it gets into drinking water – methaemoglobinemia (WHO, 2014).

1.1. Phosphorous

In the same way, P, arguably one of six unconditional ingredients for life on earth (Wolfe-Simon *et al.*, 2011; Schoepp-Cothenet *et al.*, 2011), follows a similar trend. In nature it tends to be found as the inorganic element which can be mined from the earth, or as organic constituents of animals and plants. In the latter case, it is largely found phosphorylating inositol in the form *myo*-inositolhexa*kis*phosphate (IP_6), where it can accumulate in the environment, contributing to eutrophication of watercourses (Turner *et al.,* 2002). Pigs have very limited endogenous phytase production in the stomach and small intestines, instead relying on exogenous phytase to catalyse the dephosphorylation of IP_6 (Schlemmer *et al*., 2001). The result is that 60 - 70% of dietary P from phytate containing crops, is excreted in farm slurry or manure where exogenous phytase is not supplemented (Lenis and Jongbloed, 1999). This is partly in the form of phytic acid (IP_6) or lower inositol phosphates from the incompletely digested cereals such as wheat and corn, with the remainder as inorganic P (Giles *et al*., 2011).

Total daily P intakes from pig feeds in the UK are estimated to be 60 tonnes, 83% of which is fed to fattening pigs (Table 1). With this in mind, a partial solution to the problem of P digestibility in pigs and poultry was put in to commercial practice in the 1990's in the form of dietary exogenous phytase enzymes (van Gorcom *et al*., 1991). These were initially derived from *Aspergillus ficuum* but have since been produced from a number of other sources including *Eschericha coil* and *Peniophora lycii* (Lei *et al*., 2013).

Table 1: Estimated daily phosphorus usage by UK pigs from feed sources.

Class of pig	Number of animals ($x10^3$)	DFI (kg)	Digestible P (g/kg)	Total P in feed (g/kg)	Total P intake/pig/ day (g)	Total P intake/day (Tonnes)
Pregnant sows	339	2.7	2.3	5.1	13.8	4.7
Lactating sows	68	8.0	3.2	7.1	56.9	3.9
Boars/gilts	98	2.8	2.4	5.3	14.9	4.5
All breeding pigs	**505**					**10.0**
Weaners	1080	1.01	3.8	8.4	8.5	9.2
Growers	1080	1.96	2.5	5.6	10.9	11.8
Finishers	1080	2.5	2.4	5.3	13.3	14.4
Bacon pigs	1080	2.8	2.2	4.9	13.7	14.8
All fattening pigs	**4319**					**50.1**

(Source: Adapted from DEFRA, 2014; Whittemore *et al*., 2003)

1.2. Phytase

In pig feeds, rates of 500 FTU/kg of microbial phytase are efficient at improving P digestibility, allowing for a reduction of 0.77 g/kg to 1.0 g/kg inorganic P in feed (Adeola *et al*., 2006; Jones *et al*., 2010). At spring 2015 prices, this equates to a UK wide saving of £18000/day from the substitution of dicalcium phosphate with phytase. A plateau in improved P digestibility is observed above 1000 FTU/kg supplemented phytase (Jones *et al*., 2010). Despite little extra P digestibility past this dose, 'extra phosphoric' effects have been reported, including enhanced feed conversion efficiency (Cowieson *et al*., 2011). Following a comprehensive review of phytase enzymes in pig diets, Selle and Ravindran (2008) conclude that further work to develop nutritional frameworks for phytase supplemented diets is still needed. Based on current population projections, we must continue to develop sustainable production systems that provide healthy food from healthy animals to meet the nutritional needs of humans in the 21st century. The concept of phytase super-dosing may thus be a useful strategy to incorporate in pig production systems, alongside phase feeding to reduce dietary P usage (Kebreab *et al*., 2012). Investigating phytase super dosing at weaning is of particular interest, due to the high levels of dietary P, rapid growth and changing gut environment.

1.3. Zinc oxide

Typical requirements of dietary zinc for modern EU pig genotypes is estimated to be 100 – 110 mg/kg feed (DM basis) for pigs around weaning, decreasing to 89 – 100 mg/kg feed (DM basis) during the first few weeks post-weaning (Whittemore *et al*., 2003; NRC, 2012). Pooled data from Europe (22 countries), however, suggests that the median level of zinc in piglet feeds is typically higher than this at 137 mg/kg feed (DM basis), close to the currently authorised total maximum content of zinc permitted in complete feed of 150 mg/kg (FEEDAP, 2014). At very high levels (1000 – 3000 mg/kg), zinc has been concluded to have therapeutic properties (FEEDAP, 2014) and pharmaceutical grade zinc oxide (ZnO) has therefore been licensed as a veterinary medicinal product for use in Europe. In the UK, PigZin Premix (Vm 19108/4000) and ZincoTec® (Vm 03941/4000) are both authorised for use in pig feeds on a veterinary prescription (Mounsey, 2016), and would normally be given either metaphylactically or therapeutically to prevent/treat post-weaning diarrhoea (PWD).

The bioavailability of ZnO for pigs is not well defined, with some estimates as low as 20% (Poulsen and Larson, 1995), however, availability of ZnO is typically 50 - 80% (NRC, 2012) so that the amount absorbed and retained from a typical diet supplemented with 2000 mg/kg ZnO is 27 % and 24% respectively (Buff *et al*., 2005). Based on an average feed consumption of 500 g/day (over a period of three weeks post-weaning), there would be approximately 950 mg of excreted zinc per pig per day. In a recent Danish study, excess

zinc was confirmed to be accumulating in the environment from the application of pig slurries/manure from ZnO medicated feeds to arable land (Jensen *et al*., 2016). In addition to disrupting the soil ecosystem (Duan *et al*., 2016), excess zinc poses the risk of leaching into aquatic environments, posing a risk of toxicity in some fish species. A review by FEEDAP (2014) however, suggests that while species such as trout may be able to tolerate reasonable levels of zinc in the environment, levels >20 mg/kg bodyweight may be detrimental. In addition to these risks, pharmaceutical levels of Zn supplementation has been shown to reduce the efficacy of phytase for releasing dietary P for pigs (Augspurger *et al*., 2004). Based on the stated risks and antagonistic effects, it may be desirable to restrict the use of high doses of pharmaceutical ZnO in pig production to therapeutic applications in clinical cases only.

1.4. Objective of the thesis

Based on the premise that there is now a body of evidence suggesting that super dosing phytase enzymes can enhance young pig production performance beyond that attributable to the release of additional dietary P from phytate, the aim of this thesis is to evaluate the best strategy for using phytase in newly weaned pig diets. This is done in the context of weaning being a critical time in the life of the pig, with multiple sources of stress, potentially leading to a “growth check” and/or post-weaning diarrhoea. Phytase will therefore be assessed in line with pharmaceutical ZnO as a standard, which is commonly added to pig feed immediately post-weaning in the UK to prevent adverse weaning associated events (e.g. PWD). Specifically, the hypotheses being tested in this thesis are:

1. Supplementing phytase in diets for weaned pigs at doses beyond that normally required for improving plant derived phytate phosphorus digestibility (super dosing) is an efficacious strategy for improving production performance in newly weaned pigs.

2. Metaphylactically ZnO medicated diets for seemingly healthy newly weaned pigs may need supplementation of addition digestible phosphorus or phytase to ensure optimal pig growth performance.

A schematic diagram of the experiments conducted are summarised in Figure 1.2.

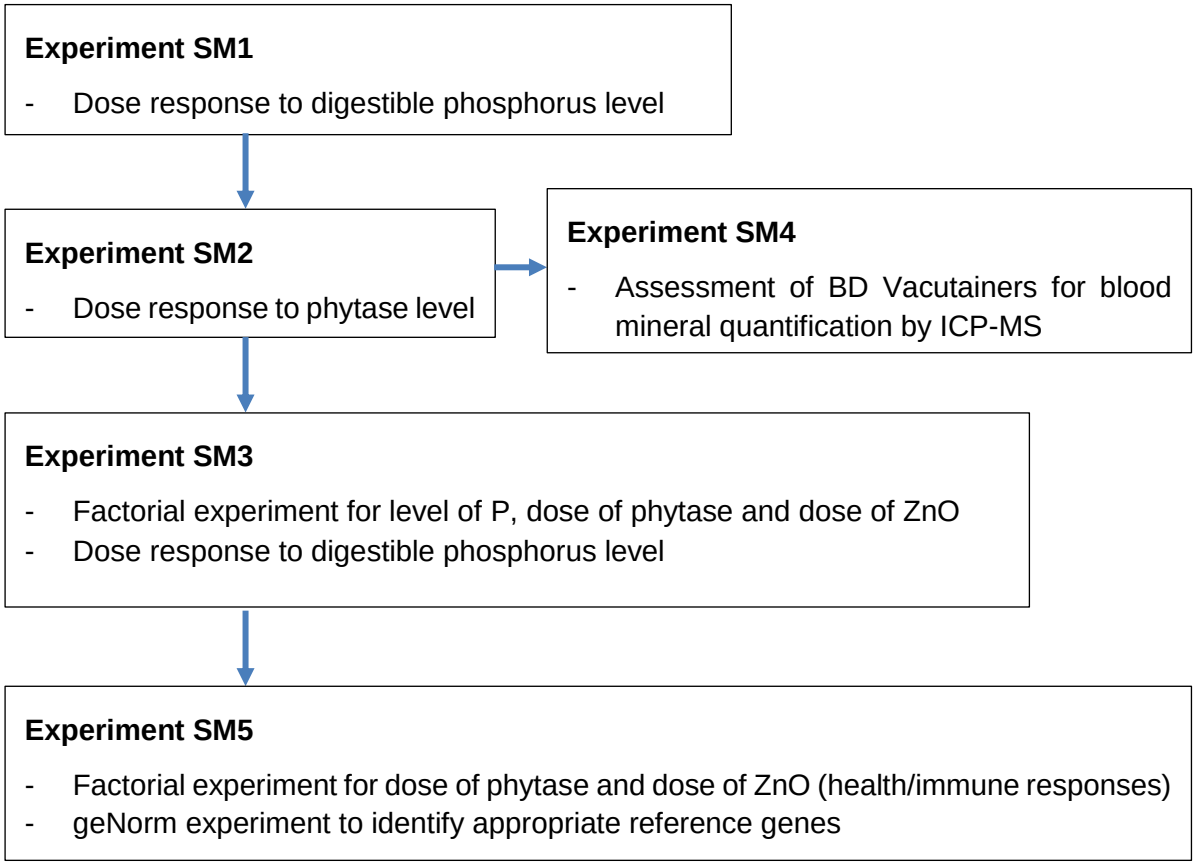

Figure 1.2: Schematic overview of the experiments conducted in this thesis.

CHAPTER TWO

Literature review

2.0. Introduction

This chapter reviews published literature on the nutritional requirements of the pig post weaning in response to the complex physiological changes and stresses during this period. It examines past and current thinking around phytase enzyme inclusion in weaner pig diets, starting with the traditional approach of reducing environmental pollution by enhancing phosphorus digestibility, and concluding with areas for developing potential new approaches.

2.1. Weaning

The definition of weaning is one of complexity and as such, has been extensively debated. In simple terms, the Concise Oxford English Dictionary (2002) states:

"**wean**[1] v. – accustom (an infant or other young mammal) to food other than its mother's milk"

This implies that weaning is unique to mammals and fails to adequately describe the behavioural and physiological changes occurring at this time. A different approach to re-define weaning was taken by Martin (1984), who also recognised the limitations of this definition and so proposed a general model based on the changes in parental care as a function of the age of the offspring (Figure 2.1). Counsilman and Lim (1985) agreed that the model proposed in Martin's revised definition was a useful concept but objected to a change from the dictionary definition. Interestingly, their objection was based on a now out-dated definition they gave from the Shorter Oxford English Dictionary (1964, 3rd ed.) which added ";to cause to cease to be suckled" to the end of the current definition.

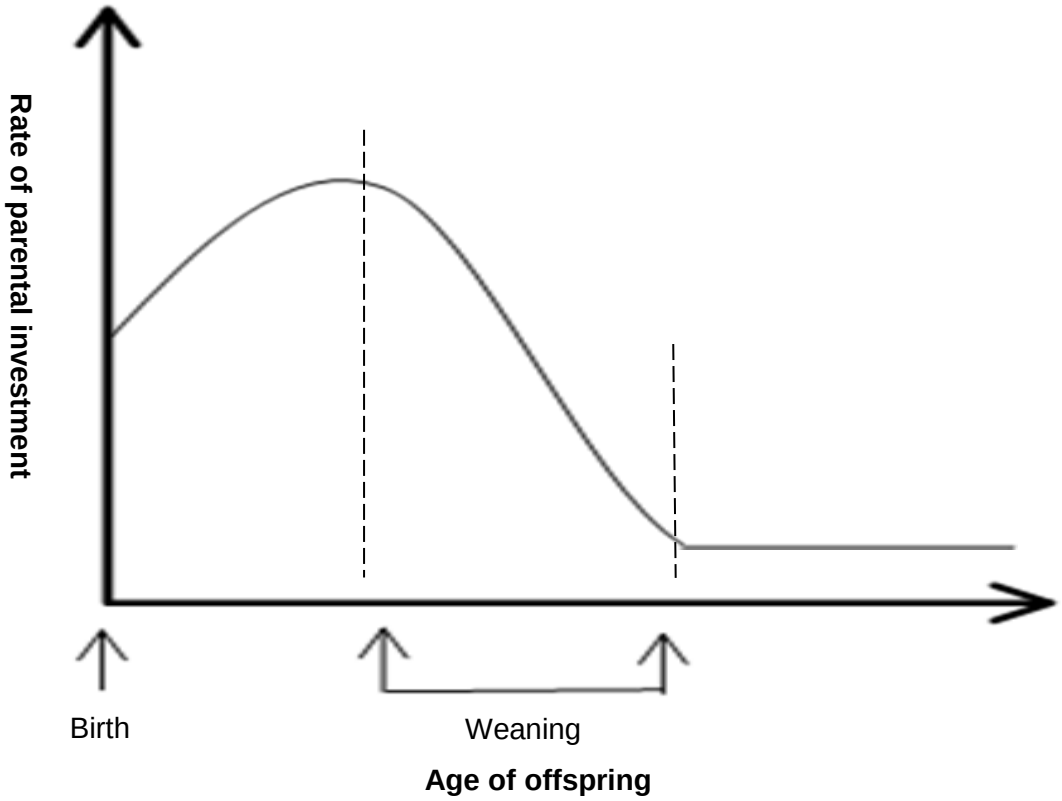

Figure 2.1: General relationship between the rate of parental investment and the age of the offspring. The period of weaning is bounded by the two principle points of inflection. (Source: adapted from Martin, 1984).

In response, Martin (1985) defended his argument that weaning could be termed as the period during ontogeny when the rate of parental investment drops most sharply. Furthermore, he criticised their focus on suckling, since his view was that parental care encompasses more than just providing milk. It is this point that complicates how we measure weaning; as Martin (1985) goes on to offer examples of parental care such as providing solid food, supporting social interactions and defence against predication. How exactly parental care is measured is therefore up for discussion but Martin (1985) also adds that weaning is accompanied by behavioural and physiological changes. Thus, Figure 2.1 would appear to be lacking in full detail. In defining the weaning process, several questions with regards to the length of transition and the general changes observed in weaner pigs must be answered.

2.1.1. Weaning the young pig

In pigs, weaning tends to be initiated by the sow, occurring gradually with a marked increase of supplemental feed intake from about week five and declining suckling periods until complete weaning at 11-12 weeks in indoor systems (Bøe, 1991). This can be even longer in semi-natural environments, with mean weaning at around week 17 (Jensen and Recén, 1989). The difference between an indoor commercial system where temperature and humidity are largely controlled and an outdoor semi-natural environment may

account for the variation in weaning length. However, in both studies it was noted that there was a cost-benefit conflict between mother and offspring. Sows increasingly terminated suckling as time progressed and preferred to be suckled lying down initially but progressively reverted to standing. This ties in with the prediction of Martin's (1984) model that parental care sharply declines during the natural weaning process.

In commercial and experimental situations it is rare that this gradual process is followed, rather, the young pig is abruptly separated from the dam at a set point in time. Segregated early weaning is a system commonly found in the USA where piglets are weaned by removal and separation from the sow at around seven – ten days of age. Performance data of early weaned pigs (at day seven) also lends weight to the idea that these systems are sub-optimal, with reportedly lighter piglets at six weeks ($p<0.05$), than those weaned at 14 or 28 days (Worobec *et al.*, 1999).

A similar study by Patience *et al.* (2000) found that at six weeks, pigs weaned at 12 days and kept on the same site as the sows were not heavier than those weaned at 21 days ($p=0.05$). While this is in good agreement with the results of Worobec *et al.* (1999), early weaned pigs (at day 12) that were moved off-site weighed more at day 56 ($p<0.05$) than those weaned at 21 days (Patience *et al.,* 2000), indicating weaning age is only one of the factors affecting growth at this time. Furthermore, Patience *et al.* (1999) report that early weaning followed by rearing off-site continues with heavier pigs ($p<0.01$) at day 56 than those weaned at 21 days. However, they also found that early weaned pigs remaining on-site end up lighter ($p<0.01$) than the later weaned pigs by 0.8 kg. Multisite production may then become a useful management tool for segregated early weaning.

It is apparent from these studies that performance is multifactorial, however, growth performance is not the only measure applicable to finding the optimal age to separate sow and piglet. Pigs weaned at seven days have also been observed to perform more belly-nosing ($p<0.05$) on pen mates than those weaned later (Worobec *et al.*, 1999), suggesting that behavioural changes should also be taken into account. Given the ethical considerations and abnormal behaviour exhibited by early weaned pigs, animal welfare legislations (The Welfare of Farmed Animals [England, 2007] and [Scotland, 2010] Regulations) require weaning to be postponed until at least 28 days of age or 21 days if the piglets are moved to clean and disinfected housing, which is separate to that of the sow. In practice, this means that the UK pig industry tends to abrupt wean around day 27, which is in line with the EU average (BPEX, 2012).

Main *et al.* (2004) lends further support to the UK strategy of later weaning as they found that multisite production in early weaning systems is still no substitute for increasing weaning age to three weeks. In their series of trials, they weaned pigs at 12, 15, 18 or 21 days of age and found a linear improvement in average daily gain ($p<0.002$) and day 42 weight ($p<0.001$). They calculated that this resulted in an increase in weight sold per pig weaned of 1.80±0.12 kg for each day weaning was extended. Moreover, when they compared simple and complex diets (containing more lactose and plasma) with weaning age, there were no differences in daily gain or 42 day weight. Extending the weaning age past four weeks to six or even eight weeks raises another set of problems. Whereas early weaning is bad for the piglet, a collaborative study by DEFRA (2007) over a three year period, predicted that later weaning effectively reduced sow yearly litter size by four pigs. While some significant improvements to piglet performance were identified immediately post-weaning, they did not find any differences by the time the pigs reached slaughter weight. On balance, weaning at 21 - 28 days would appear to be an appropriate strategy that benefits both the piglet and the sow.

2.2. Transition period

The transition from milk to solid/semi-solid feed is clearly a key feature of weaning in mammals, resulting from the reduction of parental investment. Formulated solid feed fed post-weaning is lower in fat and denser in carbohydrates than milk due to the cereal content (Table 2.1). Liquid feeds can be fed as an alternative to dry feed but must be adjusted to ensure adequate dry matter (DM) provision.

Table 2.1: The composition of sow milk, creep feed and starter diet

Composition	Sow milk[1]	Creep feed[2]	Starter feed[2]
Dry Matter %	20.7	88.0	88.0
Fat %	10.4	13.0	6.5
Protein %	4.8	20.0	21.5
Lactose %	5.8	-	-
Digestible Energy (MJ)	6.1	15.0	15.0

(Source: adapted from [1]Atwood and Hartmann, 1992 and [2]Primary Diets, not dated).

There is evidence that pigs are able to influence their DM intakes by regulating their consumption of water in relation to the DM content of their feed (Geary *et al*., 1996). This may be a natural response, since if feed is restricted post-weaning, the gut will not develop fully in terms of anatomy (smaller stomach and SI tract), morphology (shorter villi height and deeper crypts due to a lack of energy and physical stimulus) and will have lower digestive functionality due to reduced digestive enzyme activity (Kelly *et al*., 1991; Dong and Pluske, 2007). Dry matter intakes during this weaning period are thus not only key to ensuring adequate nutrient provision, but influence how the gut develops.

2.2.1. Gut physiology

Lining the whole gut lumen are long finger like projections called villi, which provide a large absorptive area for the transport of nutrients into the blood via the brush border membrane coating them (Mosenthin, 1998). Using scanning electron microscope images from birth to 21 days post-natal, Skrzypek *et al*. (2007) reported that duodenal villi at birth were folded (forming transversal furrows) but these disappeared as the length of villi increased and flattened. Jejunum villi also flattened out with active extrusion zones whereas, ileal villi started out flat with many large lysosomal vacuoles but had developed into different shapes by day seven, depending on the crossbreed examined. Polish Landrace / Pietrain showed a range from finger-like to tongue-like, but Duroc / Hampshire / Wild Boar crossbreeds developed only short finger-like ileal villi. Even after weaning, Rubio *et al*. (2010) reports that in a 15 kg pig, the duodenal and jejunal villi height are similar but that ileal villi are significantly shorter. Rubio *et al*. (2010) also found differences in overall villus height and surface area between Landrace/Large White and Iberian breeds. Taken together with the results of Skrzypek *et al*. (2007), their work suggests different crossbreeds may be better at absorbing nutrients depending on how their GI tract develops.

At the base between the villi are mucosal glands (crypts) which function to produce all the intestinal epithelia cells required to maintain a constant rate of cellular turnover (Kraehenbuhl *et al*., 1997). The emerging enterocytes are fully capable of producing a range of proteins as they emerge from the crypts (Fan *et al*., 2001) having already differentiated into one of four terminal cell types (Chandrasekaran *et al*., 1996). Protein/biological activity then increases the further the enterocytes migrate up the villus away from the villus/crypt junction (Brown *et al*., 1999; Fan *et al*., 2001), before cell death by apoptosis at the tip of the villi. The rate of migration up the villus is constant along the small intestines in the neonatal pig, however, the lifespan of these cell increases from around 4.7 days in the proximal segment to around 10.2 days in the distal region (Fan *et al*., 2001). Differences in regional functions may account for this discrepancy.

During weaning, major histophysiological changes occur in the gut of the pig (Lallès and Salmon, 1994, Pluske *et al*., 1997). The most distinctive changes were described by Boudry *et al.* (2004) who killed piglets at two, five, eight and 15 days after weaning (day 21), before dissecting and measuring various parts of their intestines. They found that villous height in the proximal jejunum significantly decreased by 40 % from two days post-weaning but began to recover by day 15 (77 % of pre-weaning height). Additionally, crypt depth in the proximal jejunum and distal ileum significantly increased by day five post-weaning. Even when weaning was postponed to weeks four and six, Miller *et al.* (1986) reported a shortening of villi and increasing crypt depth along the length of the

small intestines at day five. Similar results were presented by Spreeuwenberg *et al.* (2001) who also observed a significant decrease in the mean ratio of villus height to crypt depth (2.25 vs 1.80) over four days post-weaning.

Since Skrzypek *et al*. (2007) found that villi had fully extended by 21 days post-partition, it is likely that the reduction in villus height is attributed to an increase loss of cells as they shed into the lumen. This may also explain the increase in crypt depth as they have to expand to keep up with cell proliferation to maintain villus height. While these physical changes to the tissue structure of the small intestines post-weaning may contribute to a reduced absorptive surface area (Pluske *et al*., 1997), changes to digestive function and absorption may be a more profound problem (Miller *et al*., 1986; Kelly *et al*., 1991).

2.2.2. Digestive and absorptive functions

The intestinal brush border membrane comprises of a microvilli layer lining the apical surface of enterocytes originating from the crypts (as previously described in section 2.2.1), which acts to facilitate the transfer of nutrients from the gut lumen to the interstitial fluids and blood. The microvilli overlay a fibrillar cytoskeleton, which interacts as a support and regulatory structure (Holmes and Lobley, 1989) allowing intracellular entry of nutrients. These migrate through the cell to the basolateral membrane for absorption into the blood. A potent cocktail of enzymes and proteins are secreted on the brush border membrane (Fan *et al*., 2001; Rubio *et al*., 2010), which aid in digestion (brush border enzymes) and absorption (transporters). Activity of the digestive enzymes vary depending on location along the small intestines, with the highest activity of lactase, sucrase, maltase and isomaltase found in the jejunum of 15 kg pigs (Rubio *et al*., 2010). Both aminopeptidase and alkaline phosphatase activity were highest in the mid-distal regions. The concentration of these enzymes were also shown to vary depending on breed (Rubio *et al*., 2010), not unlike the differences in gut morphology previously described.

Passive absorption of nutrients occur by diffusion between epithelial cells via the tight junctions (Figure 2.2), as well as by endocytosis (Wijtten *et al*., 2011). Mineral ions and other nutrients (such as glucose and amino acids) can also move across the brush border membrane by active transport (Figure 2.2).

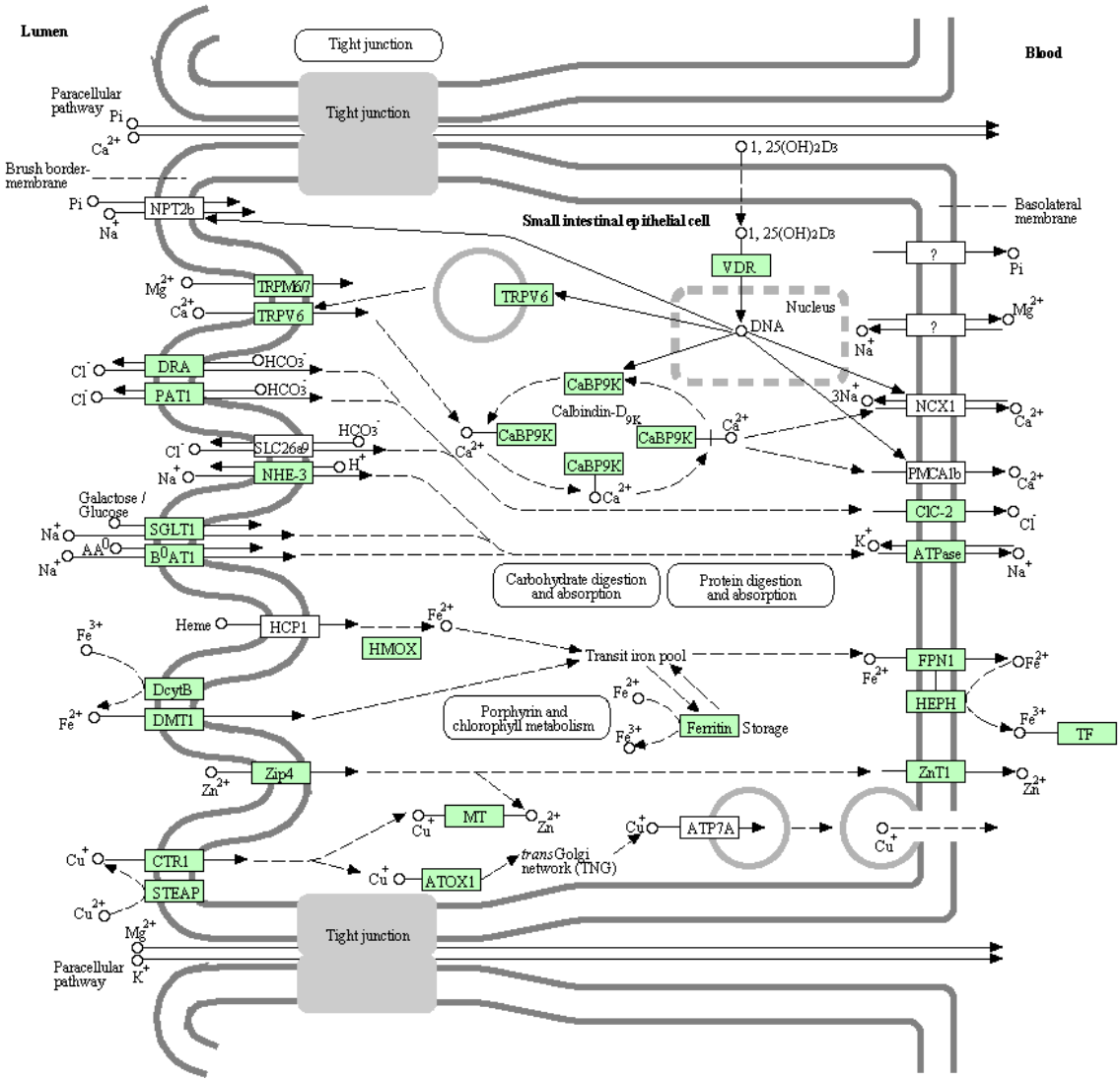

Figure 2.2: Pig nutrient absorption pathways through paracellular routes via tight junctions and intracellular routes by transporters in the small intestines.

(Source: Adapted from KEGG, 2013)

Many of the active transport mechanisms are co-dependent on multiple nutrients. For example, glucose and phosphorous (to a certain extent) are dependent on the transport of sodium (Figure 2.2) via sodium-dependent glucose co-transporter member 1 (SGLT-1) and type 2 sodium-dependent phosphorous co-transporter (NPT2b). Before weaning, there is a constant active transport of Ca and P across the brush border membrane, however, post-weaning this is dependent on calcitriol status (Schröder *et al.*, 1998). Just like the digestive enzymes, these active transporter genes are not constantly expressed along the length of the intestine (Figure 2.3), indicating they may have localised functionality.

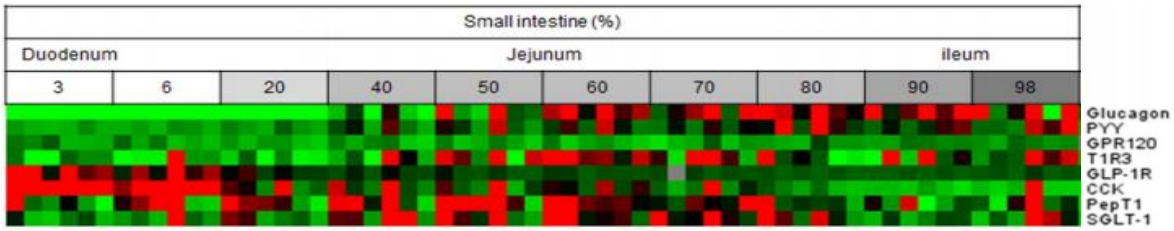

Figure 2.3: Heatmap of pig gene expression results of eight selected targets. Green and red indicate low and high gene expression respectively compared to the average expression. (Source: Adapted from van der Wielen *et al*., 2014).

Peptide transporter 1 (PepT1) shows the highest expression in the proximal and mid small intestines whereas, sodium-dependent glucose co-transporter member 1 (SGLT-1) up-regulation tends to be confined to the jejunum only (Figure 2.3). In contrast, gut hormones such as glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK) are expressed largely in the proximal small intestines but glucagon and peptide YY (PYY) are found mid to distal regions (Figure 2.3). This localisation of transporter expression level is consistent with site dependent digestive enzyme activity (Rubio *et al*., 2010), chemosensing (Mace and Marshall, 2013) and nutrient absorption site (Table 2.2).

Most of the nutrient absorption is localised to the duodenum and jejunum with the exception of magnesium, which is reported to be absorbed mainly from the hind gut (Table 2.2). New born pigs have also been shown to have a considerable net electrogenic ion transport across duodenal brush border membrane compared to those suckling or weaned (up to a 75% reduction in short circuit currents) and show a significant reduction in tissue conductance post-weaning (Schröder *et al*., 1998). This indicates that the tight junctions are more permeable post-weaning; confirmed by a significant increase in mannitol (used as a marker) transport from two days post-weaning (Spreeuwenberg *et al*., 2001).

Table 2.2: Sites of mineral absorption in the porcine intestine

Nutrient	Major site of absorption	Mechanism	Source
Phosphorous	Jejunum	Sodium dependent active transport, calcitriol and passive diffusion	1, 2, 6
Sodium	Jejunum and ileum	Sodium-dependent transporters	3, 6, 7, 11
Calcium	Active transport along duodenum and jejunum. Passive transport along whole tract.	Calcitriol dependent active transport and passive transport in high Ca diets	2, 4, 6
Zinc	Duodenum and jejunum	Zinc specific transporters and peptide co-transporters	5, 6, 8
Potassium	Whole tract	Passive diffusion via tight junctions	6, 7, 11
Iron	Duodenum	Active transporters and active transport of Heme	6, 10
Magnesium	Colon and caecum	Mainly passive diffusion, some via tight junctions but some active transporters	6, 9

[1]Saddoris *et al*. (2010); [2]Schröder *et al*. (1998); [3] van der Wielen *et al*. (2014); [4]Bronner (2003); [5]Tacnet *et al*., (1993); [6]KEGG (2013); [7]Nabuurs *et al*. (1996); [8]Hill *et al*. (1987); [9]Milinković-Tur *et al*. (2001); [10]Furugouri and Kawabata (1976); [11]Leddin (1991).

Wijtten *et al*. (2011) reviewed the literature around intestinal barrier function post-weaning and concluded that low feed intakes at weaning led to reduced villus height. Low feed intake also resulted in compromised proximal intestinal barrier function but weaning in general had no effects on distal barrier function. Supplementing solid feed during the suckling period has been shown to prevent the normal reduction in small intestine villus height and net fluid absorption four days post-weaning, compared with those left on the sow or weaned without supplemental feed (Nabuurs *et al*., 1996). In a different strategy, cow's milk has previously been used to ensure nutrient supply was maintained immediately post-weaning and also effectively prevented villus atrophy (Pluske *et al*., 1996). Both approaches support the need for adequate nutrient provision at weaning.

2.2.3. Gut immunology

The gastro-intestinal (GI) tract forms a complex interface where substances cross from the environment into the body. This is clearly vital for the absorption of nutrients but also exposes the animal to the risk of pathogens. The pig has evolved a layered defence system to protect against infection, consisting of physical barriers, generic innate mechanisms and specific pathogen acquired immunity. The physical intestinal barrier is composed of intestinal epithelial cells (as already discussed), their associated mucins (Oswald, 2006), diffuse intestinal intraepithelial lymphocytes (iIELs) and dendritic cells (Christ and Blumberg, 1997). These all play active roles in immunity above the basolateral membrane (Christ and Blumberg, 1997). They are able to sense antigens using pattern recognition receptors (PRR) such as Toll-like receptors (TLR), Nucleotide oligomerisation domain-like receptors (NLR) and RIG-I-like receptors (RLRs) (Bailey, 2009; KEGG, 2014). Ligation of PRR can up-regulate the transcription of a variety of cytokines within the micro-environment (Oswald, 2006; Bailey, 2009; KEGG, 2014). These cytokines also act to signal other classes of immune cells (Table 2.3) and can induce an acute phase response.

Table 2.3: Summary of cytokines and chemokines secreted from pig intestinal epithelial cells and their usual target for interaction

Cytokine/ Chemokine	Usual target
IL-8, TNF (α/β), CXCL1, CXCL2, CXCL5	Neutrophils
CCL28, CCL5	Eosinophils, Lymphocytes
CCL11	Eosinophils
GM-CSF, CX3CL1, CXCL9, CCL2	Monocytes
IL-1 (α/β), IL-6, IL-7, IL-10, IL-12, IL-15, IL-18, TGF-β, CXCL10, CCL22, CCL25, CCL7	Lymphocytes
CCL20	Dendritic cells

(Source: Oswald, 2006)

Mucosal layer thickness is greater in the ileum verses the jejunum or duodenum (Rubio *et al*., 2010), where in addition to the array of frontline defences, special M (microfold) cells are able to transport luminal antigens to the gut-associated lymphoid tissue (GALT), underlying the basolateral membrane (Jung *et al*., 2010). GALT, is formed of either isolated or aggregated lymphoid follicles (Peyer's patches), with the former found in the jejunum and the latter in the ileum in the pig (Pabst and Rothkötter, 1999). Since the ileum contains larger continuous Peyer's patches compared to the mid and proximal small intestine (Figure 2.4), the distal region is likely at higher risk of pathogenic bacterial challenge from the hind gut.

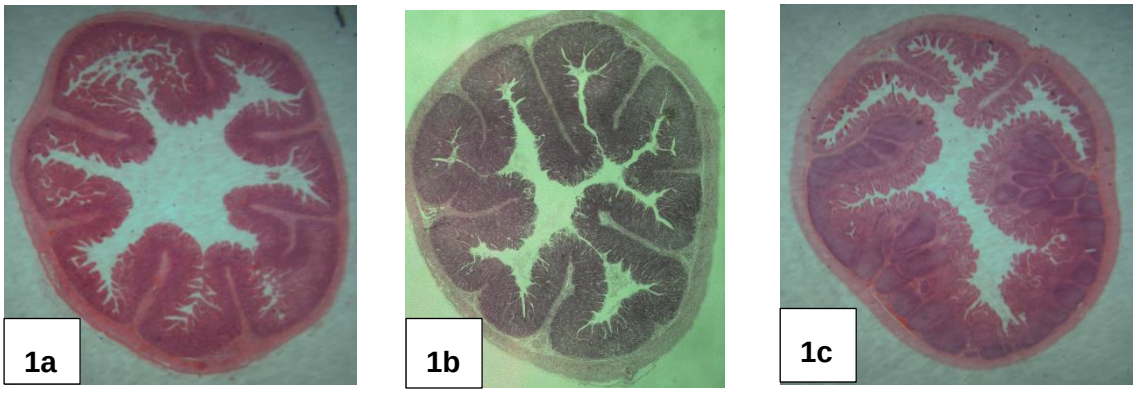

Figure 2.4: comparison of small intestines of pigs three weeks post weaning (1a = duodenum, 1b = jejunum, 1c = ileum). Villi can be seen lining the gut lumen in all three sections and Peyer's patches are visible as dark purple in the ileum sections.

In intestinal section 1c (Figure 2.4) Peyer's patches (stained purple) are easy to see as they are found as a continuous follicle in the ileum whereas, those located along the jejunum are found as discrete packages and have been missed in the 1b intestinal section. The distribution of both T cells and others such as macrophages and dendritic cells in these Peyer's patches are organised, with $CD2^+/CD3^+$ receptors seen throughout the structures but sIg receptors only expressed in the follicles (Makala *et al*., 2001). Once transported to the GALT, antigens further stimulate an immune response by the interactions of professional immune cells and antigen presenting cells (Figure 2.5).

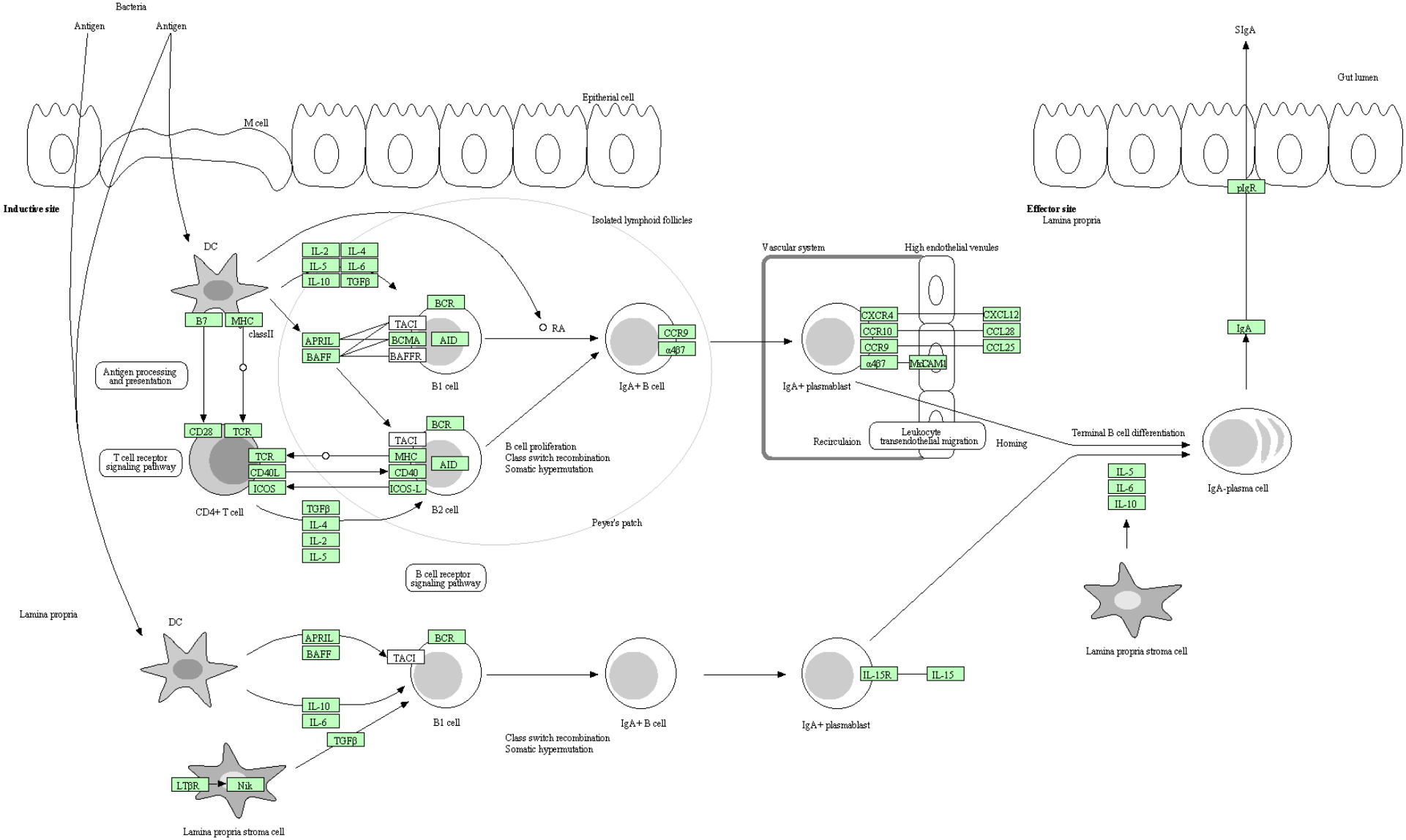

Figure 2.5: Pig Intestinal immune network for IgA production (Source: Adapted from KEGG, 2010)

The importance of interactions between the cells of both the GALT and those above the basal membrane to orchestrate an appropriate immune response is clearly seen in Figure 2.5 and relies on cell to cell communication. In fact, only capillary endothelial cells and those of the lamina propria express major histocompatibility complex (MHC) class two receptors in pig intestine, with those of the iIELs ($CD2^+$) staining negative (Wilson *et al*., 1996). Despite the lack of MHC II on iIELs, Inman *et al*. (2010) hypothesise that endothelial cells in the mucosal layer of the neonatal pig are able to interact directly with T cells, as well as with dendritic cells located in the lamina propria, which in turn, are also capable of $CD4^+$ Th cell proliferation and polarisation via MHC II. Furthermore, in the pig, most cells of the Peyer's patches are antibody producing B type as opposed to T cells, unless the pigs are kept germ free (Pabst and Rothkötter, 1999). This indicates that the presence of bacterial microbiota play a key role in conditioning the GI immune system. It is possible that the increase of B cells relative to T cells may be due to colonisation of the gut with probiotic bacteria, since oral dosing of some lactic acid bacteria in mice has been shown to increase B cells but not $CD4^+$ T cells (Perdigón *et al*., 1999).

The mechanism for B and T cell activation is, at least in part, mediated by the production of IL-6, where in mice, non-pathogenic bacteria do not stimulate enough IL-6 to induce intestinal epithelial cells to produce IFN-γ or IL-12, unlike pathogenic strains which do (Vinderola *et al*., 2005). If IL-6 production is excessive and is in combination with TGF-β and IL-23, then naïve T cells may be triggered to express the Th17 phenotype (Vernal and Garcia-Sanz, 2008). At least in mice, these cells are largely controlled by the small intestines, with the highest populations found in the duodenum, and declining numbers the further along the tract they are measured (Esplugues *et al*., 2011). This makes them an important target in the understanding of GI immunology.

Birth and weaning are both critical points in the development of the intestinal immune system as at these two points, the gut must 'learn' to distinguish between harmful antigens and safe nutrients. The neonatal pig soon develops a full complement of immune cells after birth in pre-formed Peyer's patches (Pabst and Rothkötter, 1999; Makala *et al*., 2001), and is supported by an external supply of maternal immunoglobulins (IgG) and immune cells from colostrum for the first 24 hours post-parturition (Dividich *et al*., 2005; Bandrick *et al*., 2014). Their diffuse gut immune system normally develops in stages and is complete within six weeks of life (Lallès *et al*., 2007b). At weaning, the composition and range of protein ingestion from food and gut microbiota change dramatically again from those at birth and the immune system must adapt again. Lymphocyte blastogenesis assays have previously suggested that weaning pigs below five weeks of age negatively affects pig cell-mediated immunity (Blecha *et al*., 1983).

Strong cell-mediated immunity would suggest an immune system that is able to rapidly respond to an immune challenge, thereby limiting potential damage from an invading pathogen and may explain some of the difference observed in growth performance. This is in line with commercial practice of weaning from week four.

Evidence suggests that in the first two days post-weaning, there is a significant reduction in the ratio of $CD4^+/CD8^+$ in the jejunum but this seems to recover by the fourth day (Spreeuwenberg *et al*., 2001). Interestingly, Parra *et al*. (2013) measured the expression of pro-inflammatory cytokines: TNF-α, IL-8 and IL-18 in the intestines of newly weaned pigs and demonstrated a significant increase in the expression of all three by the fifth day post weaning and a subsequent decline thereafter. They propose that the elevation in these cytokines may contribute to post-weaning diarrhea. These changes in T cell populations may be partially the result of the neonatal microenvironment, since Inman *et al*. (2010) found that in contrast to adult pig intestines, neonatal endothelial cells played a role in presentation of antigens to T cells, in addition to the classical $CD16^+$ dendritic cell/T cell presentation. Given the increased permeability of the intestines to antigens post-weaning (Spreeuwenberg *et al*., 2001), and the ability for professional and endothelial cells alike to present antigens, it is not surprising T cell phenotype ratios are dynamic. Post-weaning, small intestinal alkaline phosphatase (IAP) digestive capacity and maximal enzyme specific activity are both reduced (Lackeyram *et al*., 2010). The reason for this is unclear but Lackeyram *et al*. (2010) discuss a range of factors which together, seem likely to contribute to a down regulation of IAP transcription. The distal GI tract may then be more vulnerable to the effects of bacterial antigens stimulating TNF-α via LPS mediated TLR4 activation, since IAP plays a role in dephosphorylating LPS, thus helping maintain intestinal barrier function (Lallès, 2010).

These combined challenges at weaning often lead to a multi-factorial temporary growth check (Lallès *et al.*, 2007a), which is pronounced in abrupt weaning (Berkeveld *et al.*, 2007) but apparently mitigated in gradual weaning systems (Bøe, 1991). The transition period is thus a critical time in the life of the pig that needs careful management.

2.3. Nutrient requirements post-weaning

Nutrient requirements must be met through the appropriate selection of dietary ingredients. The availability of these ingredients tend to be influenced by crop production in the various countries (Table 2.4), since those crops not grown locally must be imported.

Table 2.4: 2010 crop production of likely pig feed ingredients in the UK and USA

Crop	United Kingdom 2010 Production (millions of tonnes)	United States of America 2010 Production (millions of tonnes)
Barley	5.3	3.9
Maize	N/A	316.2
Soybeans	N/A	90.6
Wheat	14.9	60.1

(Source: FAO, 2012)

Wheat based diets for pigs are common in the UK, whereas maize-soybean diets are prevalent in the USA, reflecting the production patterns of Table 2.4. This factor must be considered when diets are formulated, since the standards are calculated using prediction models, validated through experiments. The diets fed in these experiments are likely to differ depending on which area of the world the research was conducted in, and thus which cereal base was used.

Recommendations concerning the nutrient and energy requirements of the young pig were set out by the British Society of Animal Science (Whittemore *et al.,* 2003) and the National Research Council of the National Academes (NRC, 2012). These concise documents are drawn up using a range of scientific publications and can thus be regarded as good compilations of our knowledge and understanding of pig nutrition. Even so, the former standard is now over a decade old, and while they are of high quality information, neither takes into account the health status of the herd or the breeding of the pigs.

Aside from the key macro nutrients (fats, protein and carbohydrates) found in compound pig feeds, minerals are known to play many significant roles in body maintenance and growth. Unlike protein, carbohydrates and fats; minerals do not carry a potential physical energy (PPE) value, since they are independent of the digestible organic fraction of feed (Boisen, 2007). However, they are required to be present in significant quantities in the pig's diet (Table 2.5) to prevent deficiencies that would otherwise result in clinical disease (e.g. hypercalcemia and anaemia).

Table 2.5: Dietary mineral requirements of the weaner pig on a dry matter basis

Nutrient	BSAS recommendation (units/Kg [air dried] feed) 10 – 30 kg BW	NRC recommendation (units/Kg [DM] feed) 11 – 25 kg BW
Calcium (Ca)	8.0 g/kg	7.8 g/kg
*Phosphorus (P)	3.8 g/kg	3.2 g/kg
Sodium (Na)	2.0 g/kg	3.1 g/kg
Potassium (K)	2.5 g/kg	2.9 g/kg
Magnesium (Mg)	0.4 g/kg	0.4 g/kg
Chloride (Cl)	2.0 g/kg	3.6 g/kg
Zinc (Zn)	100 mg/kg	88.9 mg/kg
Manganese (Mn)	30 mg/kg	3.3 mg/kg
Iron (Fe)	120 mg/kg	111.1 mg/kg
Cobalt (Co)	0.2 mg/kg	-
Iodine (I)	0.2 mg/kg	0.2 mg/kg
Selenium (Se)	0.2 mg/kg	0.3 mg/kg
Copper (Cu)	6.0 mg/kg	5.6 mg/kg

*Digestible phosphorus, (Source: adapted from Whittemore *et al.,* 2003 and NRC, 2012)

The chemical form each mineral is provided in and the presence of modulators such as enzymes and anti-nutritional factors (ANF) will affect how the gut can metabolise and ultimately use these elements.

2.3.1. Phosphorus and Calcium

Phosphorus (P) and calcium (Ca) are essential inorganic elements that play diverse roles within the body. Balance studies have shown that absorption of Ca is largely regulated in the pig small intestines, since urine contains little Ca when dietary Ca intake increases, but that P is only partly regulated in the pig intestines, with urinary P output positively correlated with P intake (Fernández, 1995). However, if P is deficient for bone formation, the kidneys play a much greater role in Ca regulation (González-Venga and Stein, 2014). Further investigation has shown linear uptake of P by a Na-P co-transport system across both pig small intestinal and renal tissues (Brandis *et al*., 1987). This mechanism is independent of calcium level and is stimulated to increase P absorption in response to low dietary P intake (Saddoris *et al*., 2010). During suckling and post-weaning, Schröder *et al*. (1998) found that the kinetics (V_{max}) of the Na-P co-transporter are greatly reduced compared with new born pigs. However, P transport affinity remained constant at 0.45 – 0.63 mmol/litre in normal pigs. In their study, vitamin D_3 deficient pigs also showed normal Na-P transport when new born but this was significantly reduced in the suckling and weaning period. Once supplemented with D_3 post-weaning, the levels were not

significantly different from new born levels, suggesting active intestinal P transport is only vitamin D_3 dependent post-weaning.

Transcellular calcium absorption in the small intestine is the main route in low Ca concentrated diets via a three stage process mediated by CaT1 and vitamin D (calcitriol), followed by extrusion from the cell with CaATPase (Bronner, 2003). This is evident from the results of Schröder *et al*. (1998) who reported that the inclusion of dietary vitamin D_3 in pigs post-weaning was able to restore plasma Ca concentrations whereas, these values were 39 % lower in calcitriol deficient animals. In high Ca diets, paracellular transport over the whole length of the small intestines takes over as the main route of Ca absorption (Bronner, 2003).

Extensive reviews of Ca and P requirements for pigs have been undertaken by working committees of the Agricultural Research Council (ARC, 1981), the British Society of Animal Science (Whittemore *et al.,* 2003) and the National Research Council (NRC, 2012) in a bid to suggest dietary guidelines. The most recent recommended standard (Whittemore *et al.,* 2003) for UK weaned pigs (10 – 30 kg) suggests levels of 7.5 g/kg of total Ca and 3.4 g/kg (air dried matter basis) of digestible P. This standard is now over 10 years old but is a good starting point to formulating practical diets, since they suggest nutrient levels to meet efficient production targets. They also state that they "are mindful of nutritional welfare and taking account of the needs for environmental protection in relation to emissions of nitrogen and phosphorus". This suggests the recommended level may be a compromise between the needs of the pig and safeguarding the environment.

2.3.2. Mineral Bioavailability

Both the BSAS and NRC standards give similar recommendations for mineral requirements of newly weaned pigs, up to approximately four weeks post-weaning (Table 2.5). While total P requirements are predicted to be around 7g/kg of feed, the digestibility of this mineral must also be considered. Modelling the fate of P and Ca in the stomach/small intestines has suggested that the dietary form P and Ca are supplied in is the biggest factor for absorption (Létourneau-Montminy *et al*., 2011). In animal products, P digestibility is typically 61 – 90 % (example given for bone precipitate and skimmed milk powder respectively). This is very similar to the digestibility values for pure inorganic P sources, which are also around 64 % for dicalcium phosphate and 90 % for monosodium phosphate. Lenis and Jongbloed (1999) compare these values with those from a variety of plant sources and found P digestibility to average only ~30 % in plant material for pig diets. It is therefore more appropriate to formulate diets using the

apparent or standardised total tract digestibility (ATTD or STTD) rather than total P, since undigested phosphorus is simply excreted and is therefore not available to the animal.

Several approaches to describing P requirements for pigs have been referred to in the literature. Their use was recently re-visited by Peter Wilcock at the 1st International Phytase Summit in 2010. His conclusions indicate that digestible Ca and P are still the most appropriate expression to use when formulating pig diets but that they rely on a number of variables, making them hard to predict in practice. The main problem with using digestibility values is that they are only predictions based on experimental observation. They will naturally vary depending on factors such as the genotype of the pig (Alexander *et al*., 2010), their bio-availability and how they interact with other chemicals. Phytic acid (*myo*-inositol hexakisphosphate) for example, is a highly reactive molecule (Maenz *et al*., 1999) which has a high affinity to bind Ca into indigestible complexes (phytate) at neutral pH (Applegate *et al.,* 2007). Even for inorganic P, it is predicted that increasing dietary Ca reduces P digestibility by 2 % due to the formation of insoluble Ca – P complexes (Létourneau-Montminy *et al*., 2011; 2012).

The retention of P is also influenced by dietary Ca concentration in a quadratic relationship, rather than the linear reduction seen on digestible P (Létourneau-Montminy *et al*., 2012). This is consistent with the mineralisation of P into bone in a Ca dependent manner. In young pigs, (3.7 – 24.8 kg), a greater content of bone ash (in relation to total bone mineral content) is found in the trunk section, with lower but equally portioned content in the fore and hind legs (Mitchell *et al*., 2001). As the pig exceeds ~30 kg body weight, so the accumulation of bone ash in the trunk in relation to total bone ash starts to decrease, making way for linear increases in leg bone ash content (Mitchell *et al*., 2001).

Mineralisation of Ca in the bone is partly regulated by parathyroid hormone (PTH) which increases in response to a restriction in blood Ca supply (Hagemoser *et al*., 2000). Elevated PTH results in stimulation of vitamin D_3 from the kidneys, thereby increasing intestinal Ca and P absorption as previously described. At the same time, increased PTH results in bone demineralisation, as Ca is mobilised to restore plasma levels. This is clearly demonstrated in pigs fed diets very deficient in Ca, where a time lag of 16 days from treatment to plasma Ca response is observed (Eklou-Kalonji *et al*., 1999). This resulted in a rapid increase in blood PTH to compensate, resulting in significantly reduced bone volume and reduced mineralisation (as indicated by significantly higher osteoid thickness) compared to a Ca adequate control diet, 32 days post-treatment.

When Ca is in excess, the retention of Ca absorbed decreases in a linear fashion over the range 0.33% to 0.67% (linear, p=0.01), when a plateau becomes apparent (quadratic, p=0.01) above this level (Stein *et al*., 2011). This tends to be pronounced from the time of leg bone mineralisation noted by Mitchell *et al*. (2001), as the efficiency of retention is significantly higher in lower Ca diets around this time, having been significantly lower than Ca excess diets prior to this point (Aiyangar *et al*., 2010). This suggests there is a limit to how much Ca can be retained in the bones, with the efficiency of retention decreasing closer to this limit. Since hydroxyapatite (the main mineral structure in bone) is composed of Ca and P in a 2:1 ratio ($Ca_5(PO_4)_3$), the retention efficiency of P should also slow as described by Létourneau-Montminy *et al*. (2012) as dietary Ca increases.

In low P diets, young and growing pigs are more sensitive to a wide Ca:P, with Reinhart and Mahan (1986) reporting tendencies of lower growth performance when ratios exceeded 1.3 and 2.0 for low P and high P diets respectively. They also show linear decreases in bone ash content as the ratio widens from 1.3 – 4.0 in both starter and growing pigs. Dietary Ca and P for optimal growth performance differs from that ideal for bone mineralisation by at least 1 g/kg less (NRC, 2012). A brief survey of published scientific studies indicates a wide range of total Ca and P levels are fed as basal diets, which also differ in the Ca:P (Table 2.6).

Table 2.6: Analysed total Ca and tP of selected experimental weaner pig basal diets

Source	Ca (g/kg)	Total P (g/Kg)	Ca:P
(Woyengo *et al.*, 2009)	9.4	5.0	1.9
(Woyengo *et al.*, 2010)	10.6	5.3	2.0
(Simons *et al.*, 1990)	5.2	3.3	1.6
(Simons *et al.*, 1990)	5.7	4.1	1.4
(Mroz *et al.*, 1994)	7.3	3.6	2.0
(Lei *et al.*, 1993)	4.4	3.2	1.4
(Revy *et al.*, 2006)	9.5	7.3	1.3
(Augspurger *et al.*, 2004)	7.0	3.2	2.2
(Jones *et al.*, 2010)	7.1	4.0	1.8

While both the inclusion level and ratio of Ca and tP differ between authors (Table 2.6), they are broadly related to standard recommendations. Some of the variation may be down to the differing guidelines used (NRC vs BSAS), the age of the animals (weaning age and weight varies) and genotype/production targets the diets were designed for. The range of Ca:tP ratios described in Table 2.6 (1.3 – 2.2) were largely assessed by Qian *et al.* (1996) who used 1.2, 1.6 and 2.0 in their trial. They found that a ratio of 1.2 resulted in the best performance (in agreement with Reinhart and Mahan, 1986), P digestibility and phytase efficacy; which decreased linearly as the ratio was widened. A similar study

by Poulsen *et al.* (2010) concluded that Ca:tP ratios in the range of 0.9 – 1.8 did not significantly affect P digestibility which is in disagreement with Qian *et al.* (1996). In this latter study, all P was derived from plant phytate and revealed a Ca – phytase interaction on P utilisation, thus confirming the source of P and level of dietary phytase are both important factors to consider in addition to the total Ca:P.

2.4. Dietary phytic acid

Phosphorus derived from plant sources is predominately found as part of the organic molecule phytic acid (PA) (Table 2.8). Of the nine stereoisomers (Shears and Turner, 2007), it is the fully phosphorylated form: myo-Inositol 1,2,3,4,5,6 hexa*kis*phosphate (IP_6), that is overwhelmingly found in nature, accumulating in both terrestrial and aquatic environments (Turner *et al.,* 2002; Giles *et al.,* 2011). Plants synthesise the various inositol phosphates for many diverse and multifunctional reasons (Loewus and Murthy, 2000), resulting in a lock up of the majority of P in phytic acid salts (phytate). The highest concentrations of PA are typically found in the seeds (Table 2.7), where they act as a reserve store, mobilised through enzymatic activity during germination (Hall and Hodges, 1966; Centeno *et al.,* 2001).

Table 2.7: Analysed total P and calculated PA content of selected raw materials

Feedstuff	Total P content (g/kg)	Phytate P content (g/kg)	Phytic acid content (g/kg)	Source
Maize	3.2	2.34	8.30	1
	3.24	1.86	6.60	2
	2.6	2.2	7.80	3
	Mean = 3.0±0.36	Mean = 2.1±0.25	Mean = 7.6±0.87	
Barley	4.23	2.67	9.47	1
Wheat	3.17	2.02	7.16	1
	4.21	2.51	8.90	2
	Mean = 3.7±0.74	Mean = 2.3±0.35	Mean = 8.0±1.23	
Oats	3.7	2.2	7.80	1
Wheat bran	11.4	8.7	30.85	1
Wheat middlings	8.4	6	21.28	1
	13.05	7.99	28.33	2
	Mean = 10.7±3.29	Mean = 7.0±1.41	Mean = 24.8±4.99	
Soybean meal	6.99	4.675	16.58	1
	6.3	3.8	13.48	3
	8.36	3.95	14.01	2
	Mean = 7.2±1.05	Mean = 4.1±0.47	Mean = 14.7±1.66	
Rapeseed meal	12.4	7.3	25.89	1

Sources: 1. Weremko *et al.*, 1997; 2. Tahir *et al.*, 2012; 3. Ravindran *et al.*, 1994

In wheat seeds, PA is predominately confined to the aleurone layer as opposed to the endosperm or germ (Williams, 1970; O'Dell *et al.,* 1972), which explains why wheat bran and middlings have far greater concentrations than whole wheat (Table 2.7). Since these tend to be low value by-products, they have previously found application in pig diets, thus acting as a substantial source of dietary PA. Typical weaner pig diets in the UK contain ~45% wheat/barley and ~15% soya, providing approximately 6 – 7 g PA/kg diet. The PA in corn is almost exclusively confined to the germ (O'Dell *et al.,* 1972). However, progress has been made in terms of breeding low phytate crops (Raboy, 2002). These are particularly suited to the American corn/soybean based diets. In these low phytate plants, there does not appear to be any disadvantage in terms of germination, compared to conventional varieties, since the total P is not reduced; rather some PA is replaced with lower inositol phosphates or inorganic P (Raboy, 2007).

2.4.1. Chemical structure of inositol phosphates

IP_6 consists of a six carbon ring, populated by six phosphate groups, giving the chemical formula $C_6H_{18}O_{24}P_6$. The calculated PA concentration in plant material (Table 2.7) is thus based on the assumption that one molecule contains 28.2% phosphorus, as derived from the molecular weight of its individual components (Table 2.8).

Table 2.8: The chemical formulation of inositol 1,2,3,4,3,5,6 hexa*kis*phosphate

Symbol	Element	Atomic weight	Number of atoms	Mass %
C	Carbon	12.01078	6	10.9183
H	Hydrogen	1.007947	18	2.7488
O	Oxygen	15.99943	24	58.1765
P	Phosphorus	30.9737622	6	28.1564

Recommendations for the numbering of carbon atoms in *myo*-inositol come from the Nomenclature Committee of the International Union of Biochemistry (NC-IUB), when in 1988 they relaxed the lowest-locant rule (NC-IUB, 1989). The problem they faced is shown in Figure 2.6, where depending on the positional numbering (locants) of carbon atoms in the ring (clockwise or anticlockwise); there are two possible first carbons. This stems from the IUPAC CNOC and IUPAC-IUB CBN (1976) nomenclature of cyclitols recommendations I-4 and I-10. In the absolute configuration, rule I-10 specifies that when drawn in a vertical (Fischer-Tollens type) manor, C-1 is at the top, followed by C-2 and C-3 on the front edge. If then C-1 is projecting to the right, a clockwise numbering of the carbon atoms is used (D configuration) but if C-1 is projecting to the left, an anticlockwise numbering is enforced (L configuration).

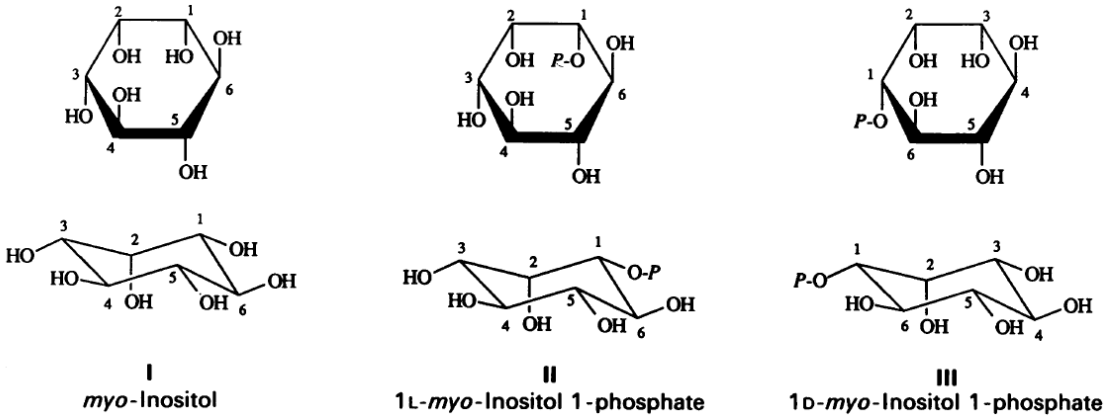

Figure 2.6: Numbering of atoms in *myo*-inositol (Source: NC-IUB, 1989)

Figure 2.6 also reveals the solution to the problem. *myo*-Inositol (I) is shown in the L-configuration where the numbering is anticlockwise (II), and also in the D-configuration where numbering is clockwise (III). The relaxation of the numbering rule (NC-IUB, 1989) means that either the L or D configuration can be used depending on convenience, thus so long as L or D is indicated, both are acceptable. The conformational structure was debated in the 1980's (Maga, 1982) though Rabinowitz and Kraut (1964) correctly reported that there is only one hydroxyl group axial, conventionally now assigned to C-2. Furthermore, they determined that in *myo*-Inositol crystals, each unit contains two asymmetric molecules. This property has recently been confirmed by Rebecca *et al.* (2012) who isolated crystals from the flesh of dragon fruit. Their approach to isolate and identify these crystals involved four different techniques (X-ray crystallography, HPLC, LC-MS/MS and NMR) which together, provide clear evidence that a considerable amount (4 g/kg) is also present in dragon fruit in addition to the traditional seeds.

2.4.2. Anti-nutritional effects

Due to the 12 ionisable protons, PA is a strong chelator of metal cations at neutral pH (Maenz *et al.,* 1999) and forms stable complexes with these metals (Figure 2.7), inhibiting their digestion/absorption over a wide pH range (3.0 – 9.0) (Kim *et al*., 2010). An equilibrium study by Odani *et al.* (2011) further confirmed that the conformational behaviour of IP_6 is pH dependent. At low pH they reported that there was a high liability of protons dissociating from the phosphate groups but that raising the pH increases the degree of polyamine protonation. Traditionally, PA has therefore been viewed as an anti-nutritional factor (ANF) in pig diets, since phosphorus and complexed metal ions are simply excreted rather than providing a nutritional value.

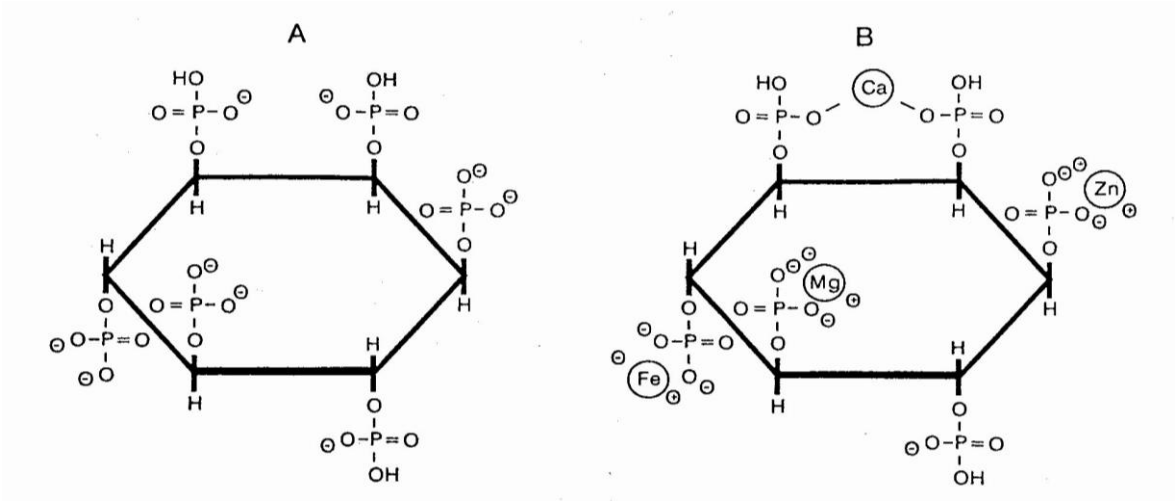

Figure 2.7: Structure of phytic acid (A) and phytic acid chelate (B)
(Source: adapted from Schinckel, not dated)

An *in vitro* experiment was conducted by Yu *et al.* (2012) which showed how these properties of PA may affect practical aspects of pig nutrition. They demonstrate that in a pH range of 2.5 to 3.8 (typical of conditions in the stomach of the pig), protein aggregates can form. However, below pH 2.0 this is not the case due to the dissociable proteins (they reported a clear solution). Additionally, they showed that as PA concentration increased in solution, so relative pepsin activity dropped sharply. Pepsin is an enzyme required to assist protein breakdown in the stomach, thus inhibition of pepsin activity may reduce protein digestion.

A review by Selle *et al.* (2012) suggests that the anti-nutritional effects of phytic acid on protein digestion may not be limited to binary complexes, but may also extend to ternary complexes formed through cationic bridges above the protein isoelectric point. These would be difficult to breakdown in the small intestines leading to reduced protein digestibility.

2.4.3. Metabolic roles of inositol phosphates

The inositol phosphates have been implicated in many metabolic pathways in both plants and animals (Holub, 1986; Loewus and Murthy, 2000; Raboy, 2003; Tsui and York, 2010; Valluru and Van den Ende, 2011). Indirect protein-protein reactions (as successive enzymes) are shown in the pathway map (Figure 2.8), based on information in the KEGG database (Kanehisa and Goto, 2000). The numbered boxes serve to identify individual EC enzymes and the reaction products are identified by hollow circles. It is clear from the array of reference metabolic pathways (Figure 2.8) that the inositol phosphates are implicated in many different reactions. This suggests that phosphate release is only the tip of the iceberg and that research should now focus on areas like cellular signalling pathways.

In the pig, not all of these metabolism pathways are active. However, the pathways in green (Figure 2.8) reveal that there are still many lower inositol phosphate products produced. It is likely that the instability of these compounds allow them to play many diverse roles in the body. Conversely, the stability of IP_6 makes it an effective precursor substrate. In fact, Barker *et al.* (2009) has described the molecule as a platform for which there are 63 theoretical stereochemical forms. Thus, not only can phosphate groups be removed, but extra P may be added, forming inositol pyrophosphates.

It can be seen from Figure 2.8 that the phytase (EC 3.1.3.26 and EC 3.1.3.8) metabolism pathways are not active in the pig. This prevents the breakdown of IP_6 to the lower esters. However, there are many other phosphate hydrolases and kinases working on the substrate. These metabolism pathways are important for cellular function with many reported roles of the active inositol phosphates (Miller *et al.,* 2008; Tsui and York, 2010). In fact, there is increasing evidence that they have anticancer activity (Bozsik *et al.,* 2007) and the ability to modulate the immune system (Liu *et al.,* 2008; Ghahri *et al.,* 2012).

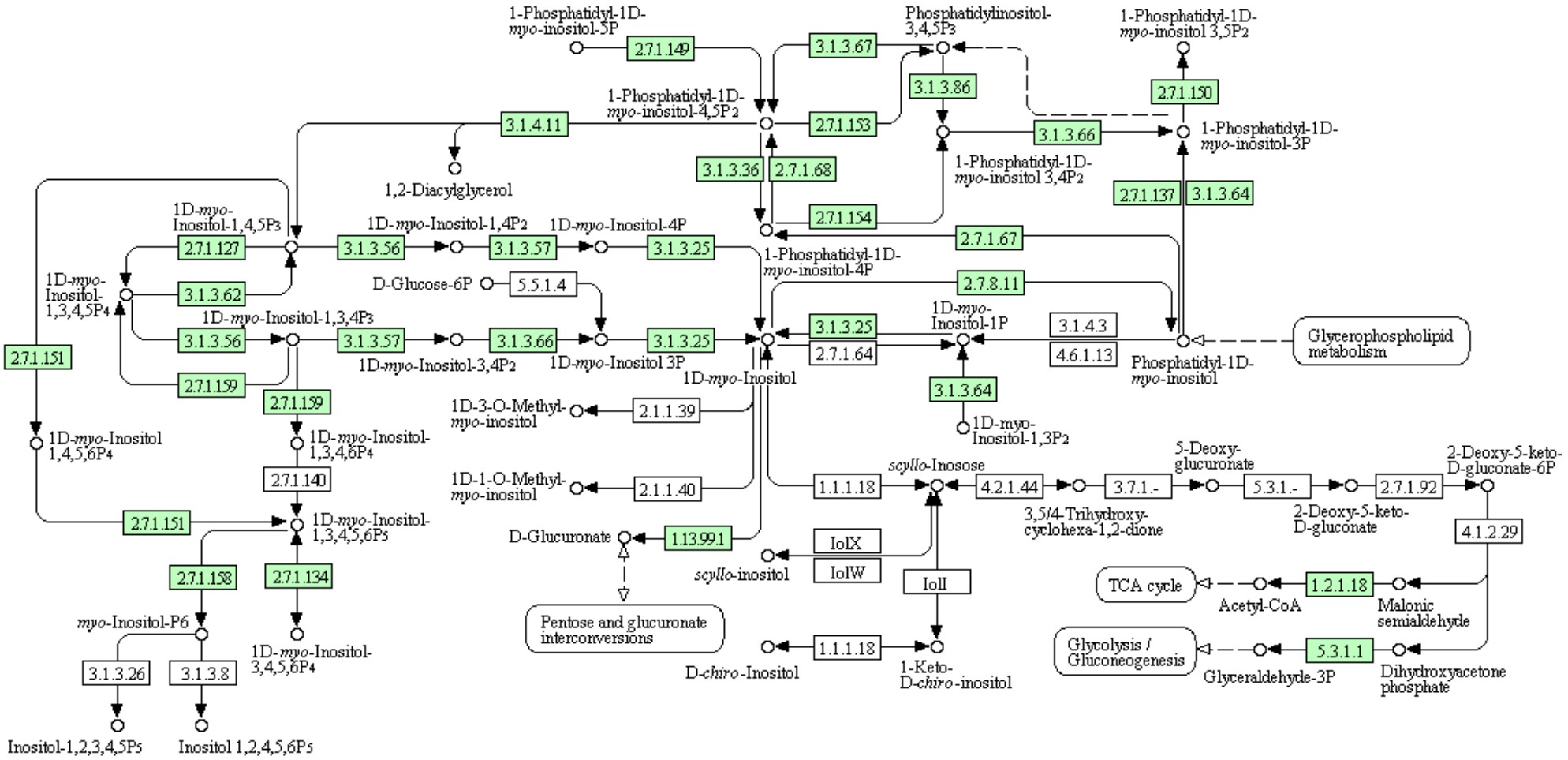

Figure 2.8: Inositol phosphate metabolism pathways of the pig. Active pathways are shown in green. (Source: KEGG, 2012)

2.5. Phytate degrading enzymes

Phytases are a class of phosphatase enzymes, specific to phytate, classified by the Nomenclature Committee of the International Union of Biochemistry (NC-IUB) as phosphoric monoester hydrolases (EC 3.1.3.). They catalyse the dephosphorylation of IP_6 (Figure 2.9) and can be split into two main categories based on their pH optima: acid phytases and alkaline phytases. Of interest in monogastric nutrition are the histidine acid phytases (HAPhy) which demonstrate optimal activity at low pH (Oh *et al*., 2004) and are thus suited to working in the stomach, where phytate complexes are most vulnerable to degradation due to the low pH. There are two other classes of acid phytases: purple acid phosphatases (PAPhy) and protein tyrosine phytase (PTPhy) (Yao *et al*. 2011; Lei *et al*. 2013).

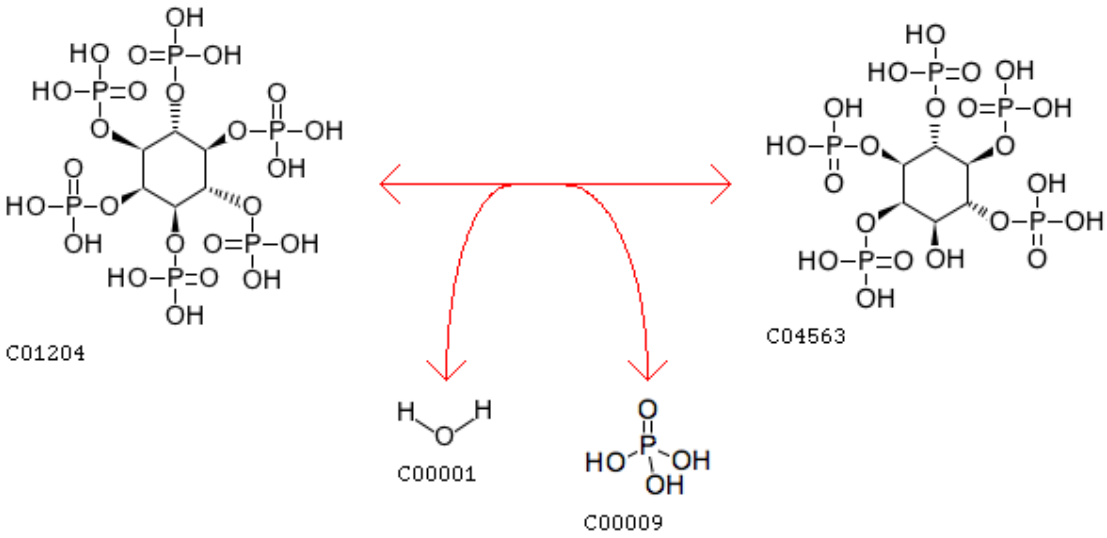

Figure 2.9: Hydrolysis of phytic acid by phytase enzymes (Source: KEGG, 2012)

Only one alkaline phytase has been characterised: β-propeller phytase (BPPhy). These have been isolated from *Bacillus* and Enterobacter bacteria and some plant seeds including *Lilium longiforum*, legume seeds, mung beans, spelt and *Typa latifolia* L (Liu *et al.,* 1998; Oh *et al*., 2004). Their major drawback is their specificity for calcium phytate, of which the end product from alkaline phytases is IP_3 and not a complete dephosphorization (Oh *et al*., 2004; Kim *et al*., 2010; Zeng *et al*., 2011). This may limit their use as the sole phytase incorporated into mono-gastric animal feeds.

Greiner and Bedford (2010) summarised the classification of phytases based on their catalytic mechanisms, pH optimum and mode of action. The latter refers to the official classifications of phytase into three classes by NC-IUBMB (2012). These sub-classes are known as 3-phytase (EC 3.1.3.8), 4-Phytase (EC 3.1.3.26) and 5-phytase (EC 3.1.3.72), depending on which carbon in the *myo*-inositol ring is hydrolysed first. Loewus and Murthy (2000) recognised that the nomenclature of inositols is a source of confusion. They point

out that that the positions of C2 and C5 are fixed but the two remaining pairs of carbon atoms (C1/C3 and C4/C6) can be numbered clockwise (L-configuration) or counter clockwise (D-configuration). Depending on which is used, 4-phytase may then be regarded as 6-phytase and 3-phytases can be called 1-phytase. While it is generally regarded that microbes produce 3-phytase and seeds of higher plants produce 6-phytase, there are some exceptions as noted by Konietzny and Greiner (2002). They propose that the D-configuration should be used so that a distinction can be made between micro-organisms producing EC 3.1.3.8 (3-phytase), higher plant seeds producing EC 3.1.3.26 (4-phytase) and a few micro-organisms not following the rule that produce EC 3.1.3.26 (referred to as 6-phytase to distinguish between that produced by seeds). It should be noted that 5-phytase (EC 3.1.3.72) also exists and is produced by a range of organisms including lily pollen (Barrientos *et al*., 1994), *Bifidobacterium pseudocatenulatum* (Haros *et al*., 2009) and *Selenomonas ruminantium* subsp. *lactilytica* (Puhl *et al*., 2008).

Interestingly, while Zeng *et al*. (2011) have identified alkaline phytase from *Bacillus* as a 4-phytase, alkaline phytase from lily pollen (*Lilium longiforum*) is a 5-phytase (Barrientos *et al*., 1994). There are very few documented cases of alkaline phytase in the literature being used for animal nutrition, the exception is fish (Liu *et al*., 2013), but both 3- and 6- histidine acid phytases have been successfully produced and marketed as feed enzymes for the last few decades. Even so, they are usually only able to dephosphorylate down to lower esters (Konietzny and Greiner, 2002). For complete destruction of *myo*-inositol phosphate to *myo*-inositol and a free phosphate, inositol-phosphate phosphatase (EC 3.1.3.25) can be used (NC-IUBMB, 2012), or one of the enhanced phytases (which have better substrate affinity for lower IPs).

At least three phytase classes (HAPhy, PAPhy and BPPhy) are known to hydrolyse phytate salts of Ca^{2+} and Mg^{2+} but have been shown not to dephosphorylate Al^{3+}, Cu^{2+}, Fe^{2+}, Fe^{3+} or Zn^{2+} phytate (Tang *et al*., 2006). The fourth class (PTPhy) was unknown at the time of the study. This reflects the order of binding strength each mineral has to PA (Maenz *et al*., 1999). Since phytate has multiple Ca binding sites, BPPhy is particularly good at degrading insoluble calcium phytate and can prevent it forming salts with other metal ions (Kim *et al*., 2010). These phytases may therefore be particularly suited for use in the neutral pH conditions of the upper digestive tract, where insoluble phytate may re-form. In the acidic conditions of the stomach, PA is no longer complexed and can easily be degraded by the acid phytases. Given that each sub-class of phytase leads to a particular species of lower inositol phosphate, it is important that the end product is considered, since they play many roles in cellular signalling pathways and other metabolic processes (Liu *et al*., 1998; Konietzny and Greiner, 2002).

2.5.2. Phytase in animal nutrition

The first widely used commercial cloning and expression technology for exogenous phytase enzymes in animal feed was patented by van Gorcom *et al*. in 1991. Their system relied on the purification of a fungal phytase (*Aspergillus*), however, they also suggested their patent could be used as a template for modified wild type phytases (second generation phytases). Alternatively, Vaara *et al*. (1992) patented the concept of steeping grains in phytase enzymes for 48 hours prior to milling for animal feed. Although, in practice, steeping in phytase prior to feeding has been found to confer little benefit over phytase added to dry feed (Columbus *et al*., 2010). By 1995 commercial enzyme preparations containing both phytase and acid phosphatase activity were patented that had enhanced efficacy through thermal treatment (Vanderbeke *et al*., 1995). More recently, methods combining at least two HAP phytases (preferably both a 3-phytase and a 6-phytase) have also been patented, in a bid to ensure complete dephosphorylation (Boze and Moulin, 2013).

Taking a different approach to treating the feed, Golovan *et al*. (2001) genetically modified the pig in such a way as to express the *E. coli* appA phytase gene in the parotid gland. The resulting phytase released in the saliva proved to be quite effective at breaking down phytate, leading to a 75% reduction in faecal P output. Despite the technology being patented in 2006 (Forsberg *et al*., 2006) and proven stability over nine generations (Forsberg *et al*., 2013), Ontario Pork ceased funding for the project in 2012 (Ontario Pork, 2014). Since the herd kept at the University of Guelph was euthanized and are not in commercial production, next generation exogenous phytase preparations are now in strong demand for treating feeds.

These third generation phytases have enhanced intrinsic thermostability and substrate affinity, making them far better and more effective than their predecessors. Within the European Union, there are currently nine preparations (except Finase EC which is second generation) authorised for use in pig diets (Table 2.9).

Table 2.9: EU authorised phytase preparations for inclusion in pig feeds

Trade name	Sub-class	Production organism	Registration holder	Date of EU authorisation
Finase (EC)	6	*Trichoderma reesei*	ROAL Oy	21.04.2010 / 26.09.2011
Quantum	6	*Pichia pastoris*	Syngenta Ltd	08.07.2008
Quantum Blue	6	*Trichoderma reesei*	ROAL Oy	11.04.2014
Natuphos	3	*Aspergillus niger*	BASF SE	01.12.2009 / 27.06.2008
Optiphos	6	*Pichia pastoris*	Huvepharma AD	28.02.2012
Rovabio PHY AP	3	*Penicillium funiculosum*	Adisseo	22.10.2007
Ronozyme NP	6	*Aspergillus oryzae*	DSM Nutritional Products Ltd	16.03.2011
Ronozyme HiPhos	6	*Aspergillus oryzae*	DSM Nutritional Products	09.10.2012
Phyzyme XP	6	*Schizosaccharomyces pombe*	Danisco Animal Nutrition	29.05.2009

(Source: adapted from EU, 2015)

The majority of those authorised are 6-phytases, with the two 3-phytases among the first to be registered (Table 2.9). In addition to the five expression systems used in the protein production, it is important to note where the original gene sequence is from. In the case of Finase EC, Optiphos, Phyzyme, Quantum and Quantum Blue, the phytase gene originates from *E. coli*. In Natuphos the original sequence was isolated from *A. niger* NRRL 3135 and the gene from *P. funiculosum* is used in Rovabio PHY AP. The phytase gene in Ronozyme originates from *Peniophora lycii*. Phytases from each of these sources will differ in pH optimum, temperature optimum and end product produced (Table 2.10).

Table 2.10: Selected characteristics of phytase found in commercial feed products

Original organism	Group	pH optimum	Temperature optimum (°C)
A. niger	HAP Phy A	2.5 – 5.0	55 – 60
P. funiculosum	-	4.0 – 5.0	50
E. coli	HAP Phy C	5.0 – 6.0	40 – 60

- Unknown (Source: adapted from Oh *et al*., 2004; Pierrard *et al*., 2008)

Phytase activity is measured as the concentration of inorganic phosphate released from a specific phytate substrate per minute, under specific reaction conditions. The original method called for phytase to be incubated with sodium phytate at 37±0.1°C and pH 5.5 and defined one phytase unit (FTU) as the quantity of enzyme that liberates 1 mol of inorganic P per minute, under the conditions of the assay (Engelen *et al*., 2001). While this method is useful for the pure phytase product, Kim and Lei (2005) suggested an improved version using spin columns, under the same conditions, specifically for determination of phytase in animal feed, due to the higher levels of background P. The official definition of phytase activity was updated by Gizzi *et al*. (2008) and subsequently published as ISO 30024 in

2009. One FTU is currently defined as “the amount of enzyme that releases 1 µmol of inorganic phosphate from 5 mM phytate/min at pH 5.5 and 37°C”.

Given that both the pH and temperature optimums for commercial phytases are different to those specified by the *in vitro* test (Table 2.10), a number of phytase manufacturers have designed their own in house tests specific for their products. For the product Quantum™, a new assay was proposed for the determination of activity in feedstuffs. The new method proposed modified conditions for testing at pH 4.5 and 60°C in replace of pH 5.5 and 37°C in the official method (CRL-FA, 2007). Under these conditions, phytase activity is expressed in Quantum™ phytase units (QPU/kg). Similarly, Optiphos® activity was submitted to the European Food Safety Authority (EFSA) in units of OTU/kg and not FTU/kg. The definition of one OTU is given as: “the amount of enzyme that catalyzes the release of 1.0 micromole of inorganic phosphate per minute from 5.1 mM sodium phytate in pH 5.5 citrate buffer at 37 °C, measured as the blue P-molybdate complex colour at 820nm” (EFSA, 2011). In this case, the buffer has been changed from acetate to citrate; the latter has been shown to be effective at solubilising some phytates (Maenz *et al*., 1999; Tang *et al*., 2006). The definition of phytase activity in authorised EU products are detailed in Table 2.11.

Table 2.11: Definition of phytase activity units authorised for animal feed in the EU

Product	Unit	Definition of phytase activity
Finase (EC)	PPU	1 PPU/g is the amount of enzyme which liberates one µmol of inorganic phosphate from sodium phytate per minute at pH 5 and 37 °C
Quantum; Quantum Blue; Phyzyme XP	FTU	1 FTU is the amount of enzyme which liberates 1 micromole of inorganic phosphate from sodium phytate at pH 5.5 and 37 °C in one minute.
Quantum (alternative units)	QTU	1 QTU is the amount of enzyme that releases 1 µmol of inorganic phosphate from 5 mM phytate/min at pH 5.5 and 37°C
Natuphos	FTU	1 FTU-unit is the amount of 3-phytase which liberates 1 µmol of inorganic phosphate per minute from sodium phytate at pH = 5.5 and 37 °C.
Optiphos	OTU	1 OTU is the amount of enzyme that catalyzes the release of 1.0 micromole of inorganic phosphate per minute from 5.1 mM sodium phytate in pH 5.5 citrate buffer at 37 °C, measured as the blue P-molybdate complex colour at 820nm
Rovabio PHY AP	RPU	1 RPU is the amount of enzyme that releases 1 µM inorganic ortho-phosphate per minute from sodium phytate as substrate at pH 5.5 and 37°C.
Ronozyme NP	FYT	1 FYT unit is the quantity of enzyme which liberates 1 micromole of inorganic phosphate per minute from sodium phytate at pH = 5.5 and 37°C
Ronozyme HiPhos	FTU	1 FYT unit is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute under reaction conditions with a phytate concentration of 5.0 mM at pH 5.5 and 37 °C.

(Source: adapted from CRL-FA, 2007; 2009; EFSA, 2007; 2008a; 2008b; 2009a; 2009b; 2011; 2012; 2013)

Although the unit of phytase activity acronym differs between products (Table 2.11), the definitions remain largely unchanged from the official ISO 30024 method. The normal dose of phytase enzymes recommended in the EU for pigs varies depending on age of the animal and the product registered are detailed in Table 2.12.

Table 2.12: Authorised phytase enzyme doses for pigs in the EU

Trade name	Class	Recommended dose (weaners)	Recommended dose (finishers)	Recommended dose (sows)	Units/kg of feed
Finase (EC)	6	250 – 1000	250 – 1000	250 – 1000	PPU
Quantum	6	100 – 2500	-	-	FTU
Quantum Blue	6	500 – 1750	250 – 1750	250 – 1750	FTU
Natuphos	3	500	100	500	FTU
Optiphos	6	250 – 500	250 – 500	125 – 500	OTU
Rovabio PHY AP	3	250 – 500	350 – 500	-	RPU
Ronozyme NP	6	500	500	750	FYT
Ronozyme HiPhos	6	500 – 4000	500 – 4000	1000 – 4000	FTU
Phyzyme XP	6	500 – 1000	500 – 1000	500	FTU

(Source: adapted from Commission Regulation (EU) No. 277/2010; 886/2011; 554/2008; 292/2014; 1269/2009; 243/2007; 505/2008; 98/2012; 1141/2007; 171/2011; 837/2012; 379/2009)

The recommended doses of phytase for pig diets range from 100 – 4000 units/kg (Table 2.12) and is derived from data submitted by the applicant to the EFSA on the safety and efficacy of the product to improve the digestibility of dietary P. Due to the variable nature of the mono-gastric digestive tract environment, *in vivo*, phytase activity may not reflect that measured *in vitro*. Studies of how phytase actually performs in live animals are therefore needed to attain this information.

2.5.3. Phytase efficacy *in vivo*

The efficacy of phytase to degrade phytate in the pig may be better expressed as the improvement in digestible P derived from the dephosphorylation of dietary phytate. Several approaches to expressing P digestibility have been previously described whereby, digestibility is measured at the terminal ileum or as ATTD. An updated description of the latter accounts for the secretion of endogenous P and is referred to as standardised total tract digestibility (STTD). There have been many studies over the years to improve P utilisation in pig diets, starting with unsuccessful live yeast cultures (Chapple *et al*., 1979) but progressing to purified commercially available phytase products (Adeola *et al*., 2006; Jones *et al*., 2010). Microbial phytase first showed efficacy to improve P digestibility in pigs from 34 % to 56 % and reduced P excretion by 33 %, 26 years ago, when fed at a 1000

FTU/kg (Simons *et al*., 1990). This was based on a purified culture of *Aspergillus ficuum* fed to only six cannulated fattening pigs. More recent and larger studies have also confirmed linear increases in P digestibility for *E. coli*, *P. lycii*, and *A. niger* derived phytases fed at similar levels (Adeola *et al*., 2006; Kies *et al*., 2006a).

An alternative approach to defining phytase efficacy *in vivo* calculates the additional release of P by phytase, based on the increase P mineralisation of bone relative to that from dietary inorganic P. This method expresses additional available P in relation to a response criteria but does not account for that excreted (Adeola and Applegate, 2010). Available P release curves based on this method have been constructed for a number of phytase products and have generally shown them to be effective at releasing P up to a plateau of 1000 FTU/kg (Figure 2.10).

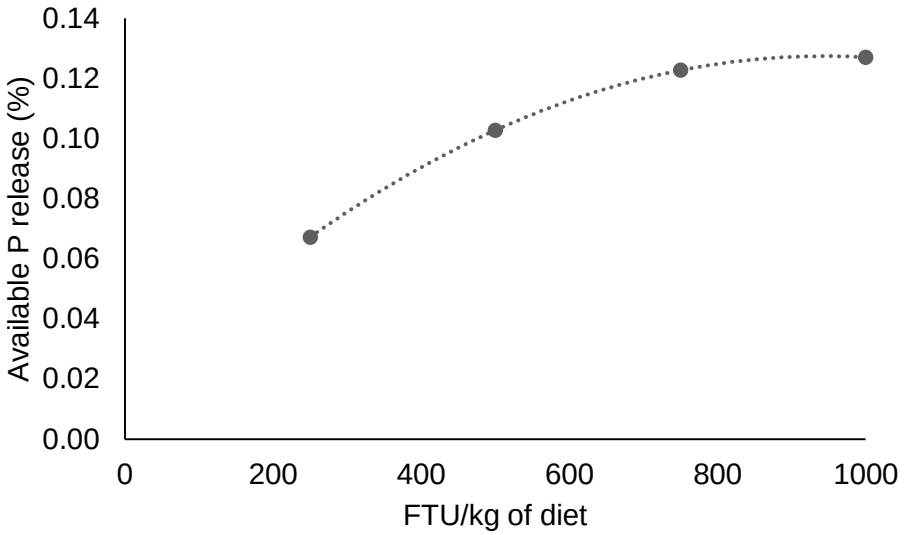

Figure 2.10: Available phosphorous release curve for *E coli* derived phytase in P deficient diets fed to weaned pigs. (Source: adapted from Jones *et al*., 2010).

The reconstructed curve in Figure 2.10 based on the equation published by Jones *et al*. (2010) shows that the 500 FTU/kg of 6-phytase is able to release the equivalent of 0.1 % aP, increasing to 0.12 % by 750 FTU/kg. Although effective up to 1000 FTU/kg, little extra benefit is gained from going beyond 750 FTU/kg. This fits well with the EU recommended doses (Table 2.12), suggesting a normal dose of phytase for pigs should be 500 – 750 FTU/kg of diet.

2.5.4. Physiological effects

As previously described, bone mineralisation due to the increased P and Ca released from phytate by phytase is routinely used to calculate available P release. The increase in bone mineralisation is also positively correlated with bone breaking strength, which has been reported to increase linearly with phytase dose (Veum *et al*., 2006; James *et al*., 2008; Zeng *et al*., 2014). Despite the increase in available P from PA at normal phytase doses, there is little evidence of improved growth rates in the first month post-weaning when compared with P adequate diets (Stahl *et al*., 2004; Veum *et al*., 2006; Zeng *et al*., 2014). However, Veum *et al*. (2006) did report that weaned pigs supplemented with 500 FTU/kg of *E. coli* or *P. lycii* phytase grew 73 g/day more than those without phytase, fed a P adequate diet, from day 28 – 42 post-weaning. In this experiment, those on the P adequate diet grew 100 g/day more in the same post-weaning period as those fed the non-phytase supplemented basal diet. Even though the phytase supplemented basal diet was formulated below the P requirement of the pig, assuming 500 FTU/kg released 0.1 % available P (based on Figure 2.10) meeting the pig's requirements, then the additional growth is likely due to the phytase and not the released P.

In poultry, the addition of both fungal and bacterial derived phytase have been shown to improve immune function. Liu *et al.* (2008) investigated the addition of up to 1000 FTU/kg of *E. coli* phytase on antibody production to Newcastle disease virus (NDV) vaccine. At seven days post-vaccination it is unclear if phytase had a significant effect, as the authors have reported non-significance but have also suggested that birds supplemented with 1000 FTU/kg phytase had significantly higher NDV antibodies than those not supplemented with phytase on a 0.44 % phytate diet. By day 21 post-vaccination, both 500 and 1000 FTU/kg phytase had increased NDV antibodies over the 0.44 % phytate control. In a second similar study by Ghahri *et al.* (2012) the addition of up to 1000 FTU/kg *A. niger* phytase also increased antibody levels to NDV vaccination by 19 days post-vaccination. In addition to B cell stimulation, Liu *et al.* (2008) also reported that the addition of dietary phytase significantly increased the number of both $CD4^+$ and $CD8^+$ lymphocytes but did not affect their ratios. They further reported that secretory IgA levels were higher in the phytase supplemented diets. Taken together, the evidence suggests that dietary phytase supplemented up to 1000 FTU/kg may enhance immune status. However, in pigs very little has been done to investigate the effects of phytase on immunity. Almeida *et al*. (2007) investigated doses of 500 FTU/kg on white blood cell differentiation and secretory immunoglobulins of finishing pigs (100 kg) and concluded there were no effects from this dose. It is known however, that T lymphocyte mediated response (to phytohemagglutinin) is linearly increased in weaned pigs as dietary available P increases from deficient to adequate (Kegley *et al*., 2001). This raises the possibility that doses >500 FTU/kg may influence immunity in young pigs, similar to that found in chickens.

2.6. Super dosing

It is clear that normal doses of phytase (500 – 1000 FTU/kg) are able to restore growth performance in the first few weeks post weaning in negative P diets, back to the level of those fed the recommended dose of P (Zeng *et al*., 2014; Vigors *et al*., 2014). However, although Veum *et al*. (2006) showed that pigs supplemented with phytase up to 500 FTU/kg did not grow significantly different to, or tended ($p<0.10$) to perform slightly less than an adequate P control diet, when increased to above 2500 FTU/kg, pigs grew 64 g/day more than a P adequate diet ($p=0.03$). This also reflects the findings of Kies *et al*. (2006a) who found that it was not until >750 FTU/kg that growth rates matched a 1.5 g/kg inorganic P supplemented positive control (PC) diet and tended ($p<0.08$) to exceed them past 1500 FTU/kg (Figure 2.11).

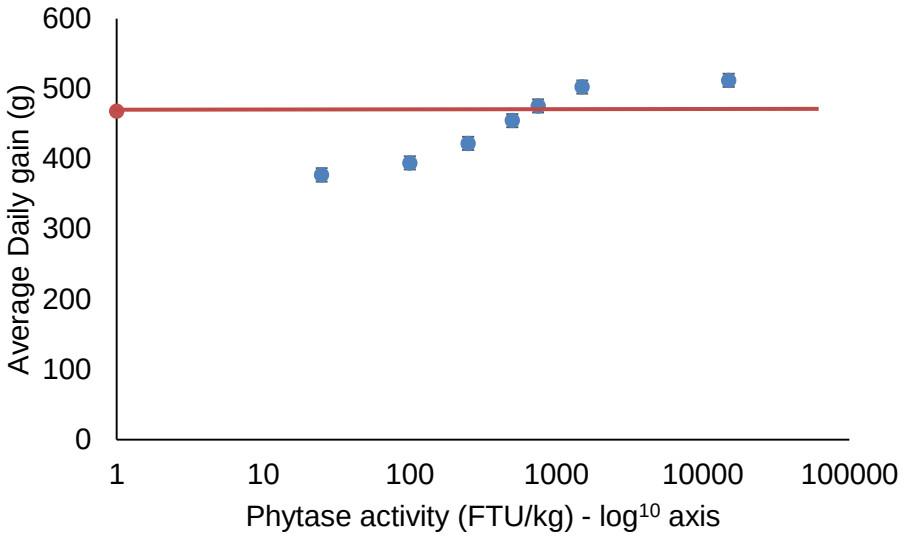

Figure 2.11: The effect of phytase on the growth rate of weaned pigs. The threshold line represents growth from a positive control diet containing adequate digestible P. (Source: adapted from Kies *et al*., 2006a).

The findings of Kies *et al*. (2006a) highlight the importance of meeting the digestible P requirement of the pig because even in their PC diet and at their highest phytase dose, they still have not met the BSAS recommendations of 3.4 g/kg (Whittemore *et al*., 2003), explaining why the pigs have not met their full growth potential by 15,000 FTU/kg. Given that Zeng *et al*. (2014) found no difference in the growth performance of weaned pigs fed 20,000 FTU/kg over those fed a P adequate diet (4.2 g/kg), the evidence for 'extra phosphoric' effects on growth rates due to phytase super-dosing in pigs unclear. Phytase super dosing in poultry however, has recently been reviewed (Cowieson *et al*., 2011), showing clear benefits in growth performance. Although a number of mechanism have been proposed, particularly the release of amino acids (Selle and Ravindran, 2008), further work is needed to unravel how these work in pigs.

2.6.1. Binary phytate – protein complexes

It is possible that these 'extra phosphoric' benefits may be related to the release of chelated minerals and nutrients as phytate complexes are destroyed (Kies *et al*., 2006a), or from physical changes in the gut, such as prevention of crypt shrinkage and expression of gut transporters (Woyengo *et al*., 2011; Vigors *et al*., 2014) and other gut hormones (Liu *et al*., 2009). Cooper and Growing (1983) found endogenous phytase in the brush border membrane of rat intestines and hypothesised that because of its location, it may play a role in facilitating metal absorption from dietary phytate. Very low levels of phytase have also been found in the gut of humans (Iqbal *et al*., 1994). Similar observations were made by Maenz and Classen (1998) in chickens, who found brush border endogenous phytase activity mostly occurs only in the pH range 5.5 to 6.5. Interestingly in all studies, the authors reported the highest intestinal phytase activity taking place in the upper sections of the small intestines, though levels they observed were very low, given that extra phosphoric effects have mostly been associated with super dosing phytase (>1500 FTU/kg).

Kemme *et al*. (2006) investigated phytate degradation in the upper gastrointestinal tract of the pig using up to 900 FTU/kg of a 3-phytase derived from *Aspergillus niger*. Their evidence, based on using cannulated weaned pigs fed a maize/soybean diet, indicated a difference in the quantitative pattern of lower inositol phosphate products. They noted high formation of IP_5 and IP_4 in the duodenum at low phytase levels (186 FTU/kg) which shifted to destruction of higher inositol phosphate products (IP_{6-4}) but high formation of IP_2 at a phytase dose of 1000 FTU/kg. It has been demonstrated that lower IPs do not have the same, if any, protein binding capacity at low pH (Yu *et al*., 2012) and are thus less likely to reduce protein digestibility through binary phytate-protein complexes formed at low pH (Kies *et al*., 2006b).

Evidence shows that *in vitro*, phytase significantly reduces phytate-protein complexes (Yu *et al*., 2012; Kies *et al*., 2006b) and conversely *in vivo*, high dietary phytic acid reduces protein digestibility to a greater (Liao *et al*., 2005a; Lehnen *et al*., 2011) or lesser extent (Woyengo *et al*., 2009). Despite this, *in vivo* experiments indicate that supplemental phytase of ~500 FTU/kg marginally increase crude protein (CP) digestibility by ~2 % (Mroz *et al*., 1994; Liao *et al*., 2005b) but super dosing phytase provides no additional benefits for pigs (Traylor *et al*., 2001; Liao *et al*., 2005a) or chickens (Augspurger and Baker, 2004). This may be due to the presence of high dietary mineral concentrations, since a meta-analysis by Lehnen *et al*. (2011) indicates that high Ca and P can reduce phytase activity. Alternatively, the diet matrix may also play a role as Liao *et al*. (2005b) only found a phytase effect on CP in wheat-soybean meal/canola meal diet and not in corn-soybean, wheat-soybean or barley-pea-canola meal diets.

2.6.2. Ternary phytate – mineral – protein complexes

In vitro experimentation by Yu *et al*. (2012) has indicated that doses of microbial phytase of between 1000 - 2000 FTU/Kg degrade phytate in just 10 - 20 minutes but lower doses of 250 - 800 FTU/Kg require considerably more time to work (3 - 7+ hours). A rapid breakdown of phytate complexes in the stomach by using high doses of phytase (1000+ FTU/Kg) may thus still be of great use in practical pig diets. In particular, the smaller dimensions of the weaner stomach in relation to adult animals lends itself well to a rapid breakdown of phytate complexes by using higher doses of better performing (second/third generation) phytases. This may allow for the vast majority of IP_6 to be reduced to lower esters before they enter the small intestines, therefore maximising the opportunity for nutrient absorption by minimising the probability of ternary phytate-mineral-protein complexes described by Champagne (1988) formed at intermediate pH. Estimates of additional digestible mineral generation from exogenous phytase based on a meta-analysis of the literature are presented in Table 2.13.

Table 2.13: Generated digestible minerals (per kg of diet) from microbial phytase for growing pigs

Microbial phytase (FTU/kg)	dCa (g/kg)	dMg (g/kg)	dP (g/kg)	dNa (g/kg)	dK (g/kg)	dCu (mg/kg)	dZn (g/kg)
0	0.00	0.00	0.00	0.00	0.00	0.0	0.0
100	0.46	0.04	0.49	0.04	0.25	1.3	2.2
250	0.62	0.05	0.67	0.06	0.33	1.8	3.0
400	0.71	0.06	0.76	0.06	0.38	2.0	3.4
500	0.75	0.07	0.80	0.07	0.40	2.1	3.6
750	0.83	0.07	0.88	0.07	0.44	2.4	4.0
1000	0.88	0.08	0.94	0.08	0.47	2.5	4.3
1250	0.92	0.08	0.99	0.08	0.49	2.6	4.5
1500	0.96	0.08	1.02	0.08	0.51	2.7	4.6
2000	1.01	0.09	1.08	0.09	0.54	2.9	4.9
5000	1.18	0.10	1.27	0.11	0.64	3.4	5.8

(Source: adapted from Jongbloed and Bikker, 2010)

The relationships between added phytase level and generated digestible mineral is quadratic and positive for all minerals analysed (Table 2.13). Phytase super dosing may thus require a re-appraisal of dietary formulations, given that a release of minerals and some proteins from phytate complexes can be expected. Many of these liberated mineral ions have also been shown to play essential roles in biochemical pathways and immune function. Taken together, it can be speculated that super dosing effects may be particularly useful in mitigating some of the symptoms associated with the post-weaning growth check, however, there is no clear consensus regarding the underpinning mechanisms. Currently, there is no data on the maximum dose of phytase that is effective at producing increased

growth performance in pigs, since Veum *et al*. (2006) did not reach a growth plateau at 12,500 FTU/kg.

2.7. Zinc interactions

Zn supplied in various forms is well known to help reduce post-weaning challenge due to its roles in gut integrity (Hu *et al*, 2013; Han *et al*, 2014), and immune function (Kidd *et al.*, 1996). Depending on the form it is fed, absorption varies from up to 100 % from zinc sulphate ($ZnSO_4$) and 50 – 80 % in zinc oxide (ZnO) (NRC, 2012), although a lower estimate for ZnO of only 20 % has been reported (Poulsen and Larson, 1995). Recommendations for inclusion in weaner pig diets vary depending on dietary ingredients but 75 – 100 mg/kg is generally considered to be sufficient (Whittemore *et al.*, 2003; NRC, 2012; Hill *et al.*, 2014). In cases of post-weaning diarrhoea, pharmaceutical supplementation of zinc (1000 – 5000 mg/kg) is commonly prescribed. In general, ZnO supplementation post-weaning improves growth rates, feed intake and feed conversion ratios (Sales, 2013), although these have not been seen in all cases (Walk *et al.,* 2013; Chai *et al.*, 2014). This may be explained by zinc's antimicrobial action against some pathogens (Bratz *et al.*, 2013) but not against others (Li *et al.*, 2001; Šperling *et al.*, 2014; Chai *et al.*, 2014). This mixed response to the effectiveness of high zinc supplementation for newly weaned pigs needs further research given that excess Zn is excreted via faecal matter in high levels and is well known to pollute land and watercourses.

Phytate is a strong chelator of Zn which forms phytase resistant complexes (Maenz *et al*., 1999) with endogenous zinc secreted in the upper GI tract (Harland and Oberleas, 2010). Exogenous phytase activity thus needs to primarily occur in the stomach due to the solubility of phytate at low pH (Schlemmer *et al.*, 2001). Zn dependent phytase has been reported to be localised to the brush boarder membrane of the duodenum of the rat (Davies and Flett, 1978), suggesting this is an important site for phytase and Zn interactions. At normal doses of phytase, zinc bound to phytate complexes is released, allowing for the recommended inclusion levels in the diet to be substantially lowered from ~100 mg/kg to 35 mg/kg (Revy *et al.*, 2006). However, at pharmaceutical levels Zn supplementation has been shown to reduce the efficacy of phytase for releasing dietary P for pigs (Augspurger *et al*., 2004). Recent research has, however, suggested that super doses of phytase enzymes may be an alternative strategy to high levels of zinc supplementation post-weaning to boost growth rates (Walk *et al.*, 2013). Research should therefore focus on comparing super dosing of phytase to a ZnO positive control in seemingly healthy pigs, to establish if they have similar growth promoting effects. Of equal interest is the possibility that pharmaceutical ZnO may reduce the effects of phytase super dosing due to suppression of phytase activity by high zinc concentrations.

2.8. Conclusions and research gap

Numerous studies have assessed the impact of nutrition around weaning on the lifetime performance of pigs. Abrupt weaning is clearly a critical control point in the life of the pig when changes in environment and diet lead to altered intestinal architecture, gut microbiota and gut immunity. The removal of dietary anti-nutritional factors, in particular phytate, at this time, may help the pig to mitigate some of the factors culminating in the commonly observed post-weaning growth check. There is some evidence to suggest that super doses of phytase enzymes can be used post-weaning to almost completely breakdown dietary phytate and to enhance mineral digestibility, especially zinc. It is also known that pharmaceutical doses of zinc oxide help reduce pathogenic microbial loading in the gut but only for some microbes. High levels of ZnO, such as those currently stated on the POM-V label (3100 mg/kg), may however suppress phytase activity, meaning that further information is needed on the optimal inclusion rates of phytase post-weaning and its effect in combination with zinc oxide. This needs to be focused on wheat based diets for the UK market, to help inform guidance for feed manufactures and nutritional consultants.

CHAPTER THREE

General materials and methods

3.0. Introduction

Common materials and methods used throughout this thesis are reported in this chapter. In addition, developmental works are also included, on the selection of vacutainers for trace element analysis (section 3.4.2) and the selection of suitable reference genes for gene expression analysis (section 3.6.1). Specific information regarding the choice of techniques are detailed in the relevant experimental chapters.

3.1. Animals

All experimental procedures were approved by Harper Adams University Ethics Committee and regulated procedures were conducted in compliance with UK regulations (Animals [Scientific Procedures] Act 1986). Protocols were designed in accordance with the ARRIVE (Kilkenny *et al.,* 2010) and MIQE (Bustin *et al*., 2009) guidelines. Pigs were sourced from the Harper Adams University Pig Unit, which maintains a high health status and conforms to the Red Tractor Assurance for Farms - Pigs Scheme (AFS, 2013).

3.1.1. Reduction

Three components were assessed in order to reduce the number of pigs required for each experiment:

1. Control of inter-subject variation
2. Sample size determination
3. Integration of experiments

Healthy animals were used at all times to reflect baseline parameters found in commercial pig production and to reduce variation in the measured parameters, thus helping to control inter-subject variation.

The sample size required to give adequate statistical power for each experiment was based on data obtained under similar conditions from previous experiments, using the procedure of Berndtson (1991), to detect an n % difference, with 80 % power, at the 95 % level of probability. Justification for the level of type one and type two error come from the requirements of FEEDAP (2011) for designing studies to assess tolerance and efficacy of zootechnical feed additives. Pigs are social animals and prefer to live in herds rather than in isolation, potentially becoming more easily stressed if individually housed, in part due to a lower maximum corticosteroid binding capacity (Barnett *et al*., 1981). Grouping animals

into pens rather than separating individuals allows the animals to express normal social behaviour. Pen studies also provide better quality information, since each replicate is an average measure of several pigs, thus reducing variation associated with individuals. Given the nature of the trials, the diets or housing conditions were not expected to produce any adverse effects. Balancing this with the stress that individual housing may cause, pens of animals were thus the most appropriate method to meet objectives in these studies.

3.1.2. Refinement

Environmental conditions in all experiments were close to those found in commercial practice and were designed to maximise performance by reducing stress and maintaining healthy stock. In addition, trained and highly experienced technicians were in attendance to ensure adequate care was provided. Protocols were refined following review by a qualified veterinary surgeon.

3.1.3. Replacement

The use of pigs in this thesis was unavoidable, as other animals would not adequately represent the conditions of the pig GI tract. Furthermore, other species require different dietary levels of nutrients that would not be appropriate for pig production. Consideration was given to non-animal models and *in vitro* studies where appropriate.

3.1.4. Genotypes

Pigs used in this thesis were from two dam lines (JSR Genpacker 90 [LWxLR] and PIC Camborough [[LWxLR] x White Duroc]) mated to various terminal sire lines, specified in each chapter. Both genotypes are representative of the UK national herd and were balanced across all treatments. One day prior to weaning, all progeny in the farrowing batch (n=~32 sows) were weighed, tagged in the ear with a unique number and the sex and genotype were recorded for selection purposes.

3.1.5. Housing

Fully slatted weaner accommodation with environmental controls were used in all experiments. Housing type A (Figure 3.1 a) consisted of twelve pens per building and was used in experiments SM1, SM3 and SM5, whereas housing type B (Figure 3.1 b) consisted of eight pens per building and was used in trial SM2.

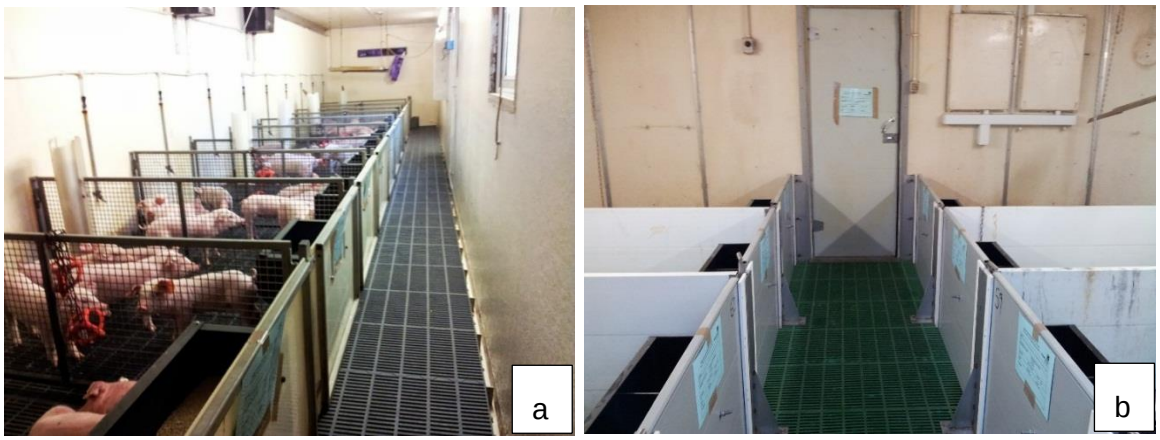

Figure 3.1: Housing type A (a) used in experiments SM1, SM3, SM5 and housing type B (b) used in experiment SM2

Single sex and genotype pens were established for trials SM1, SM3 and SM5, with mixed pens used in trial SM2 due to building limitations. Building temperature was set at 28 ^{0}C during week one and stepped down by 1 ^{0}C every two days until a final temperature of 24 ^{0}C was achieved by the end of week three post-weaning.

3.2. Feed analysis

Approximately 1 kg of feed was collected into plastic sample bags from unused 25 kg bags at the start of each batch. These were immediately frozen and stored at -20 ^{0}C until analysis.

3.2.1. Premix formulation

All experimental diets contained a premix sourced and mixed by Primary Diets Ltd (Rippon, UK) providing (per kg of complete diet as fed): 11,500 IU of vitamin A, 2,000 IU of vitamin D3, 100 IU of vitamin E, 4 mg of vitamin K, 27.5 µg of vitamin B12, 15 mg of pantothenic acid, 25 mg of nicotinic acid, 150 µg of biotin, 1.0 mg of folic acid, 160 mg of CU ($CuSO_4$), 1.0 mg of Iodine (KI, Ca(IO3)2), 150 mg of Fe ($FeSO_4$), 40 mg of Mn (MnO), 0.25 mg of Se (BMP^{TM}-Se), 110 mg Zn ($ZnSO_4$).

3.2.2. Minerals

Inorganic elements were quantified in feed using inductively coupled plasma mass spectrometry (ICP – MS) based on the method of Cope *et al*. (2009). 0.5 g of freeze dried, milled sample was weighed into plastic DigiTubes and digested in 1 ml concentrated analytical grade HCL (Fisher Scientific) + 6 ml concentrated analytical grade HNO_3 (Fisher Scientific), using a DigiPREP heating block with the following program (Table 3.1):

Table 3.1: DigiPREP heating program for acid digestion of compound pig feed destined for mineral analysis by ICP-MS

Program	Time (minutes)	Temperature set point (^{0}C)
To temperature:	30	45
At temperature:	01	
To temperature:	25	65
At temperature:	05	
To temperature:	15	100
At temperature:	45	

In addition to the samples, a tube containing only the acid mix was also heated in each batch as a reagent blank. Following digestion, all samples including the reagent blanks were made up to 50 ml volume with Purite water and stored at ~8 ^{0}C until analysis. Duplicate 250 µl of digested sample was vortexed with 4.75 ml of Ga (Romil) spiked diluting acid (1000 ppb Ga + 20 ml concentrated HNO_3 + 10 ml methanol + 1 ml Triton X + Purite water to volume) into 5 ml plastic sample tubes.

The 1:20 diluted samples were run on an X Series 2 ICP-MS (Thermo Fisher Scientific Inc) in standard operating mode. Before analysis, mass calibration was run to ensure the machine was operating within normal parameters using 5 ppb Tune A (Thermo Electron Corporation, Bremen, Germany) with ten reference elements (Figure 3.2).

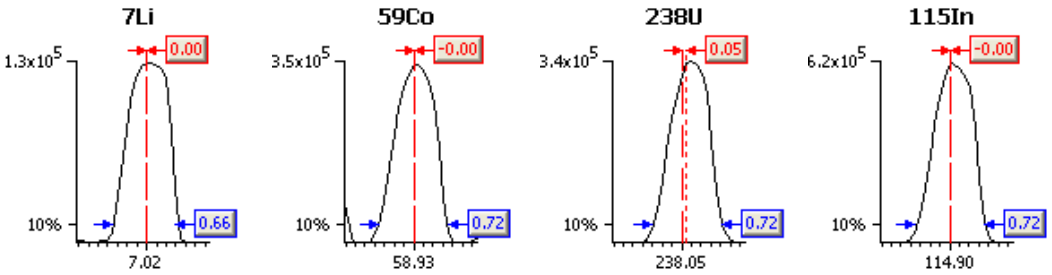

Figure 3.2: Typical mass calibration peaks of 5 ppb Tune A using X Series 2 ICP – MS

Accepted peak width (measured at 10 % of peak maximum) range was 0.65 – 0.85 and maximum peak error was below 0.1 %. The ratio of ^{156}CeO to ^{140}Ce was not allowed to exceed 0.02. A cross calibration and voltage detector setup was run post the successful pass of the mass calibration, using 50 ppb Tune F solution (Thermo Electron Corporation, Bremen, Germany).

Standard calibration curves were established for each element using a five point serial dilution (see relevant chapter for the range of elements analysed) and mathematically forced through the blank acid. The paired technical duplicate aliquots were loaded into an auto-sample rack for analysis with a Ga acid blank and calibration standard run after every ten samples. A mean value for each sample was calculated from three readings of each of the technical sample aliquots. These were normalised in relation to the Ga internal standard

and the reagent blank was subtracted. Final values were calculated by adjusting for the original sample weight. Each run of analysis was validated against two European Commission certified reference materials (Hay powder - BSR® 129 and Dairy feed - BSR® 708) for feed and faecal samples. Liver samples were validated against one European Commission certified reference material (Bovine liver - BSR® 185R).

3.2.3. Inositol

Inositol was measured by pulsed amperometry at the School of Biological Sciences, University of East Anglia. Filtered samples were diluted 50-fold in 18.2 MOhm.cm water. Aliquots (20 µL) were injected onto a 4 mm x 250 mM CarboPac MA1 column (Dionex) fitted with a 4 mm x 50 mm guard column of the same material. The column was eluted isocratically with 150 mM NaOH delivered at a flow rate of 0.4 mL/minute from a Dionex GP50 HPLC pump. Inositol was detected on a gold electrode with a Dionex ED40 electrochemical detector. The column and electrode were maintained at 35 °C in a Dionex LC25 oven. The following pulse protocol was applied to the electrode (Table 3.2.):

Table 3.2: Pulse amperometry protocol for HPLC quantification of inositol

Time (min)	0:0	0:20	0:40	0:41	0:42	0:43	0:44	0:50
Voltage (V)	0.1	0.1	0.1	-2.00	-2.00	0.60	-0.10	-0.10

Signal was integrated in the 0.2 – 0.4 minute interval and sampled at a frequency of 0.5 Hz. Calibration curves for inositol measurement were afforded by injection of 20 µL aliquots containing 0.25 – 4 nmol of inositol (Sigma-Aldrich Co LLC).

3.2.4. Inositol phosphates

Inositol phosphates were extracted from freeze-dried, milled feed material or digesta (0.1 g) at the School of Biological Sciences, University of East Anglia, with 5 mL of 200 mM disodium EDTA, 100 mM NaF, adjusted to pH 10.0 with NaOH in 15 mL polypropylene centrifuge tubes (Corning CentriStar, part no. 430791). Samples were incubated on a rotating platform (250 rpm) for 30 minutes at room temperature and then transferred to a bath sonicator and extracted for a further 30 minutes at 4 °C. The resulting samples were centrifuged at 9,000 x g for 15 minutes. 2 mL of the supernatant was filtered by hand through a 13 mm, 0.45 µm PTFE syringe filter (Kinesis, part no. ESF-PT-13-045) into 2 mL borosilicate glass auto-sampler vials. Aliquots of the filtrate (typically, 20 – 40 µL) were injected onto the HPLC system.

Inositol phosphates were then separated on a 3 mm x 250 mm CarboPac PA200 column (Dionex) and 3 mm x 50 mm guard column of the same material. The column was eluted with a gradient of methanesulfonic acid delivered from solvent reservoirs A and B containing 60 mM and 600 mM methane sulfonic acid, respectively. The gradient was delivered at a flow rate of 0.4 mL/minute according to the following programme (Table 3.3):

Table 3.3: Methanesulfonic acid delivery protocol for HPLC quantification of inositol phosphates

Time (minutes)	0	22	25	38
% B	10	60	100	100

Solvents were delivered using a Jasco PU-2089i Plus inert quaternary pump and samples were injected at 49 minute intervals using a Jasco AS-2055i Plus inert auto-sampler. The solvent stream eluting from the column was mixed, using a mixing T, with a solution of 2% perchloric acid containing 0.1% w/v ferric nitrate delivered at a flow rate of 0.2 mL/minute using a Jasco PU-1585 pump, before passage through a knitted reaction coil (200 uL) and detection of peaks at 290 nm in a Jasco UV-2077 Plus UV detector.

3.2.5. Total phytic acid

Samples from trial SM2 were analysed by Enzyme Services & Consultancy (ESC), UK using a modified Megazyme method K-PHYT 08/14 (Megazyme International Ireland) designated SAM074. Samples were extracted in 0.66 M HCL (> four hours/overnight with a ratio of liquid to sample of 20:1) for inositol phosphates followed by treatment with a phytase that is specific for phytic acid (IP_6) and the lower inositol phosphate forms. Subsequent treatment with alkaline phosphatase ensured the release of the final phosphate from *myo*-inositol phosphate (IP1) which is relatively resistant to the action of phytase. The total phosphate released was measured using a modified colorimetric method. This was converted to phytic acid by dividing the values by 0.282 (it is assumed that phytic acid contains 28.2 % P). Samples from trial SM2 and SM3 were analysed by ESC using a Near Infrared (NIR) spectrometer (FOSS, Cheshire, UK), with the Phytate-P levels predicated using AUNIR calibration standards (designated by ESC as method: SAM120). The correlation coefficient between the same samples from experiment SM2 analysed by the Megazyme method and NIR was 0.988 ($p<0.001$), suggesting that either method was appropriate.

3.2.6. Phytase activity

Feeds were analysed for phytase activity by Enzyme Services & Consultancy (ESC), UK using an ELISA method with Quantiplate Kits for Quantum Blue® (Envirologix, USA) based on Envirologix method AP181, Rev. 12-28-11 with the following modifications:

1. Sample extraction was carried out by automated stirring, as opposed to the technique of shaking by hand as described in the Envirologix method and in the ratio of ~1:10 sample:buffer, as described in the wet chemistry method, rather than ~1:4 as described in the Envirologix method.

2. Results were standardised against a standard curve prepared in-house at ESC, from a sample of Quantum Blue® enzyme with a known activity in FTU/g, as determined by the Quantum product method, rather than using the standards supplied with the ELISA kits. Dilutions of the Quantum Blue® enzyme product and of the sample extracts were carried out in acetate buffer (pH 5.5) as described in the Quantum product method, as opposed to the Quantum Assay Diluent supplied in the kits.

3. Samples were also analysed in duplicate (duplicate extractions), with each extraction also tested in duplicate wells, whereas the kit method normally applies a single extraction and with a single well test.

In addition to the ELISA test, mega doses of phytase were double checked using a wet chemistry method, based on phytase activity using phytic acid (Sigma-Aldrich Co LLC, Product code: P8810) from rice as a substrate, according to the Quantum feed assay (extraction for 30 minutes in 25 mM Borate, pH 10.0, analysis at pH 4.5 and 60 ^{0}C). The method is based on the end-point determination of phosphate using a molybdate-vanadate colour system. Units were measured in QPU and then converted to FTU by dividing the QPU by a conversion factor of 5.67.

3.3. Nutrient digestibility

Nutrient digestibility was estimated using live pigs by the index method (Adeola, 2001). The feed marker of choice was $Ti0_2$ (Titanium (IV) oxide anatase [Sigma-Aldrich Co LLC, Product code: 248576]), added to all experimental diets at a rate of 3 g/kg (as fed). Digesta was collected from individual pigs immediately post-mortem and fresh faecal samples were collected immediately post defecation off a slatted floor from at least three animals per pen of five, into 100 ml plastic pots. In either case, these were frozen at -20 ^{0}C until freeze drying at -45 ^{0}C for ~10 days (until a constant weight). Samples were then manually ground using a pestle to avoid milling contamination. Following acid digestion (as described in section 3.2.2), the titanium was quantified using ICP-MS in standard operating mode, as an additional element per run, in parallel to the mineral analysis. Isotope ^{49}Ti was measured instead of the common ^{47}Ti isotope due to possible interference with the argon carrier gas. The digestibility of each element was then calculated according to the following formulas:

Dry matter digestibility:

$$\frac{\text{Indicator in faeces or digesta (g/kg) - Indicator in food (g/kg)}}{\text{Indicator in faeces or digesta (g/kg)}} = \text{Dry matter digestibility}$$

Nutrient digestibility coefficients:

$$1 - \left[\frac{Marker\ in\ food\ (mg\ per\ g\ DM) * Nutrient\ in\ waste\ (mg\ per\ g\ DM)}{Marker\ in\ waste\ (mg\ per\ g\ DM) * Nutrient\ in\ food\ (mg\ per\ g\ DM)}\right] = Nutrient\ digestibility$$

*Waste denotes faeces or digesta

3.4. Haematology

Blood samples were collected by jugular venepuncture, into suitable vacutainers, using 25 mm (20 gauge) needles for pigs sampled during the first or second week post-weaning and 25 mm (18 gauge) needles for those sampled in the third week post-weaning. Pigs were restrained using a V-shaped board of an appropriate size throughout the procedure.

3.4.1. Cell differentiation

Blood was collected by jugular venepuncture into a 4.0 ml BD Vacutainer® containing 7.2 mg K_2EDTA (as described in 3.4.). Blood was refrigerated overnight and analysed in technical duplicates using an $MS4_5$ impedance counter (Melet Schloesing Laboratories, Osny, France), following the manufacture's protocol and validated with a normal level control sample (Woodley Equipment Company Ltd, Lancashire, UK). This provides a five cell differentiation for the white blood cells (basophils, eosinophils, neutrophils, monocytes and lymphocytes) and also reports red blood cell count (RBC), MCV, Hct, MCH, MCHC, RDW, Hb, THR, MPV, Pct and PDW.

3.4.2. Plasma and serum minerals

The use of mineral concentration as an indicator of health is a tool widely used by veterinarians and animal scientists. The optimal choice of sample type and collection protocol is dependent on the mineral under investigation (Herdt and Hoff, 2011). If blood is to be used, it can either be allowed to clot (for serum) or prevented from clotting (for plasma) prior to analysis. BD Vacutainer® tubes contain a range of anticoagulants and have a product line specifically for trace element analysis. A pilot study (SM4) was conducted to assess differences in determined blood mineral concentration dependent on the choice of collection tube and internal coating.

Blood was collected by jugular venipuncture from four weaned pigs (see section 3.4.), fed on a wheat based commercial diet, in three different BD Vacutainer® tubes (Becton, Dickinson and Company: Oxford, UK). These were one serum trace element tube (CAT), one trace element tube spray coated with K_2EDTA and one standard tube spray coated with lithium heparin (LH). Tubes were allowed to stand at room temperature for two hours before centrifugation at 1000 x g for ten minutes at 4 °C. Plasma and serum were pipetted into 2.0 ml microtubes and stored at 4 °C overnight prior to storage at -20 °C. Defrosted plasma and serum was vortexed and diluted 1:20 with 1 % HNO_3 prior to elemental analysis using an X series 2 ICP-MS in standard mode, running Plasma Lab software (Thermo Scientific Inc, USA). Calculations were performed in Excel 2010 (Microsoft Corporation) and analysed in GenStat sixteenth edition (VSN International Ltd) using ANOVA blocked by pig and followed by Tukey test (Tukey, 1949) where significance was indicated.

There were no differences in blood mineral P, Ca, Mn, Fe, Cu, Se or Mo concentrations when obtained from serum or plasma. Zinc concentrations were lower in serum than in either of the plasma samples ($p=0.01$). Magnesium concentrations were lower in the LH tubes over the two trace element tubes ($p=0.002$). K concentration was elevated in the K_2EDTA coated tubes ($p=0.001$) as expected due to the K present in the preservative but was also different between serum and LH plasma ($p<0.05$). The concentration of Na was higher in the K_2EDTA coated tube compared with the serum tube ($p=0.04$) but neither were different to LH plasma. Concentrations of Co obtained from the serum samples were lower ($p=0.03$) than those found in LH plasma but not from K_2EDTA plasma. Variation between pigs was generally low (<20%) with the exception of Fe (41.8%) and Co (31.8%). The biological ranges measured were within the reported ranges from the literature (Table 3.4), with the exception of Fe, Zn and Cu.

Table 3.4: Deviation in blood minerals between BD Vacutainer® collection tubes and normal reference ranges for pigs

Element	Unit	Normal range[1]	Deviation of vacutainer means[2]	Biological sample range[3]
Na	mmol/l	14	2.16	4.61
Mg	mmol/l	0.82	0.22	0.55
P	mmol/l	2.25	0.36	0.89
K	mmol/l	2.7	1.25	1.90
Ca	mmol/l	1.12	0.04	0.38
Mn	µmol/l	-	0.09	0.17
Fe	µmol/l	12	14.9	46.67
Co	µmol/l	-	0.00	0.01
Cu	µmol/l	1.3	1.26	13.71
Zn	µmol/l	1.2	2.88	7.47
Se	µmol/l	-	0.08	0.38
Mo	µmol/l	-	0.09	0.33

[1]Min – max of literature quoted normal values; [2]The difference between the highest and lowest vacutainer means; [3]Range calculated for all replicate tubes (not just mean values).

Despite the statistical differences, when the variation between vacutainer means are compared (Table 3.4), there is little practical difference for most minerals. The exceptions are Fe, Zn and Cu which have larger biological ranges than expected.

As a result of SM4, plasma samples for experiments SM2, SM3 and SM5 were collected into lithium heparin vacutainers. These were centrifuged at 1000 x g for 10 minutes at 4 °C, prior to plasma storage at -20 °C until analysis. Defrosted plasma was vortexed and diluted 1:20 with 1 % HNO_3 prior to elemental analysis using an X series 2 ICP-MS in standard mode, running Plasma Lab software (Thermo Scientific Inc, USA). The results in ppm or ppb were then divided by their molecular weight for conversion to mmol/L or µmol/L.

3.5. Gut histology

Gut histomorphology was based on the method of Pluske *et al*., (1996). Over days 22/23 post-weaning, one mean average weight pig from each pen was euthanized by captive bolt followed by destruction of the brain stem. Carcases were briefly washed in hot water and a vertical incision made along the belly from sternum to the pubis (Figure 3.3 a). The small intestines were clamped at the junction of the stomach/duodenum and the terminal ileum/colon, before being removed and carefully spread out on a clean surface. Plastic cable ties were used to divide the intestines into three equal sections representing the duodenum, jejunum and ileum (Figure 3.3 b).

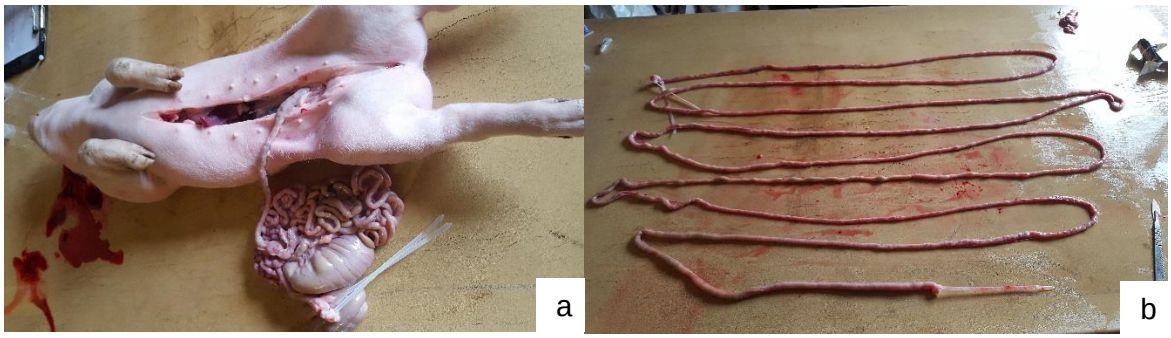

Figure 3.3: Dissection of small intestines out of pig abdomen (a) and sectioning of pig small intestines into duodenum, jejunum and ileum, using cable ties (b)

A three centimetre sample of each section was excised and immediately washed and stored in 10 % neutral buffered formalin. To represent the duodenum, this was taken ~15 CM posterior to the stomach/duodenum junction, from the centre of the small intestines to represent the jejunum and at ~15 CM anterior to the ileum/colon junction for the ileal section. On arrival at Finn Pathologists (Norfolk, UK), the sections were cut through the middle and placed on a trimming block for tissue processing using the following program (Table 3.5).

Table 3.5: Tissue processing procedure for embedding intestinal samples into wax

Reagent	Time (minutes)	Temperature (^{0}C)
70 % Industrial Mentholated Spirit	90	Ambient
95 % Industrial Mentholated Spirit	30	Ambient
95 % Industrial Mentholated Spirit	45	Ambient
100 % Industrial Mentholated Spirit	30	Ambient
100 % Industrial Mentholated Spirit	60	Ambient
100 % Industrial Mentholated Spirit	120	Ambient
Xylene	30	Ambient
Xylene	60	Ambient
Xylene	90	Ambient
Wax	30	61
Wax	60	61
Wax	60	61

The blocks were sectioned to 5 µm thick, baked for 30 minutes and then stained with Heamatoxylin and Eosin using an automated Leica ST4040, before being mounted on slides. Slides were examined by optical microscopy (Olympus CX31, equipped with an Olympus V-CMAD3 digital camera), calibrated with a graduated scale bar (C. Baker, London) and running Infinity Analyse version 6.2 (Lumenera Corporation, 2012) software to measure villus height, crypt depth and length of an average mucosal gland.

3.6. Gene expression

Absolute quantification (copies per reaction) of gene expression was used for trial SM2, whereas relative quantification was considered sufficient for experiment SM5. Intestinal sections (duodenum, jejunum and ileum) were macroscopically dissected (approx. 500 mg) from pigs post-mortem (see section 3.5) and immediately stabilised in RNA*later*® (Sigma-Aldrich, St. Louis, Missouri, USA) for 24 hours, prior to removal of excess RNA*later*® and long term storage at -80°C.

3.6.1. Selection of reference genes

Reference genes for normalisation of data were selected using the geNorm™ method (Hellemans *et al*., 2007) with six reference genes (Primer Design Ltd, Southampton, UK), from experiment SM5. Samples equally represented the treatment control group (pigs fed a 5.8 g/kg dgP basal diet), dietary ZnO group, dietary phytase group and those fed a ZnO and phytase combination (see chapter six for further details).

3.6.2. geNorm analysis

RNA was extracted and purified using the SV total RNA isolation system (Promega Corporation, Madison, Wisconsin, USA) from ~20 mg of macro-dissected duodenum tissue (Table 3.6). This was previously stabilised in RNA*later*® (Sigma-Aldrich, St. Louis, Missouri, USA), prior to storage at -80°C for five months. Genomic DNA was removed in a 15 minute digestion with DNase 1 (Promega Corporation, Madison, Wisconsin, USA).

Table 3.6: Yield and purity of geNorm RNA

Diet	ZnO (mg/kg)	Phytase (FTU/kg)	RNA yield (ng/ul)	Standard Deviation	260/280	260/230
1	-	-	272	185.7	2.1	2.1
2	-	2500	408	56.8	2.1	2.0
3	2500	-	209	67.2	2.1	1.9
4	2500	2500	421	111.1	2.1	2.1

Reverse transcription (RT) from RNA to cDNA was completed using *Precision NanoScript*™ 2 (Primer Design Ltd, Southampton, UK), with a random nonamer primer, since RNA integrity could not be checked. No more than 2 µg of total RNA was used in each 20 µl reaction. RT conditions involved a five minute annealing step at 65°C, followed by five minutes incubation at room temperature and then a 20 minute extension at 42 °C. Finally, the reaction was stopped by heating to 75 °C for 10 minutes. cDNA was diluted to a final concentration of 2.5 ng/µl and kept on ice for two hours until qPCR.

Three biological replicates per treatment group were analysed in duplicate technical replicates at the qPCR stage. Each 96 well white plastic plate (Bio-Rad, Hemel Hempstead, UK) contained all of the samples for that gene; with a total of three genes plus controls in each run, which negates the need for inter-run calibration (Hellemans *et al*., 2007). qPCR was performed using a 20 µl reaction, loaded with 12.5 ng of cDNA, nuclease free water, primer (300 nM) and PrecisionPLUS™ 2x qPCR Mastermix containing SYBR green 1 (Primer Design Ltd, Southampton, UK). Plates were run in immediate succession on a Bio-Rad CFX96 PCR system (Bio-Rad, Hemel Hempstead, UK), under the following reaction conditions (Table 3.7):

Table 3.7: qPCR reaction conditions for geNorm analysis for use with Bio-Rad CFX96

	Step	Time (minutes)	Temperature (°C)
Cycling (x50)	Enzyme activation (hot start)	2:00	95
	Denaturation	0:15	95
	Data collection	1:00	60
	Melt curve	70	60 – 95

While the primer sequence for the assays are not available, Table 3.8 gives details of the anchor nucleotide and the context sequence length, which are acceptable replacements (Bustin *et al*., 2011).

Table 3.8: geNorm™ Reference gene Selection Kit (pig) assay details

Gene name	Accession number	Anchor nucleotide	Context sequence length
ACTB	DQ452569	189	182
GAPDH	AF017079	918	188
GSR	AY368271	100	99
GPI	NM_214330	685	105
GPX1	NM_214201	265	158
PPIA	NM_214353	137	152

(Source: PrimerDesign Ltd, 2014)

Specificity of the target was confirmed by a unified peak in the melt curve at the expected amplicon temperature (Figure 3.4). The presence of gDNA was excluded by no template controls (NTC), which tended to form primer dimers after ~38 cycles, with a characteristic melting temperature of ~75 °C (Figure 3.4).

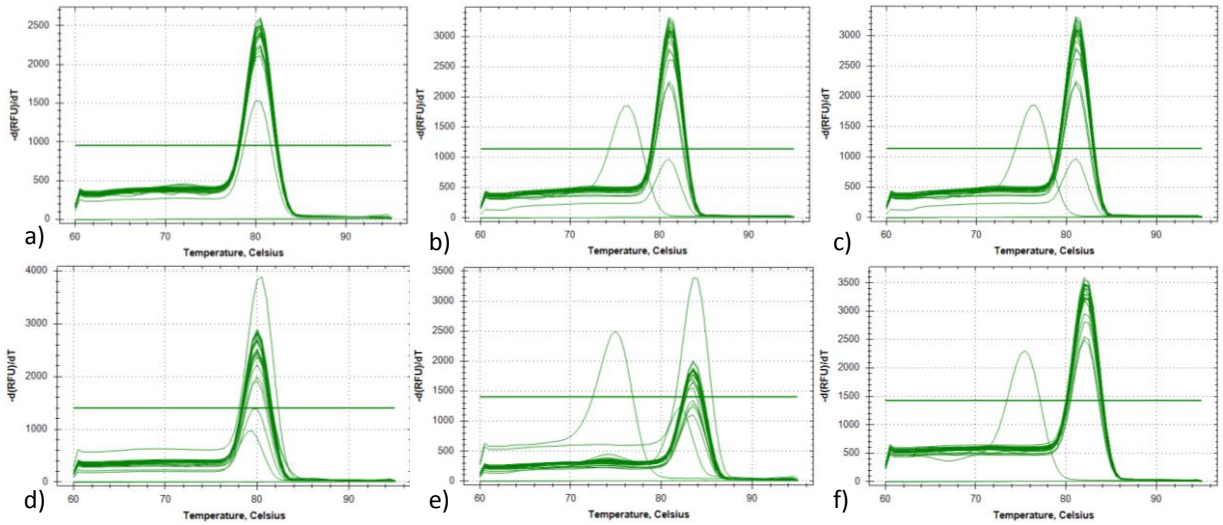

Figure 3.4: Melt curve analysis of geNorm reference genes. a) GSR, b) GAPDH, c) ACTB, d) PPIA, e) GPX1 and f) GPI. NTC are characterised by a melting temperature of ~75 °C, while target amplicons exhibit a melt peak ≥ 80 °C.

Raw fluorescence data without baseline correction was exported from CFX manager (Bio-Rad, Hemel Hempstead, UK) into LinRegPCR version 2014.2, where Cq values were set by amplicon group and PCR efficiencies were calculated using a four point window-of-linearity (Ramakers *et al*., 2003; Ruijter *et al*., 2009). Calculated Cq values were analysed using qbase+ software (Biogazelle, Zwijnaarde, Belgium) in the method of Hellemans *et al*., (2007), adjusting for PCR amplicon efficiency (Table 3.9). Outliers were excluded from data analysis if they were in excess of 0.5 cycles apart from their technical replicate, resulting in an 86.1 % pass rate.

Table 3.9: PCR amplification efficiencies and amplicon melting temperature

Amplicon	PCR Efficiency (%)	Melting temperature (°C)
ACTB	93.8	81.0
GAPDH	80.7	86.0
GPI	92.9	82..0
GPX1	90.9	83.5
GSR	92.1	80.5
PPIA	91.6	80.0

The stability of the candidate reference genes were calculated and showed high reference target stability (average geNorm M ≤ 0.5), usually associated with homogenous samples (Figure 3.5).

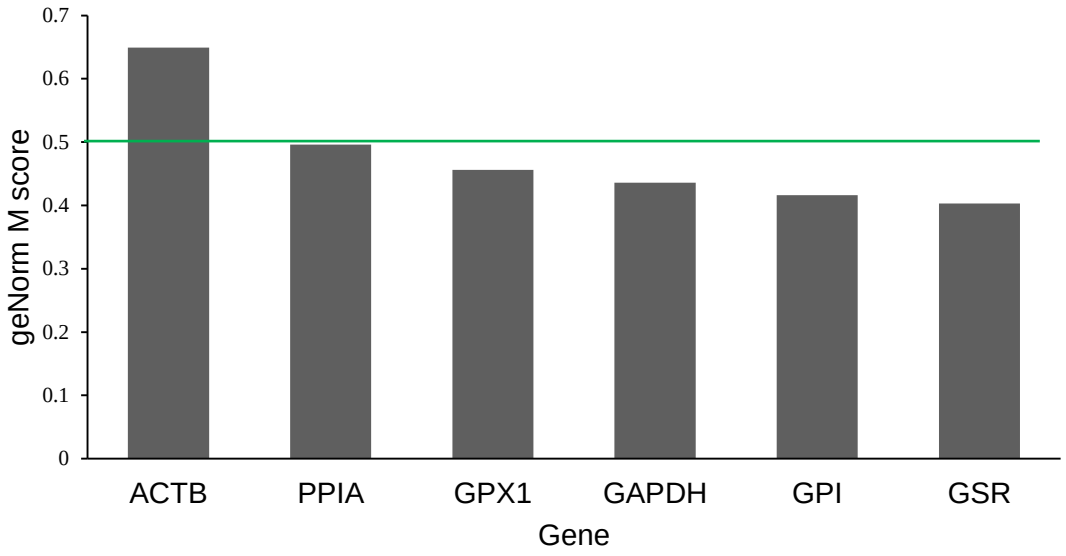

Figure 3.5: Expression stability score (geNorm M) of six candidate reference genes ranked from lowest (left) to highest (right), tested on 12 samples of duodenum tissue from weaned pigs. Horizontal line indicate gene stability threshold.

Finally, the best combination of reference genes was calculated by taking the two most stable reference genes and sequentially adding the other reference genes (Figure 3.6).

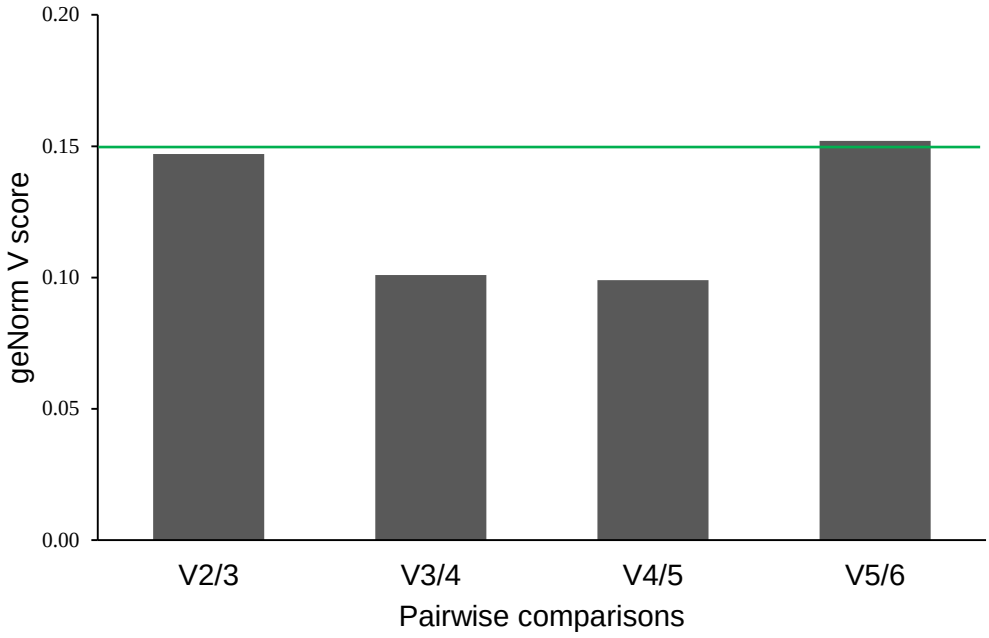

Figure 3.6: Pairwise comparisons (geNorm V) of six candidate reference genes tested on 12 samples of duodenum tissue from weaned pigs. Horizontal line indicate gene stability threshold.

A score of < 0.15 (geNorm V) was considered to be stable (Figure 3.6). Based on this, the optimal normalization factor could be calculated as the geometric mean of reference targets GPI and GSR. These were therefore used in experiments SM2 and SM5 to normalise the gene expression values.

3.6.3. Gene expression analysis

For experiment SM2: RNA was extracted and purified from intestinal tissue samples (see section 3.6) using an RNeasy kit (Qiagen Ltd, Manchester, UK). Purified RNA was then checked for integrity using a bioanalyzer (Agilent Technologies, Inc.) and yield/purity with a Nanodrop 2000 (Thermo Fisher Scientific Inc.), prior to RT. 800 ng of RNA were reverse transcribed using the Qiagen Quantitect reverse transcription kit (Qiagen Ltd, Manchester, UK), in a 10 uL reaction, according to the manufacturer's instructions. This RT kit includes a mandatory gDNA wipe-out step, which was extended to 4 minutes at 42 °C for these samples. RT duplicate reactions were performed for four samples. The completed reaction was diluted 10-fold with 5 ug/mL tRNA in water. Two microliters of cDNA were amplified in a 10 uL reaction using Agilent Brilliant III SYBR Green mix with each primer at a final concentration of 500 nmol/L. The no-template control reaction contained 2 uL of tRNA (diluted to 5 ug/mL). DNA standards (10^7-10^1 copies/reaction) for each gene were included in each run. Reactions were pipetted robotically using a Qiagility. The PCR amplifications were performed using a Rotor-Gene 6000 (Qiagen Ltd, Manchester, UK) under the following condition (Table 3.10):

Table 3.10: qPCR reaction conditions for gene expression analysis for use with Roto-Gene 6000

	Step	Time (minutes)	Temperature (°C)
Cycling (x40)	Enzyme activation	3:00	95
	Denaturation	0:10	95
	Data collection	0:20	60

Melt curves were checked for product specificity (single peak) and the presence of primer dimers. All primers were designed by qStandard (Middlesex, UK) to be intron-spanning and therefore unable to amplify gDNA. Assay specify was checked by agarose gel electrophoresis of PCR products and these were used to produce standards of known copy numbers. Linearity, sensitivity and efficiency of the standards were assessed by running a 7 log standard curve. Copy numbers/reaction were derived from the standard curves using the Rotor-Gene software and normalised to GPI and GSR (as identified in section 3.6.2). The primers for GPI and GSR for experiment SM2 were designed by qStandard and summarised in Table 3.11.

Table 3.11: Assay details for qPCR primers designed to detect SGLT1, ALPI and ZIP4 mRNA transcripts from pig (Sus Scrofa) intestinal tissue

Gene symbol	Gene	Accession number	Primer sequences	Product length
GPI	GPI	NM_214330	S – TAGGACAGCCAGTGAGGAGT A – TTCTCCTCTGCCACAGATGTC	99
GSR	GSR	XM_003483635	S – CCAGCCTAGGAATTACCAGTG A – GGATTCCAGCTATCTCCACAG	105

(Source: qStandard, 2015)

For experiment SM5: RNA was extracted and purified from intestinal tissue samples (see section 3.6) prepared from section 3.6.1, using the SV total RNA isolation system (Promega Corporation, Madison, Wisconsin, USA). Genomic DNA was removed in a 15 minute digestion with DNase 1 (Promega Corporation, Madison, Wisconsin, USA). Purified RNA was then checked for integrity using an E-gel (Thermo Fisher Scientific Inc.) and yield/purity assessed using a Nanodrop 2000 (Thermo Fisher Scientific Inc.), prior to RT. Reverse transcription from RNA to cDNA was completed using *Precision NanoScript*™ 2 Reverse Transcription kit (Primer Design Ltd, Southampton, UK) with random nonamer primers in a 20 µL reaction. No more than 2 µg of total RNA was used in each 20 µl reaction. cDNA was diluted to 5 µL/ml and genes of interest were amplified in 20 µL reactions, using custom SYBRgreen gene specific primers with a Bio-Rad CFX96 PCR system (Bio-Rad, Hemel Hempstead, UK), under the following reaction conditions (Table 3.12):

Table 3.12: qPCR reaction conditions for cytokine gene expression analysis for use with Bio-Rad CFX96

	Step	Time (minutes)	Temperature (°C)
Cycling (x50)	Enzyme activation (hot start)	2:00	95
	Denaturation	0:15	95
	Data collection	1:00	60
	Melt curve	70	60 – 95

Melt curves were checked for product specificity (single peak) and the presence of primer dimers. Cq values were set by amplicon group and PCR efficiencies were calculated using a four point window-of-linearity (Ramakers *et al*., 2003; Ruijter *et al*., 2009). Normalisation of data was carried out using the geNorm™ method with six reference genes (Primer Design Ltd, Southampton, UK) and analysed using qbase+ software (Biogazelle, Zwijnaarde, Belgium).

3.7. Bone analysis

Following being euthanized by captive bolt and destruction of the brain stem (as described in 3.5), the pig's front right side leg was removed and frozen at -20 °C. Legs were defrosted overnight (at room temperature) and the 3rd and 4th metacarpal bones carefully dissected (Figure 3.7).

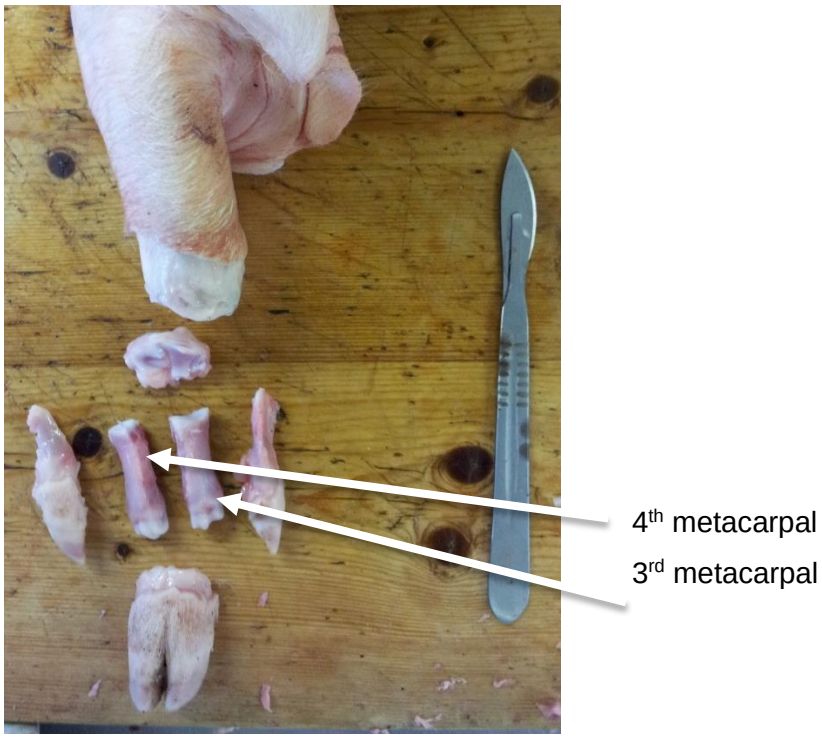

Figure 3.7: Dissection of the third and fourth metacarpals from the right side leg of seven week old pigs.

All bones were re-frozen at -20 °C in clear plastic bags for further testing. All the 4th metacarpals were freeze dried before the strength test and the 3rd metacarpals tested fresh, after being defrosted in a fridge overnight.

3.7.1. Bone strength

The lengths of all bones were measured at the longest point and the midpoint using a digital calliper (Mitutoyo), which was then marked with a marker pen to identify the centre of the bone. Shear strength measurements were obtained using a Sans SHT4035 300 kN testing machine at room temperature, in a modified method based on Combs *et al*. (1991), where the area of the bone was estimated by tracing the mid-point of the bone on 1 mm^2 graph paper. The instrument was calibrated and verified for compression for increasing forces to BS EN ISO 7500-1:2004, using verification equipment calibrated to BS EN ISO 376: 2004. Force was applied to the bone at 10 mm/minute until complete bone structural failure (Figure 3.8).

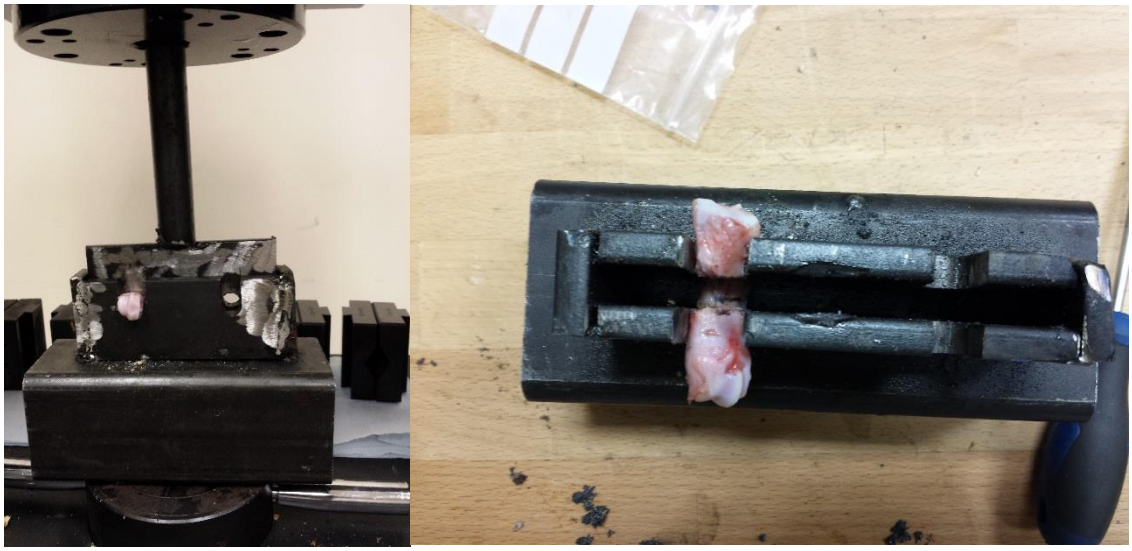

Figure 3.8: Shear strength testing of 3rd pig metacarpal bone using a SANS SHT4035 testing rig. A constant force of 10 mm/minute was applied to the bone (left image) until shearing was complete (right image).

The shear failure point was determined from a load graph to define the maximum load required for bone failure. Shear stress was calculated as:

$$\text{Shear stress } [\text{N/mm}^2] = \frac{\text{Force [N]}}{\text{Area [mm}^2]}$$

All bones were replaced into the labelled bags/pots and the 3rd metacarpals were frozen and freeze dried prior to ashing (see section 3.7.2.). Femurs and tibia bones were dried for four hours at 70 °C and assessed for compressive strength as detailed by Morris (2016).

3.7.2. Bone ash analysis

Porcelain crucibles were prepared by washing in *Aqua regia* (1 part HNO_3 and 3 parts HCl), neutralised in sodium bicarbonate and then washed in distilled water prior to bone ashing. Freeze dried whole bones recovered from shear testing were ashed in a muffle furnace at 800 °C for 18 hours (CEM, 2011), at a ramp rate of 3 °C per minute.

Ash percentage was defined as:

$$Ash\ [\%] = \frac{(Ash\ [g] - crucible\ weight\ [g])}{(dried\ bone\ weight\ [g])} * 100$$

Ash samples (0.25 g) were then digested for mineral analysis by ICP-MS in the procedure described in section 3.2.2.

3.8. Liver mineral analysis

Over days 22/23 post-weaning, one mean average weight pig from each pen was euthanized by captive bolt followed by destruction of the brain stem. Carcases were briefly washed in hot water and a vertical incision made along the belly from sternum to pubis (Figure 3.3 a). Liver samples were taken from the bottom of the liver lobe and frozen (-25 °C) prior to 0.25 g of being dried in a forced air oven (Phillip Harris LTD, Shenstone, UK) at 60 °C overnight without a lid. The tubes were cooled and re-weighed to calculate dry matter prior to digestion of the liver in 6 ml of nitric acid (HNO_3) at 60 °C overnight. Samples were diluted to 50 ml with Purite water and thoroughly mixed before being stored (4 – 6 °C) until analysis by ICP – MS. Mineral analysis was carried out according to section 3.4.2.

3.9. Sialic acid determination

Sialic acid standard (N-Acetylneuraminic acid {N-AM}) prepared from *Escherichia coli* was purchased (Sigma-Aldrich, UK, CAT No. A-2388). A fresh 1 mM stock solution was prepared by accurately weighing out 0.0309 g and dissolving this in 100 ml distilled water, before storing at a temperature of -20 ºC until required. A total of seven N-AM standards were prepared, covering a range of concentrations between 0 and 0.3 µmol. The standards were prepared in 10 ml screw-capped glass tubes, where 0, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 ml stock solution of N-AM were added to each tube in sequence. The tubes were then made up to a final volume of 0.5 ml, with the addition of 0.5, 0.45, 0.4, 0.35, 0.3, 0.25 and 0.2 ml distilled water.

Samples of freeze dried ileal digesta were analysed for their content of sialic acid (N-acetylneuraminic acid) according to a modified version of the method of Jourdian *et al*., (1971). A mass of 0.02 g of samples were weighed accurately into 10 ml screw-capped tubes (VWR Ltd., UK) and 0.5 ml distilled water was added to each, to make the tube volumes equivalent to that of the standards. The tubes for both samples and standards were treated equally for the remainder of the assay. A volume of 0.1 ml periodic acid (0.4559 g periodic acid (VWR Ltd, UK, CAT No. 20593.151) was weighed accurately, transferred to a 50 ml volumetric flask and made up to volume with distilled water to prepare a 0.04 M solution. This was added and the tubes mixed well, before the tubes were immediately placed in an ice bath for a period of 20 min, to allow the production of chromogens by oxidation of N-AN.

With the tubes still standing in ice to slow the reaction, a volume of 1.25 ml resorcinol solution (7 mg of $CuSO_4.5H_20$ [VWR Ltd., UK] was weighed accurately and placed into a beaker along with 0.6g resorcinol granules [Sigma-Aldrich, UK, CAT No. R-5645]. Using a measuring cylinder, 40 ml distilled water and 60 ml of 25 % HCl were each dispensed, and these were also added to the beaker and mixed thoroughly) was added and the tube contents mixed well. The tubes remained in the ice bath for a further 5 min. After this time, the outer tube surfaces were dried using tissue and tubes were placed in a forced air oven at 105 ºC for a period of 30 min. On removal, the tubes were instantly cooled in tap water and a volume of 1.25 ml tert-butyl alcohol pipetted into the tubes, vortexed thoroughly to achieve a single-phase solution. The tubes were then set into a water bath at 37 ºC for a period of 3 min, to stabilise the colour achieved in the reaction before cooling to room temperature. The sample tubes were centrifuged for 5 min at 1500 x g to remove solid particles from suspension. The supernatant from each tube was pipetted into plastic 1 ml macro-cuvettes and absorbances in the sample cuvettes were read at 630 nm in a spectrophotometer. A standard curve prepared from the standard tubes (Figure 3.9) was used to calculate the unknown sample concentrations.

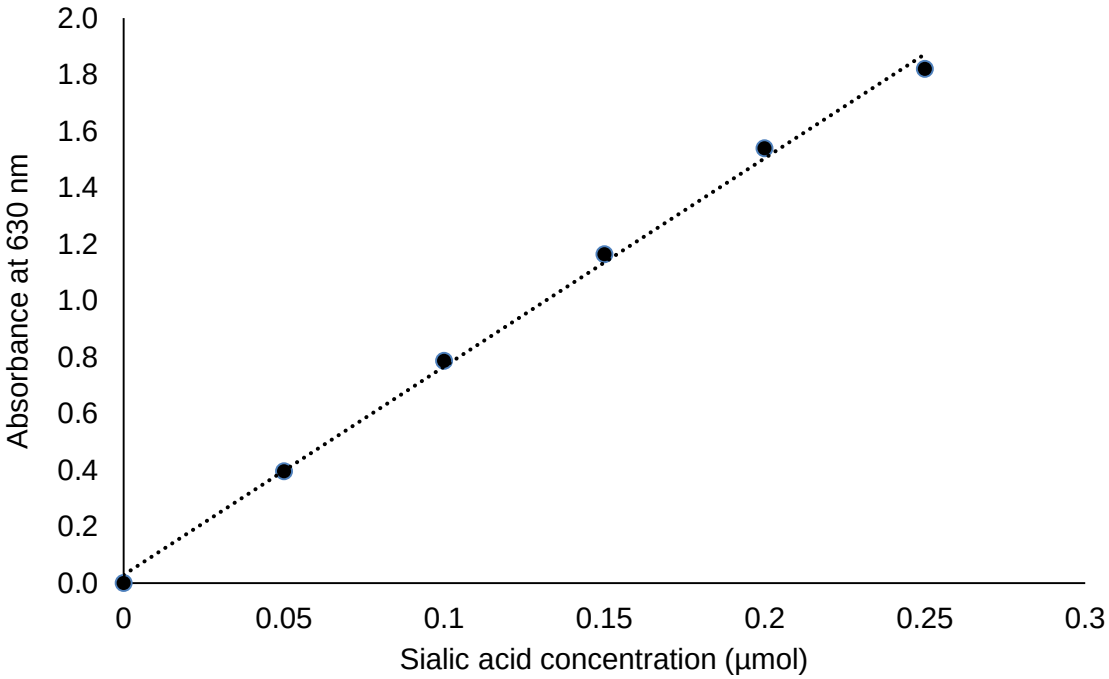

Figure 3.9: Standard curve for the determination of sialic acid concentration in samples of biological origin (y = 7.3749x + 0.0291), R^2=0.997).

CHAPTER FOUR

Phosphorus requirements of the weaner pig

4.0. Introduction

Phosphorus (P) requirements of pigs are revised regularly on the basis of improved scientific data (ARC, 1981; NRC, 1998; Whittemore *et al*., 2003; NRC, 2012). This has led to a gradual change in the way requirements are expressed, in addition to the levels recommended. Available P is typically measured as changes in bone ash P content in relation to that fed, representing the amount of P digested and metabolised (*i.e.* available for the animal to use). Refinements to this procedure may involve radioisotopic determination of ^{32}P in the femur to exclude P from endogenous sources (Whittemore and Thompson, 1969). In the UK however, digestible phosphorus (dgP) is the expression unit of choice, reflecting the extent to which the total P contained within plant, animal and mineral dietary constituents are apparently digested and absorbed across the GI tract. This can be expressed in two ways.

Firstly, a sample of digesta can be taken directly from the ileum, thus excluding any P modifications associated with the hindgut, resulting in a measure of apparent ileal digestibility (AID). This is hard to achieve in practice, as it either requires the animals to be sacrificed or the ileum to be canulated. Furthermore, there may be high amounts of P from endogenous sources present in the ileum compared to those in the faeces (Fan *et al*., 2001), possibly due to additional P absorption in the cecum (Liu *et al*., 2000). Alternatively, P digestion can be expressed as apparent total tract digestibility (ATTD), calculated as the difference between total P entering the digestive tract verses that contained in the faeces. However, no account of endogenous losses are considered using the ATTD method. Several methodologies have been developed to correct for this and estimate either true P digestibility or apply a standard correction factor. These are based on either linear regression (Fan *et al*., 2001) or P free diets (Petersen and Stein, 2006) respectively.

Extensive statistical modelling suggests that the form dietary P is fed in, is an important factor governing the pig's ability to digest and retain the element (Létourneau-Montminy *et al*., 2012). In the UK, wheat and soybeans form the basis of most commercial pig feeds, each containing approximately 60 % of dietary P in the form of phytate (Ravindran *et al*., 1994). Hydrolysis of P from phytate is strongly influenced by stomach pH (Létourneau-Montminy *et al*., 2011), where under very acidic conditions (pH 2.0), phytate disassociates from cleated minerals and proteins (Yu *et al*., 2012). Feed tends to increase stomach pH due to a buffering effect, reducing stomach acidity (pH 2.5 – 3.5), whereby binary phytate-protein aggregates can form (Yu *et al*., 2012). This reduces the ability of phytases to release

orthophosphate in the stomach. Once in the duodenum, the addition of bile salts increase the pH to near neutral conditions (pH 6.5), allowing phytate to precipitate out of solution and form ternary complexes through cationic bridges with minerals (Selle *et al*., 2012). This makes P from plant phytates largely unavailable to pigs.

In the absence of exogenous phytases, inorganic P has been used to provide all the dietary P required by the animal. The optimal amount of digestible P needed is strongly negatively correlated to the bodyweight of the animal (NRC, 2012) and is regulated through several mechanisms. Around 60 – 80 % of P is directly stored in skeletal tissue, bound with Ca, 2.2:1 (Ca:P) as hydroxyapatite ($Ca_5[PO_4]_3$) (Crenshaw, 2001). Given that 99 % of Ca in the body is found locked up with P within the bone matrix, regulation of P metabolism is heavily influenced by Ca intakes. Increasing dietary Ca negatively affects daily live weight gain (Létourneau-Montminy *et al*., 2012); the extent of which is more severe as Ca:P widens in marginal dietary P compared with P adequate diets (Reinhart and Mahan, 1986). This results from the reduction in P absorption across the small intestine, as excess Ca forms insoluble complexes in the chyme (Heaney and Nordin, 2002). Several studies have confirmed that intakes of Ca and P should be supplied in the diet at a ratio of around 1.0 to 1.2 for maximal intestinal P absorption (Liu *et al*., 2000) and growth performance (Qian *et al*., 1996).

Requirements for dietary digestible Ca are hard to predict since the digestibility is difficult to model, however, digestible P models are more accurate (R^2 = 0.78 for Ca verses 0.90 for P) (Létourneau-Montminy *et al*., 2011). Appropriate levels of dgP for newly weaned pigs are estimated to be 3.8 g/kg of diet (DM basis) (Whittemore *et al*., 2003), though this is formulated taking into account the risk of excess P output on the environment. Providing an appropriate Ca to P ratio is maintained, increasing dietary dgP above this level should provide additional weight gain up to the genetic potential of the pig. Conformation of where the limit of growth response to increasing dietary dgP is, in fast growing modern genotypes, would allow characterisation of non-P related effects (described by Selle and Ravindran, 2008) in a subsequent phytase super dosing experiment.

4.1. Objective

This chapter aims to evaluate the growth performance response to increasing dietary digestible P above the recommended level of 3.8 g/kg in newly weaned pigs. This data will act as a platform for investigating ‘extra phosphoric’ effects of phytase. In addition, baseline data on mineral digestibility will characterise the typical response of modern UK genotypes. The first null hypothesis is that increasing dietary dgP above 3.8 g/kg will have no effect on pig growth performance or mineral ATTD. Secondly, widening Ca:P from 1.12 to 1.60 will have no effect on pig growth performance or mineral ATTD.

4.2. Materials and methods

General materials and methods applicable to this section are described in chapter three. A dose response experimental design (graded levels of dgP) was set up to measure the performance and nutrient digestibility of pigs from weaning to three weeks post (designated trial SM1). 360 commercial type pigs (JSR Geneconverter 700 x JSR Genepacker 90 and PIC Camborough x PIC 337 genotypes) from three farrowing batches were sourced from the Harper Adams University high health status herd at weaning (~26 days of age) and balanced across treatments for genotype (50:50), sex (50:50) and selection weight. Animals were housed in pens of five pigs under commercial indoor conditions and fed one of eight treatment diets (Table 4.3) for 23 days. All diets were isoenergetic and isonitrogenous.

4.2.1. Treatments

Experimental diets (Table 4.3) were formulated to include seven levels of dgP, keeping a Ca:tP ratio of 1.12 (Primary Diets Ltd, UK). The lowest level of dgP was set at 2.8 g/kg (as fed), which is below the recommended level of 3.8 g/kg (air dried) suggested by Whittemore *et al.* (2003). Previous experimental results (not published) suggest that increasing levels to 5.8 g/kg dgP gives continuous improvement in pig performance. The diets were designed to increase incrementally by 1 g/kg up to a maximum of 8.8 g/kg. The final level was set to reflect the assumption that mega doses of phytase may release 2.83 g/kg additional inorganic P from diets containing 1 % PA, on top of the 5.8 g/kg dgP in the basal diet. It was important to establish that dgP at the max 8.8 g/kg does not provide any better performance due to the extra phosphorus than a lower 5.8 g/kg diet if extra phosphoric effects are to be detected.

The relationship between Ca and P is such that P absorption is dependent upon Ca:P in P adequate diets, thus it would not be possible to keep Ca at a constant level and change only the dgP. It was therefore decided that a constant Ca:P should be maintained at 1.12, which would then allow the effects of the dgP level to be assessed. One problem with this treatment structure is that both Ca and P increase in step, making it impossible to tell if treatment differences are attributable to the rising level of Ca or P. An additional treatment (B) was proposed to help solve this paradox (Table 4.1), which had the same level of P (dgP) as treatment C (but a higher level of Ca) and the same level of Ca as treatment E (but a different level of P). This would allow a direct comparison of treatments where Ca remained static (but different levels of dgP) and where P remained static (but different levels of Ca).

Table 4.1: Summary of treatments B, C and E (as fed basis) fed to weaned pigs in experiment SM1

Diet	B	C	E
Level of dgP (g/kg)	3.8	3.8	5.8
Level of P (g/kg)	6.3	6.3	8.9
Level of Ca (g/kg)	10.0	7.1	10.0
Ca:P	1.60	1.12	1.12
Number of pens	12	12	8

Treatment B serves a further purpose in that it can be used to indicate how widening the ratio from 1.12 to 1.60 affects pig performance and nutrient digestibility, when compared to diets C and E.

A description of the treatments for the main experiment can be found in Table 4.2. Two batches of pigs (four replicate pens in each per treatment) were used to assess levels of dgP ranging from just below the recommended level (treatment A, 2.8 g/kg) up to the maximum level expected to be released through super and mega doses of phytase (treatment J, 8.8 g/kg). Increments of 1 g/kg were used for all intermediate treatments to give a total of seven levels of dgP with eight replicates per treatment (12 in the case of diet C), all with a Ca:P of 1.12.

Table 4.2: Summary of dietary dgP treatment levels at Ca:P 1.12 (as fed basis) fed to weaned pigs in experiment SM1

Diet	A	C	D	E	G	H	J
Level of dgP (g/kg)	2.8	3.8	4.8	5.8	6.8	7.8	8.8
Level of P (g/kg)	5.0	6.3	7.6	8.9	10.2	11.5	12.8
Level of Ca (g/kg)	5.6	7.1	8.5	10.0	11.4	12.8	14.3
Ca:P	1.12	1.12	1.12	1.12	1.12	1.12	1.12
Number of pens	8	12	8	8	8	8	8

Pig performance was assessed by recording individual liveweight (LW) at day 0 (weaning), day 7, day 14 and day 23. These periods were designated as P1, P2 and P3 respectively. Feed disappearance was also recorded at these time points in order to estimate mean daily feed disappearance (DF), daily liveweight gain (DLWG) and the food conversion ratio (FCR) on a pen basis.

Table 4.3: Composition of raw ingredients (g/kg) for experimental diets fed to weaned pigs in trial SM1

Raw ingredients	A	B	C	D	E	G	H	J
Micronized barley	76	76	76	76	76	76	76	76
Raw wheat (whole meal)	173	163	169	164	159	155	150	145
Micronized wheat meal	174	163	169	164	159	155	150	145
Micronized maize	25	25	25	25	25	25	25	25
Supertherm oats	50	50	50	50	50	50	50	50
Herring meal	31	31	31	31	31	31	31	31
Soya (hypro)	100	100	100	100	100	100	100	100
Full fat soyabean meal	122	122	122	122	122	122	122	122
Premix[1]	5	5	5	5	5	5	5	5
Skimmed milk powder	25	25	25	25	25	25	25	25
De-lactosed whey	25	25	25	25	25	25	25	25
Whey powder	123	123	123	123	123	123	123	123
Sugar/sucrose	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
L-lysine HCl	5.1	5.2	5.2	5.2	5.2	5.2	5.3	5.3
Dl-methionine	2.6	2.6	2.6	2.6	2.7	2.7	2.7	2.7
L-threonine	2.7	2.7	2.7	2.7	2.7	2.8	2.8	2.8
L-tryptophan	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
L-valine	1.2	1.2	1.2	1.3	1.3	1.3	1.3	1.4
Crina 693[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin E	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Sucram®[3]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Benzoic acid (VevoVitall®)	5	5	5	5	5	5	5	5
Limestone flour	5.15	12.1	4.29	3.43	2.58	1.72	0.86	0
Dicalcium Phosphate	0.0	7.3	7.3	14.5	21.8	29.0	36.2	43.5
Soya oil	32.8	39.1	35.4	38.5	41.5	44.3	47.2	50.0

[1]Premix detailed in chapter three; [2]Flavouring made from essential oils manufactured by DSM Nutritional Products (UK) Ltd; [3]Sweetener manufactured by Pancosma SA

4.2.5. Chemical analysis

Samples of the experimental diets were taken over several periods during the nine weeks of the trial period and analysed as detailed in chapter three. Faeces were collected fresh from observed animals (minimum of three animals per pen) on days 22 and 23 of the trial and stored at -20 °C until analysis, described in chapter three.

4.2.6. Data analysis

All data was collated and calculations performed using Excel 2010/2013 (Microsoft Corporation, 2010). Data points ± 3 SD were considered outliers and removed from the analysis. Data was subject to orthogonal polynomial contrasts based on the formulated level of dgP, using the general ANOVA procedure of GenStat (16th edition, VSN International Ltd), with genetic line as a blocking factor. Treatments B & C, B & E and C & E were then compared using contrast comparisons, blocked by genetic line. Mineral digestibility data was analysed without blocking, due to the unbalanced treatment structure.

4.3. Results

During the trial, one pig in batch one on treatment H (7.8 g/kg feed dgP) died of an unknown cause at day 22 of the trial. One pig in batch two on treatment D (4.8 g/kg feed dgP) was removed from trial due to ill health (yellow scours).

4.3.1. Diet analysis

Following ICP-MS analysis, results for the certified reference material (Dairy feed, BER-708) were shown to be within range, including for P and Ca. The P and Ca composition of the diets are detailed in Table 4.4.

Table 4.4: Analysis of phytic acid, phytate-P, P and Ca (g/kg as fed basis) of experimental diets fed to pigs in trial SM1

Diet	A	B	C	D	E	G	H	J
Measured phytic acid content	5.7	5.9	5.4	4.8	5.2	4.4	4.7	4.1
Measured phytate P	1.6	1.7	1.5	1.4	1.5	1.2	1.3	1.2
Formulated total P	5.0	6.3	6.3	7.6	8.9	10.2	11.5	12.8
Measured total P	5.4	6.9	6.8	8.1	9.1	10.9	12.6	12.9
Formulated total Ca	5.6	10.0	7.1	8.5	10.0	11.4	12.8	14.3
Measured total Ca	6.7	12.4	9.0	10.7	11.3	13.8	15.4	16.1
Formulated Ca:tP	1.12	1.60	1.12	1.12	1.12	1.12	1.12	1.12
Calculated Ca:tP	1.24	1.81	1.33	1.32	1.24	1.26	1.22	1.25

Phytic acid content of the diets were typical and broadly similar at between 0.41 to 0.59%. Analysed tP was in general close to the formulated tP, though there was a 1.1 g/kg discrepancy for diet H (Table 4.4). Ca was consistently higher than that formulated by between 1.1 and 2.6 g/kg. Ca to tP ratios were similar to expected, with the desired wide ratio in treatment B compared to all other diets.

4.3.2. Mineral digestibility

There were four missing faecal samples out of 72 (unattainable collections). A further two samples (one in D and one in J) exceeded 3 SD and were excluded. Calculated digestible P and Ca based on the concentration of Ti added to the feed is shown in Table 4.5.

Table 4.5: Formulated and analysed digestible P and Ca content (g/kg diet as fed) of diets fed to weaned pigs in experiment SM1

Diet	A	B	C	D	E	G	H	J
Formulated digestible P	2.8	3.8	3.8	4.8	5.8	6.8	7.8	8.8
Calculated digestible P	3.2	4.0	4.2	4.8	5.4	6.8	7.7	6.9
Calculated digestible Ca	4.4	7.7	6.3	6.5	6.6	8.4	8.8	8.2

Calculated dgP was very similar to formulated levels, except for diet J which was 1.9 g/kg lower than predicted (Table 4.5). When P output was expressed as g/kg DM of diet intake, there was a linear relationship with dietary P input ($p<0.01$) (Figure 4.1).

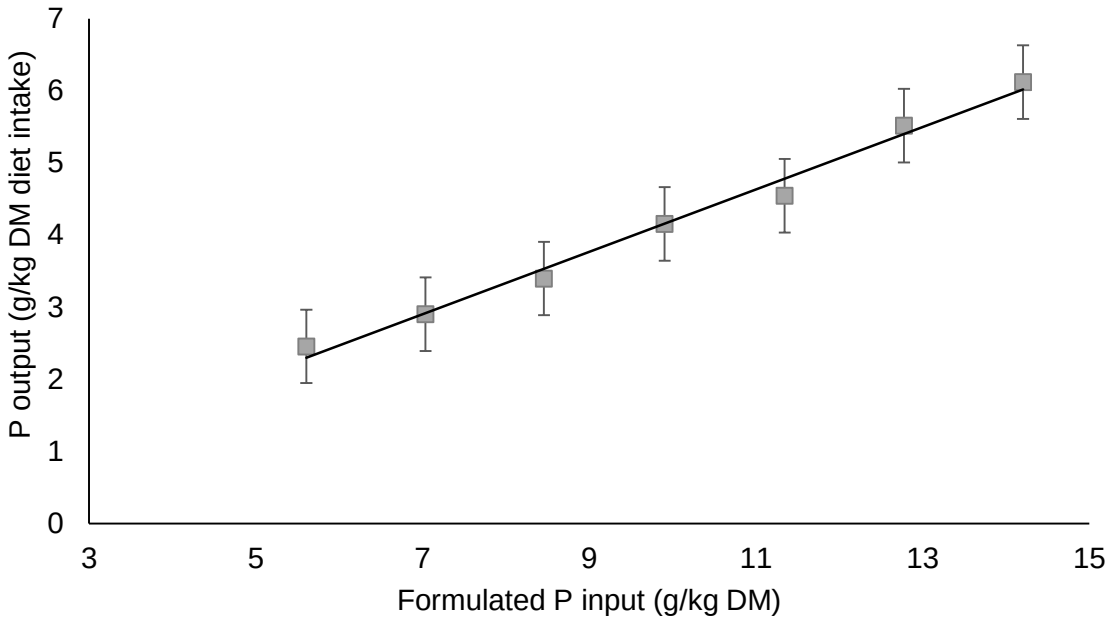

Figure 4.1: Linear relationship between total faecal P output (g/kg DM diet intake) and dietary P input (g/kg DM) in pigs three weeks post weaning. Y=0.41*x*-0.18 (r^2=0.89). Error bars indicate SEM.

The regression analysis (Figure 4.1) had good explanatory power, accounting for 89% of the variation of the raw dataset. The Y intercept did not differ from zero ($p=0.40$) at -0.18. Apparent total tract digestibility (ATTD) of measured minerals are detailed in Table 4.6.

Table 4.6: The effect of increasing digestible phosphorus on mineral apparent total tract digestibility in weaned pigs

Formulated level of dgP (g/kg diet)	2.8	3.8	4.8	5.8	6.8	7.8	8.8	S.E.D.	dgP	L	Q	D
Replication/treatment	8	11	8	7	7	8	7					
P	0.59	0.62	0.62	0.59	0.63	0.61	0.57	0.021	0.12	0.31	0.05	0.25
Ca	0.66	0.70	0.63	0.59	0.61	0.57	0.54	0.022	0.00	0.00	0.48	0.01
K	0.82	0.83	0.84	0.81	0.86	0.85	0.85	0.019	0.15	0.03	0.96	0.33
Mg	0.37	0.37	0.39	0.35	0.43	0.36	0.36	0.028	0.07	0.92	0.25	0.03
Fe	0.35	0.26	0.11	0.10	0.27	0.17	0.32	0.040	0.00	0.21	0.00	0.00
Cu	0.14	0.04	0.13	-0.07	0.19	0.11	0.06	0.033	0.00	0.90	0.09	0.00
Zn	0.06	0.19	0.19	0.12	0.30	0.24	0.18	0.036	0.00	0.00	0.00	0.00
Co	0.27	0.30	0.22	0.22	0.34	0.35	0.46	0.037	0.00	0.00	0.00	0.05
Mn	0.13	0.15	0.29	0.25	0.32	0.34	0.24	0.037	0.00	0.00	0.00	0.02
Se	0.54	0.49	0.68	0.43	0.51	0.49	0.54	0.031	0.00	0.27	0.93	0.00
Mo	0.72	0.68	0.77	0.78	0.76	0.76	0.77	0.052	0.29	0.08	0.51	0.43

S.E.D. represents that between minimum replication treatments; dgP = p-value for effect of dgP; L= linear contrast; Q = quadratic contrast; D = deviations contrast

The level of dietary dgP significantly affected the ATTD of all minerals measured, except P, K and Mo (Table 4.6). These were complex relationships (deviations P≤0.05) in all cases. For Ca, there was an initial increase in digestibility, followed by a steady linear decrease, as dietary dgP increased. Mg digestibility also tended (p=0.07) to be affected by increasing dgP, peaking at 43 % in diets containing 6.8 g/kg dgP. The digestibility of Fe was the highest in the 2.8 g/kg dgP diet (35 %), steadily reducing to 10 % digestibility by 5.8 g/kg dgP, before increasing to 32 % in diets containing 8.8 g/kg dgP. ATTD of Co tended to be complex (p=0.05) but overall, increased in line with dietary dgP level from 27 % to 46 % in a linear/quadratic fashion. Mn digestibility was lowest in the 2.8 and 3.8 g/kg dgP diets (13 – 15 %), increasing to a peak of 34 % in the 7.8 g/kg dgP diet, before reducing to 24 % at 8.8 g/kg dgP. The ATTD of Cu remained low at <20 % and went negative in the 5.8 g/kg dgP diet, suggesting the animal was losing more Cu than the diet provided. In the lowest dgP diet, Zn ATTD was only 6 %, but increased up to 30 % in the 6.8 g/kg dgP diet, before reducing below 20 % as dgP increased further. Se ATTD fluctuated between 43 – 54 % in all treatments except 4.8 g/kg dgP, where it increased to 68 %.

4.3.1. Growth performance

In general, growth performance was as expected, with no evidence of a treatment effect on weight gain over the three week period (Table 4.7). Two pens in the first week of the trial and one pen in the third week had a higher than normal FCR (>3 SD), and were removed from the statistical analysis. Table 4.7 shows a dose response of dietary dgP on feed disappearance in the third week post-weaning (p=0.05), increasing from 633 g/day at 2.8 g/kg dgP, up to 688 g/day at 5.8 g/kg dgP, before declining to 570 g/kg at 8.8 g/kg dgP (p=0.03). There also tended to be a treatment effect on feed disappearance over the whole trial period (p=0.05), with feed disappearance highest at the 5.8 dgP/kg feed and lowest at 8.8 g/kg dgP feed.

Table 4.7: The effect of increasing digestible phosphors on the growth performance of weaned pigs

Formulated level of dgP (g/kg diet)	2.8	3.8	4.8	5.8	6.8	7.8	8.8	S.E.D.	dgP	L	Q	D
Number of replicates	8	12	8	8	8	8	8					
Mean weaning weight (kg)	8.0	8.2	8.0	8.1	8.1	8.1	8.0	0.120	0.85	0.44	0.76	0.75
Mean weight at day 7 (kg)	9.2	9.2	9.0	9.2	9.0	9.0	9.0	0.209	0.81	0.21	0.81	0.86
Mean weight at day 14 (kg)	11.5	11.5	11.3	11.7	11.1	11.3	10.9	0.290	0.12	0.04	0.31	0.29
Mean weight at day 23 (kg)	15.5	15.6	15.5	16.3	15.0	16.0	14.7	0.560	0.11	0.32	0.12	0.13
Average DF (g)												
Day 0 – 7	195	187	189	206	184	197	182	16.10	0.78	0.77	0.59	0.59
Day 7 – 14	417	375	371	422	345	386	342	34.30	0.14	0.10	0.84	0.14
Day 14 – 23	633	631	646	688	618	674	570	35.90	0.05	0.37	0.03	0.11
Overall	434	418	421	460	403	441	382	24.10	0.05	0.20	0.14	0.06
Average DLWG (g)												
Day 0 – 7	162	151	146	167	141	138	141	21.80	0.79	0.26	0.93	0.77
Day 7 – 14	333	322	316	357	289	329	273	32.20	0.19	0.12	0.32	0.25
Day 14 – 23	440	462	468	504	439	468	422	41.10	0.53	0.63	0.11	0.68
Overall	323	325	313	357	303	325	291	24.30	0.21	0.24	0.20	0.24
FCR												
Day 0 – 7	1.25	1.38	1.26	1.30	1.39	1.65	1.35	0.206	0.50	0.24	0.92	0.42
Day 7 – 14	1.26	1.19	1.17	1.19	1.21	1.20	1.26	0.078	0.83	0.82	0.15	0.97
Day 14 – 23	1.45	1.40	1.40	1.32	1.43	1.46	1.38	0.081	0.65	0.87	0.48	0.46
Overall	1.35	1.30	1.37	1.30	1.34	1.36	1.33	0.068	0.90	0.89	0.86	0.72

S.E.D. represents that between minimum replication treatments; dgP = p-value for effect of dgP; L= linear contrast; Q = quadratic contrast; D = deviations contrast

4.3.2. Effect of dgP and Ca on growth

Comparisons of growth performance between diets B, C and E were used to investigate whether increasing Ca influenced growth performance or if differences are likely due to increasing dgP (Table 4.8).

Table 4.8: The effect of increasing dgP or Ca on the performance of weaned pigs

Diet	B	C	E	p-value	C1	C2	C3	SED
Formulated level of dgP (g/kg)	3.8	3.8	5.8					
Formulated level of Ca (g/kg)	10.0	7.1	10.0					
Mean weaning weight (kg)	8.1	8.2	8.1	0.83	0.81	0.52	0.39	0.12
Mean weight at day 7 (kg)	9.2	9.2	9.2	0.84	0.91	0.86	0.94	0.21
Mean weight at day 14 (kg)	11.2	11.5	11.7	0.16	0.28	0.06	0.35	0.30
Mean weight at day 23 (kg)	14.8	15.6	16.3	0.07	0.10	0.01	0.23	0.57
Average DF (g)								
Day 0 – 7	198	187	206	0.83	0.41	0.61	0.21	16.5
Day 7 – 14	369	375	422	0.17	0.83	0.09	0.13	33.5
Day 14 – 23	608	631	688	0.05	0.44	0.02	0.09	36.2
Overall	411	418	460	0.08	0.71	0.03	0.06	24.6
Average DLWG (g)								
Day 0 – 7	152	151	167	0.86	0.97	0.45	0.44	21.5
Day 7 – 14	287	322	357	0.14	0.19	0.02	0.24	32.0
Day 14 – 23	403	462	504	0.23	0.08	0.01	0.26	40.8
Overall	291	325	357	0.12	0.10	0.01	0.16	24.6
FCR								
Day 0 – 7	1.35	1.38	1.30	0.53	0.82	0.78	0.63	0.194
Day 7 – 14	1.31	1.19	1.19	0.44	0.06	0.09	0.99	0.075
Day 14 – 23	1.48	1.40	1.32	0.43	0.19	0.03	0.27	0.077
Overall	1.42	1.30	1.30	0.49	0.03	0.05	0.97	0.068

C1 = Diet B verses C; C2 = Diet B verses E; C3 = Diet C verses E; S.E.D. represents that between minimum replication treatments.

There was a tendency (p=0.07) for pigs on the diets containing the same quantity of Ca to weigh an average of 1.5 kg heavier by day 23 post-weaning (p=0.01), when dgP was increased from 3.8 to 5.8 g/kg (Table 4.8). The same effect was not observed when Ca:tP was equalised and dgP increased from 3.8 to 5.8 g/kg (p=0.23). Feed disappearance was higher in the third week post-weaning when dgP was increased to 5.8 g/kg and Ca:tP was reduced from 1.60 to 1.12 (p=0.02). There was also a tendency (p=0.09) for a dgP effect, since when the ratio remained constant at 1.12 Ca:tP, daily feed disappearance increased 57 g/d when dgP increased from 3.8 to 5.8 g/kg (Table 4.8). This pattern was similar, though slightly weaker, when the data was analysed over the three week trial.

4.4. Discussion

The levels of dgP fed in this experiment were considerably higher than typical commercial recommendations (Ekpe *et al*., 2002; Whittemore *et al*., 2003). This was to allow for the detection of any dgP associated growth performance that may explain the improved growth sometimes seen by super dosing phytase enzymes (Selle and Ravindran, 2008) in a subsequent trial. Formulating to excessive P levels has a major problem in that high levels of P are excreted into the environment from faeces and urine. In low P diets, P transport by Na-P co-transporter II is increased across the intestines, independent of Ca, into the blood (Crenshaw, 2001; Saddoris *et al*., 2010). Simultaneously, P excretion through the kidneys, also via a Na-P co-transport system (Werner *et al*., 1998), is reduced (Crenshaw, 2001). As dietary P becomes excessive, however, absorption across the intestine shifts mainly to paracellular transport via the tight junctions (Breves *et al*., 2007).

Disposal of absorbed excess P is through the kidneys (urine), in addition to the faecal route for unabsorbed P (Fernández, 1995). While moderately restricted P diets stimulate active transport (Saddoris *et al*., 2010), apical P_i uptake into the enterocytes would appear to be the most limiting factor for P absorption, where P concentrations in the gut lumen are high (Breves *et al*., 2007). Getting P levels correct is thus a fine balancing act, since for rapidly growing pigs, a growth ceiling can be reached where P is deficient, but is easily overcome when P becomes adequate (Sands *et al*., 2001). Absorbed P beyond that needed for maintenance and growth can then either be excreted via urine or can be channelled into bone. This luxury bone store of P allows the pig to develop a robust skeletal frame, which is useful for breeding animals. Assessments of the effects of minerals on pig physiology can be conducted in a number of ways (Ammerman, 1995). For P and Ca, mineralisation of bone is widely used as a response criterial (Soares, 1995), whereas for Cu and Fe, liver concentration and biomarkers (e.g. superoxide dismutase) tend to be favoured (Hill and Link, 2009). Measurements of bone mineralisation as a response criteria would have been a useful addition to this experiment, but would have involved sacrificing pigs and would not have met the primary objective of this trial.

4.4.1 Effects of Ca:tP ratio on pig growth

Digestibility of P depends somewhat on the concentration of Ca in the lumen. As the ratio of Ca to P widens, so the digestibility of P significantly decreases, reducing growth performance (Reinhart and Mahan, 1986; Qian *et al*., 1996). This is largely because P absorption (but not Ca) in the small intestines is reduced (Liu *et al*., 2000), probably due to insoluble Ca-P complexes (Heaney and Nordin, 2002). These effects are more severe in low P diets, where ratios of 1.0 to 1.3 Ca:tP are recommended (Reinhart and Mahan, 1986; Qian *et al*., 1996). In high P diets, this ratio has been increased up to 2.0 before growth was

reduced (Reinhart and Mahan, 1986), though modern genotypes may not respond the same.

Due to the design of this experiment, Ca levels increased in parallel to tP, to maintain a constant low ratio, thus facilitating absorption and preventing any adverse effects of high Ca doses at the top end of the range. The results showed there were no significant differences in DF or DLWG when calcium was varied but P was fed at the same level (Table 4.8). This is a good indication that increasing dietary calcium (at least from 7.1 to 10.0 g/kg) does not seem to affect growth when P is fed at a reasonable rate (3.8 g/kg). Numerically the mean FCR (calculated over the whole experiment) was higher (1.42) in the wide Ca:tP diet compared to the narrow (1.12 Ca:tP) diet (1.3). While the contrast for this was significant ($p=0.03$), the overall F ratio was not high enough ($p=49$) to indicate a treatment effect in the dataset. An increase in FCR was expected based on the evidence of Qian *et al.* (1996), though given that dgP was adequate (3.8 g/kg), the magnitude of response may have been weak enough to not be detected statistically in the data (Reinhart and Mahan, 1986).

Reducing the ratio from 1.6 to 1.12 Ca:tP by keeping Ca the same (10.0 g/kg) but increasing phosphorus, resulted in a number of significant differences in growth performance. The most striking was an increase of 1.5 kg in pig body weight at the end of the 23 day trial. This change in body weight was statistically supported as the ratio reduced ($p=0.01$) but not when the ratio was kept at 1.12 and dgP was increased by 2 g/kg (Table 4.8). This suggests there was no effect on bodyweight due to increasing dgP, but there was a ratio effect with pigs fed a diet with the narrower Ca:tP growing significantly better. Differences in feed disappearance became apparent during the second week of the trial ($p=0.05$) and tended to persist in the data when calculated over the whole three week period ($p=0.08$). These were noted to be a significant increase due to the reduction in ratio associated with increasing P (but not when P was 3.8 g/kg and Ca reduced to widen the ratio) and a tendency to increase due to higher dgP (Table 4.8). Although the DLWG contrast between the wide and narrow ratio diets (through increasing P) reflected that of DF, the overall statistical strength of the F test was not enough to support a treatment difference. Taken together, keeping the ratio of Ca to tP the same, should have allowed for maximal P absorption, giving this experiment the best possible chance of detecting growth responses to increasing dietary dgP levels.

4.2.2. Digestibility of phosphorus

There was a linear relationship between dietary P input and P output expressed as g/DM diet intake. This is in good agreement with Fan *et al*. (2001), however, true P digestibility was unable to be estimated, since endogenous losses could not be accurately predicted, as the intercept did not differ from zero (Figure 4.1). This may be a feature of the linear regression model, since other modelling techniques give differing estimates of endogenous losses, depending on how liner the line is (Schulin-Zeuthen *et al*., 2007). Extrapolating back to zero using a straight line model for high P diets (above 4.3 g/kg total P), may thus be inappropriate, as negative values would not be possible and are likely due to measurement and calculation associated error. Instead it would be expected that the intercept would be significantly greater than zero, representing endogenous losses, so that a diet containing zero P input would still contain traces of P from cellular and secretory sources (Fan *et al*., 2001).

The analysis of ATTD of P was as expected, showing no differences (Table 4.6), reflecting the constant bioavailability of dicalcium phosphate (DCP). Dietary total P concentration increased as formulated (Table 4.4) but no assessment could be made of its fate. It is likely that as more P was absorbed with increasing dietary concentration, some was incorporated into bone (as previously described), with the rest excreted via the kidneys. Providing all other nutrients were adequate and the pig was not exposed to stressors such as extremes of temperature (Weller *et al*., 2013) and pathogenic challenge (Pastorelli *et al*., 2012), additional P retention from higher dgP diets would have allowed the pig to meet its genetic potential for growth and bone development. The digestibility of Ca, while initially increasing from 66 to 70 %, showed a subsequent linear decline as dgP increased. Increasing Ca concentration in itself should not have reduced Ca ATTD (Poulsen *et al*., 2010) but could have reduced P ATTD (Stein *et al*., 2011). This is dependent on the form of P fed, as Poulson *et al*. (2010) found no effect of increasing dietary Ca on P ATTD when P was formulated from plant phytate, whereas Stein *et al*. (2011) found a linear decrease in P digestibility when formulated with monosodium phosphate. In this study, Ca and P levels were adjusted with a mix of DCP and limestone, which may have influenced Ca digestibility in the presence of increased P.

4.2.3. Effect of increasing dgP on growth performance

Increasing the content of dgP tended ($p=0.05$) to increase feed disappearance quadratically ($p=0.03$) in the final growth period (Table 4.7). Though the pigs in this study were only ~15 kg at the end of this period, Arouca *et al*. (2012) also report this trend for pigs of between 15 – 30 kg. Despite the effect on feed disappearance, there were no differences found in DLWG or body weight. This differs from Arouca *et al*. (2012) who report a quadratic effect

in line with their growth results. There was however, a strong correlation in our results between overall DF and DLWG ($r=0.84$, $p<0.001$), also reflected in the statistically non-significant FCR values, so that numerically, the mid-range dose of 5.8 g/kg dgP gave best performance. At the top end of the scale, 8.8 g/kg dgP numerically gave the worst performance, with FD being low, resulting in poor DLWG. This would indicate that dietary dgP was so excessive at this level, it was depressing appetite.

Since there was no statistical effect of increasing dgP on growth performance, it might be assumed that the animals' genetic potential had been met at 2.8 g/kg dgP, which does not reflect the data of Arouca *et al*. (2012). Their pigs had, however, been selected to have genetically high meat deposition characteristics and as such were commercial hybrids. Differences in genetic potential are well known to influence P requirements (CEFIC, not dated), making it all the more important that genotype is considered when setting P standards. In another study, the authors only began to notice an effect of increasing available P on DLWG from day 22 post-weaning (Kegley *et al*., 2001). As this study ended by day 23 post-weaning, it is possible to have missed a dgP effect that may have been detected if the study had been extended. This trial was designed to only focus on the three weeks post-weaning period, since subsequent phytase experiments will investigate alleviating the post-weaning growth check, based on this time period.

The form P is fed in is well known to influence pig growth rates. When Sands *et al*. (2001) fed diets containing 4.0 and 5.5 g/kg tP formulated with monosodium phosphate (MSP), they found an increase in plasma P concentration, as expected, but no effect on growth performance. In the same experiment, when the diets were formulated to the same level of P using high available P corn, both DLWG and daily feed intake were significantly improved. This was observed despite the higher bioavailability of MSP they reported. Arouca *et al*. (2012) found a quadratic effect on weight gain and feed intake of 15 – 30 kg pigs with diets formulated with DCP. Weight gain in their study increased from 526 g/d to 839 g/d in diets containing 1.03 g/kg to 4.24 g/kg available P with maximal growth response calculated to be at 5.34 g/kg. Thereafter growth plateaued up to their maximal inclusion level of 6.38 g/kg available P. A quadratic relationship was also reported by Wu *et al*. (2008) using DCP but they found peak DLWG occurred at only 3.1 g/kg available P, though there pigs were in measured over the body weight range of 21 – 40 kg.

Pharmaceutical levels (~2500 mg/kg) of zinc oxide (ZnO) fed post-weaning are beneficial in reducing the incidences of post weaning diarrhoea and improving DLWG (Poulsen and Carlson, 2008; Sales, 2013). At levels exceeding 1500 mg/kg, Zn can, however, inhibit phytase activity and reduce the available P levels in the diet through the reduction in phytate hydrolysis (Augspurger *et al*., 2004). There is also suggestion of Ca-Zn-P precipitate

forming in the small intestine at pharmaceutical ZnO levels, as seen indirectly by reduced serum P and Ca due to increasing ZnO dose (Walk *et al*., 2013). Together, this may mean more supplemental inorganic P is required to maintain growth – hence a growth response to increasing dgP would become apparent. In this study, ZnO was not included in the diet, which may have influenced our growth response to supplemental dgP. For example, the quadratic response in DLWG seen in the experiment of Arouca *et al*. (2012) was in the presence of 2790 mg/kg Zn. Although pharmaceutical levels of Zn were not present in the study of Wu *et al*. (2008), who also report a quadratic DLWG response, they only saw a response to below 2.3 g/kg available P before it plateaued. These diets in experiment SM1 began at 2.8 g/kg dgP and thus should have already plateaued at that point, if this data followed that pattern.

4.2.4. Digestibility of other minerals

The polynomial contrasts allowed for an assessment of the relationship complexity between increasing level of dgP and the digestibility of minerals under test. In this study, most minerals did not follow any recognisable pattern in relation to increasing dgP level (Table 4.6). In reality, there are a number of complex biological processes being undertaken in the gut of the pig during digestion and absorption. For example, phytic acid is well known to influence the ATTD of minerals (Woyengo *et al*., 2009) by binding them into insoluble phytate salts. The formation of insoluble phytate-mineral complexes are influenced by pH and by the concentration of other nutrients (the molar ratios of Zn and Cu to phytate in particular) in the chyme and digesta (Champagne, 1988). Additionally, mineral absorption is often dependent on active transporters (Hill and Link, 2009), some of which rely on co-transport mechanisms, thus requiring the availability of other minerals and nutrients (such as the Na-P co-transport system). Endogenous losses into the lumen is a particular problem that generally requires labelling minerals of feed origin with either a radio or stable isotope in order to distinguish dietary minerals from endogenous. The measure of ATTD is therefore largely nonsensical for trace minerals with high endogenous losses such as Zn and Cu and the complex digestibility patterns observed (Table 4.6) in relation to increasing dietary dgP are therefore not surprising. Importantly, the statistical evidence for P effects noted in this study suggest that the concentration of dgP is important for the digestion of trace minerals, but other factors are also involved and balance studies would be required to obtain meaningful absorption and retention values.

4.5. Conclusions

In conclusion, pig growth performance was not influenced by increasing dgP for the first three weeks post-weaning, when a narrow ratio of Ca to tP was maintained and all diets were adequate in P. The nature of mineral digestibility in pigs is complex and may depend on the matrix and availability of other minerals and nutrients available for co-transport, endogenous secretions and enzyme function. The mechanisms of improved growth performance associated with increasing dgP through phytase enzymes remain unclear and warrant further investigation.

CHAPTER FIVE

Effects of mega dosing dietary phytase on the growth performance, nutrient digestibility and physiology of weaner pigs

5.0. Introduction

Chapter four clearly showed there were no significant effects of increasing dietary digestible P on the growth performance of weaned pigs. In the last decade, studies into the use of modern phytases have begun to highlight other beneficial effects, including improved growth performance, when phytases are fed at super doses (Augspurger and Baker, 2004; Veum *et al.,* 2006; Pirgozliev and Bedford, 2013). These studies have mainly been conducted in P deficient diets and compared to a positive control, as the authors tended to investigate P bioavailability. Several studies, however, have shown significant improvements in growth performance in diets where P and Ca were adequate (Walk *et al.,* 2013; dos Santos *et al.,* 2013), suggesting that phytase has benefits in addition to its primary use of enhancing P digestibility. Currently, there is no data on the maximum dose of phytase that is effective at producing increased growth performance, since Veum *et al.* (2006) did not reach a growth plateau at 12500 FTU/kg.

Below the isoelectric point of cereal proteins, such as in the conditions found in the stomach of the pig (pH range 2.5 to 3.8), dietary protein aggregates can form binary complexes with phytic acid (Kies *et al*., 2006b). Given that Yu *et al.* (2012) clearly demonstrated an inverse relationship between phytic acid concentration and relative pepsin activity, it is likely that protein digestion could thus be impacted. A review by Selle *et al.* (2012) suggests that the anti-nutritional effects of phytic acid on protein digestion may not be limited to binary complexes, but may also extend to ternary complexes formed through cationic bridges above the protein isoelectric point. These would be difficult to breakdown in the small intestines leading to reduced protein digestibility. The formation of lower inositol products occurring in the lumen of the duodenum in high phytase diets may therefore ensure a sustained release of divalent cations and prevention of ternary phytate-mineral-protein complexes.

Fluctuations in the free nutrient contents of digesta within the lumen is associated with modulations in intestinal nutrient transporter expression. In particular, there are significant increases in ileal peptide transporter 1 and expression changes in several genes relating to Ca transport/binding (Vigors *et al*., 2014) and sodium/glucose transport (Woyengo *et al*., 2011). In both studies, responses were shown to differ depending on the site measured along the small intestines; a feature of the intestines which has now been mapped (van der Wielen *et al*., 2014). These differences may be, in part, an explanation for the complex ATTD of minerals other than P, Ca, K and Mo reported in chapter four, based on differing concentrations in the digesta. Furthermore, there is strong evidence that dietary phytic acid not only binds up dietary minerals but also increases endogenous mineral loss, through at least four different mechanisms (Woyengo and Nyachoti, 2013). These relate to increases in bile salts to neutralise stomach acid, the binding of enzyme co-factors, direct binding to endogenous minerals and binding of mineral co-transporters. When phytase is super dosed, the release of chelated minerals from phytate will thus modulate the digesta mineral composition, in addition to preventing endogenous mineral interactions, thereby changing the amount of mineral digestion and absorption.

It is possible that 'extra phosphoric' benefits may be related to the release of chelated minerals and nutrients (Kies *et al.,* 2006b; Woyengo and Nyachoti, 2013) or from physical changes in the gut, such as prevention of crypt shrinkage (Woyengo *et al.,* 2011). Cooper and Growing (1983) found endogenous phytase in the brush border membrane of rat intestines and hypothesised that because of its location, it may play a role in facilitating metal absorption from dietary phytate. Similar observations were made by Maenz and Classen (1998) in chickens, who found brush border phytase activity mostly occurs only in the pH range 5.5 to 6.5 – the normal range for digesta in the small intestines. In both studies, the authors reported the highest phytase activity taking place in the upper sections of the small intestines. The levels they observed were, however, very low given that extra phosphoric effects have mostly been associated with super dosing phytase (>1500 FTU/kg).

Alternatively, the far greater levels of phytase activity associated with dietary supplementation may offer another explanation for the extra phosphoric effects. Kemme *et al.* (2006) investigated phytate degradation in the upper gastrointestinal tract of the pig using up to 900 FTU/kg of a 3-phytase derived from *Aspergillus niger*. Their evidence, based on using cannulated weaned pigs fed a maize/soybean diet, indicated a difference in the quantitative pattern of lower inositol phosphate products. They noted high formation of IP_5 and IP_4 in the duodenum at low phytase levels (186 FTU/kg), which then shifted to the destruction of higher inositol phosphate products (IP_{6-4}) and consequently, high formation

of IP_2, at a phytase dose of 1000 FTU/kg. This leads to another hypothesis to explain 'extra phosphoric' effects associated with super dosing phytase, that lower inositol phosphates may influence cellular signalling pathways in mammalian cells (Miller *et al*., 2008; Watson *et al*., 2012; Porciello *et al*., 2016).

The gut is a major interface between antigens and the blood, thereby assuming a major role in immunity. As such, there are a number of features that provide this functionality (*e.g.* Peyer's patches full of immune cells, production of cytokines and a network of dendritic cells). Several studies have looked into the role of dietary phytase on immune function in chickens (Liu *et al*., 2008; Ghahri *et al*., 2012) and pigs (Almeida *et al*., 2007), with mixed conclusions. These authors only fed up to 1000 FTU/kg phytase, looking at various immune responses (*e.g.* antibody production, cell mediated response and ratios of immune cells) but not specifically in the gut. Following a recent review (Heyer *et al*., 2015), it seems that there are negative consequences of feeding low P diets to pigs but a very unclear picture as to the role of the lower inositol phosphates generated from phytase supplemented diets. For example, there is certainly evidence that IP_3 in particular is involved with T lymphocyte regulation and function (Porciello *et al*., 2016), though whether this can be modulated directly through dietary derived IP_x is unknown. This is therefore an area for further investigation.

5.1. Objective

The objective of this chapter is to investigate the 'extra phosphoric' effects on growth performance of newly weaned pigs associated with super dosing phytase, now that chapter four has ruled out increasing dgP as a cause. Specifically, mechanisms of intestinal nutrient transporter, gut histomorpholgy, enhanced immunity and phytate destruction will be considered. The general hypotheses to be tested are:

1. Increasing dietary phytase inclusion level will significantly improve pig growth performance in a dose response manner, above negative and positive controls.
2. Increasing dietary phytase inclusion level will significantly modify mineral digestibility and bone mineral content in a dose response manner, above a zero phytase control.
3. Increasing dietary phytase inclusion level will significantly modify mineral transporter gene expression and immune status in a dose response manner, above a zero phytase control.

5.2. Materials and methods

General materials and methods relating to housing and analytical techniques can be found in chapter three. A dose response design (graded levels of phytase) was set up to measure growth performance, nutrient digestibility and immune response of pigs from weaning to three weeks post (designated trial SM2). 240 commercial type pigs (JSR Geneconverter 400 x JSR Genepacker 90 and PIC Camborough x PIC 337 genotypes) from two farrowing batches were sourced from the Harper Adams University high health status herd at weaning (~26 days of age) and balanced across treatments for genotype (50:50), sex (50:50) and selection weight. Animals were housed in pens of five pigs (mixed sex) under commercial indoor conditions and fed one of eight treatment diets (Table 5.1) for 23 days. All diets were isoenergetic and isonitrogenous.

5.2.1. Treatments

Experimental diets (Table 5.2) were formulated to include seven levels of phytase, based on diet E of chapter four, as this diet had given the best numerical growth performance. This base diet was thus formulated to contained 5.8 g/kg dgP with a Ca:tP of 1.16, with no adjustment for P released by the exogenous phytase. An eighth diet (B) containing high dgP (7.8 g/kg) was included as a control to ensure effects were not related to increasing dietary P content.

Table 5.1: Overview of dietary treatments relating to a phytase dose response experiment, plus a high P control, designated trial SM2

Diet	A	B	C	D	E	G	H	J
Phytase (FTU/kg)	0	0	125	500	1000	2000	8000	32000
dgP (g/kg)	5.8	7.8	5.8	5.8	5.8	5.8	5.8	5.8
Ca:tP	1.16	1.16	1.16	1.16	1.16	1.16	1.16	1.16
Number of replicates	6	6	6	6	6	6	6	6

The treatment diets (Table 5.1) were formulated to contain: only intrinsic phytase activity (<50 FTU/kg), low levels (125 FTU/kg), commercial levels (500 and 1000 FTU/kg), super doses (2000 and 8000 FTU/kg) and a mega dose (32000 FTU/kg) of a third generation *Escherichia coli* derived 6-phytase enzyme (EC 3.1.3.26) expressed in *Trichoderma reesei* (Quantum Blue, AB Vista Feed Ingredients, Marlborough, UK). Permission to use Quantum Blue in this experiment was granted by the Food Standards Agency and all experimental procedures were approved by Harper Adams University Research Ethic Committee. Two batches of pigs (three replicate pens in each batch per treatment) were used to assess the effects of phytase dose.

Pig growth performance was considered the main measure to detect 'extra phosphoric' effects in this experiment and was assessed by recording individual liveweight (LW) at day 0 (weaning), day 7, day 14 and day 21. These periods were designated as P1, P2 and P3 respectively. Feed disappearance was also recorded at these time points, in order to estimate mean daily feed disappearance (DF), daily liveweight gain (DLWG) and the food conversion ratio (FCR) on a pen basis. Over days 22 and 23 post weaning, one pig (closest to the mean average weight for that pen) from each pen was blood sampled alive, then euthanized by captive bolt, followed by destruction of the brain stem. Carcases were briefly washed in hot water and tissue samples collected as detailed in chapter three.

Table 5.2: Composition of raw ingredients (g/kg) for all experimental pig diets relating to chapter five (experiment SM2)

Ingredients	A	B	C	D	E	G	H	J
Micronized barley	76	76	76	76	76	76	76	76
Raw wheat (whole meal)	159	150	159	159	159	159	157	150
Micronized wheat meal	159	150	159	159	159	159	159	159
Micronized maize	25	25	25	25	25	25	25	25
Supertherm oats	50	50	50	50	50	50	50	50
Herring meal	31	31	31	31	31	31	31	31
Soya (hypro)	100	100	100	100	100	100	100	100
Full fat soybean meal	122	122	122	122	122	122	122	122
Premix[1]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Skimmed milk powder	25	25	25	25	25	25	25	25
De-lactosed whey	25	25	25	25	25	25	25	25
Whey powder	123	123	123	123	123	123	123	123
Sugar/sucrose	13	13	13	13	13	13	13	13
L-lysine hydrochloride	5.2	5.3	5.2	5.2	5.2	5.2	5.2	5.2
DL-methionine	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
L-threonine	2.7	2.8	2.7	2.7	2.7	2.7	2.7	2.7
L-tryptophan	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
L-valine	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Crina 693[2]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Vitamin E	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Quantum Blue phytase	0.000	0.000	0.025	0.100	0.200	0.400	1.600	6.400
Sucram®[3]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Benzoic acid (VevoVitall)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Limestone flour	2.6	0.9	2.6	2.6	2.6	2.6	2.6	2.6
Dicalcium phosphate	22	36	22	22	22	22	22	22
Soya oil	42	47	42	42	42	42	42	44

[1]Premix detailed in chapter three; [2]Flavouring made from essential oils manufactured by DSM Nutritional Products (UK) Ltd; [3]Sweetener manufactured by Pancosma SA

5.2.2. Mineral and phytate digestibility

Detailed methodology on all procedures can be found in chapter three. The digestion of a range of minerals were of high interest, given that the main role of phytase is to break down phytate, releasing complexed minerals and possibly preventing the re-formation of other phytates. Digestibility of minerals were therefore measured as ATTD and in ileal digesta, using TiO_2 (3 g/kg) as an insoluble marker (see section 3.3). The increase in P digestibility is the result of IP_6 destruction, leading to lower inositol phosphate product formation. Inositol phosphates (IP_{6-3}) and inositol concentration were thus also quantified in freeze dried ileal digesta using HPLC, to characterise this degradation (sections 3.2.6 and 3.2.7). Lower inositol phosphates (IP_{2-1}) were not determined due to the unreliability of the HPLC method in this range. Mineral absorption was assessed through stores in blood plasma, bone ash and liver samples (see sections 3.2.5 and 3.4.2). Bone strength is a response criterion often associated with bone mineralisation (Veum *et al*., 2006; James *et al*., 2008; Zeng *et al*., 2014) and was used to verify P absorption (see section 3.7.1).

5.2.3. Gut histomorphology and function

Data from Woyengo *et al*. (2011) indicated that phytic acid supplementation (2 %) likely restricted nutrient supply in the small intestine, resulting in significantly lower crypt depth in the jejunum. This was not remedied when 500 FTU/kg phytase was added to the diet, however, super dosing might have a statistically stronger effect. The physical structures of the gut were therefore assessed by fixing and staining (Heamatoxylin and Eosin) a sample from each area of the small intestines, and viewing under optical light microscopy. Each histological sample was measured for villus height, crypt depth and length of an average mucosal gland, using digital software, calibrated with a certified scale bar (see section 3.5). Sialic acid concentration as a marker of endogenous losses was also measured in ileal digesta according to the method described in section 3.9.

There are also records of phytic acid influencing active transporters (Woyengo *et al*., 2011; Vigors *et al*., 2014), another indication of gut disruption. While 500 FTU/kg increased the expression of SGLT1 from high PA supplemented diets (Woyengo *et al*., 2011), it is unclear if this may be the case in diets containing naturally occurring quantities of phytate. Gene expression of SGLT1 was therefore chosen as a target in this experiment, in addition to ZIP4, since zinc should be more available in phytase supplemented diets and ZIP4 is the main zinc transporter into the enterocytes. The expression of alkaline phosphatase (ALPI) was also measured, as Lei *et al*. (1993) found interactions between blood plasma ALP and zinc, in phytase supplemented diets. Intestinal ALP is mainly implicated with the release of P from lower inositol phosphates in the small intestines (Schlemmer *et al.*, 2001), but also

plays a role in intestinal homeostasis and gut immunity (Lallès, 2010), making it a target of interest. Quantification of gut transporters (SGLT1 and ZIP4) and intestinal ALP (ALPI) was performed on whole intestinal wall samples, using RT-qPCR (see section 3.6.3, experiment SM2) with the following assays (Table 5.3) and normalised to GPI (glucose phosphate isomerase) and GSR (glutathione reductase) as specified in Table 3.11.

Table 5.3: Assay details for qPCR primers designed to detect SGLT1, ALPI and ZIP4 mRNA transcripts from pig (Sus Scrofa) intestinal tissue in experiment SM2

Gene symbol	Gene	Accession number	Primer sequences	Product length
SLC5A1	SGLT1	NM_001164021	S – TTCACCAAGCCCATTCCAG A – CATCCAGGTCAATACGCTCC	85
ALPI	ALPI	XR_131141	S – AAGACATACAACGTGGACAGAC A – CGACTTCCCTGCTTTCTTGG	186
SLC39A4	ZIP4	XM_001925360	S – AGACCTGGTGGAAGAAGAGAG A – AGACCATCAGCGAAGTTGTG	126

S = Sense primer, A = Anti-sense primer (Source: qStandard, 2015)

5.2.4. Gut health and immunity

Immune status of the intestines was assessed by measuring the expression levels of key cytokines at three points (duodenum, jejunum and ileum), using RT-qPCR (see section 3.6.3). These were the same whole intestine samples as used for SGLT1, ZIP4 and ALPI. The interleukins (IL) are a family of cytokines that play important roles in mediating responses between immune cells (Tizard, 2013). In particular, IL-2 secretion is associated with a Th1 polarisation and IL-4 with a Th2 response (Mosmann and Sad, 1996), responsible for eradication of intracellular pathogens and activation of B cells respectively (Vernal and Garcia-Sanz, 2008). These cytokines were therefore chosen to characterise the type of immune response, if any, observed in the intestine which may be modulated through increasing dose of phytase. In addition, there is evidence that IL-17 may be a major player in gut inflammation (Katoh *et al*., 2004; Mensikova *et al*., 2013), and thus useful as another measure of gut health. Details of the cytokine assays are given in Table 5.4., which were normalised to GPI and GSR as specified in Table 3.11.

Table 5.4: Assay details for qPCR primers designed to detect, IL-2, IL-4 and IL-17A mRNA transcripts from pig (Sus Scrofa) intestinal tissue in experiment SM2

Gene symbol	Gene	Accession number	Primer sequences	Product length
IL2	IL-2	NM_213861	S – TGGATTTACAGTTGCTTTTGAAGG A – CCCTCCAGAGCTTTGAGTTC	148
IL4	IL-4	NM_214123	S – ACCTTGAACATTCTCACAGCG A – GTGTCTGTAGATGTGCCGAAG	138
IL17A	IL-17A	NM_001005729	S – GAGAAACTACGATGACTCCTGTG A – ATGCTGAGGGAAGTTCTTGTC	143

S = Sense primer, A = Anti-sense primer (Source: qStandard, 2015)

Natural levels of cytokines are very low (Katial *et al*., 1998), though transcripts are still detectable through RT-qPCR (Parra *et al*., 2013) and protein through ELISA (Katial *et al*., 1998), but increase many times when stimulated (Katial *et al*., 1998). For this experiment it was important to assess the basal levels of the immune system, to determine if increasing phytase dose protected against inflammation, rather than modifying cell mediated response. Tissue cells were therefore not cultured/stimulated. The criterion for an immune response was defined as a significant increase in the $\log_{10}$ gene expression (see section 3.6.3, experiment SM2).

5.2.5. Data analysis

All data was collated and calculations performed using Excel 2010/2013 (Microsoft Corporation, 2010/2013). Data points ± 3 SD were considered outliers and removed from the analysis. Data was subject to orthogonal polynomial contrasts based on the formulated level of phytase, using the general ANOVA procedure of GenStat (16th edition, VSN International Ltd), with batch as a blocking factor. Treatments A and B, were then compared using contrast comparisons, blocked by batch. The control diet verses those containing phytase were also analysed using a contrast comparison. Body weight change at the end of each period for the comparison data were adjusted for starting weight as a covariate but this was not possible for the dose response data.

5.3. Results

All animals completed the trial in good health and were assessed by a qualified veterinary surgeon prior to release from the Animals (Scientific Procedures) Act 1986.

5.3.1. Diet analysis

Following ICP-MS analysis, results for the EU certified reference materials (Dairy feed, BER-708 and Hay powder BCR-129) were shown to be within range, except P which was marginally higher than the expected range (0.6 g/kg higher). The P, Ca and phytase analysis of the diets are detailed in Table 5.5.

Table 5.5: Analysis of phytic acid, phytate-P, P, Ca (g/kg as fed basis) and phytase activity (FTU/kg) of experimental diets fed to pigs in trial SM2

Diet	A	B	C	D	E	G	H	J
Measured phytic acid	6.5	4.5	5.8	6.8	5.8	5.5	4.9	4.5
Calculated phytate P	1.8	1.3	1.6	1.9	1.6	1.6	1.4	1.3
Formulated total P	8.9	11.5	8.9	8.9	8.9	8.9	8.9	8.9
Measured total P	11.2	13.1	10.3	9.6	10.8	10.8	10.1	11.0
Formulated total Ca	10.3	13.4	10.3	10.3	10.3	10.3	10.3	10.3
Measured total Ca	10.4	11.8	9.3	8.1	11.0	11.0	10.6	10.7
Formulated Ca:tP	1.16	1.16	1.16	1.16	1.16	1.16	1.16	1.16
Calculated Ca:tP	0.93	0.90	0.90	0.85	1.02	1.02	1.05	0.98
Formulated phytase activity	0	0	125	500	1000	2000	8000	32000
Measured phytase activity	<50	<50	262	532	1060	2030	6320	32500

The analysed tP was consistently higher than the formulated tP, by ~1.6 g/kg when analysed by ICP-MS (Table 5.5). There was, however, a 2 g/kg difference between diets A and B, as expected. Phytase activity was similar to that formulated, though there was twice the activity expected in diet C and diet H was lower than expected. Despite the deviation from that formulated, the phytase activity still represented low, normal, high, super and mega doses. The analysed IP content of the diet is detailed in Table 5.6.

Table 5.6: Analysis of inositol phosphate content of experimental diets (nmol/g DM) fed to pigs in experiment SM2

Diet	A	B	C	D	E	G	H	J
IP_6	9262	9915	9937	9676	9405	10152	10931	6785
IP_5	270	329	318	356	303	402	1266	1129
IP_4	1400	1344	1630	1166	1421	1519	1493	1637
IP_3	134	73	113	108	127	113	212	124
Inositol	321	428	430	334	451	285	397	396
Phytic acid:Inositol	29	23	23	29	21	36	28	17

While there was a variation in phytic acid content of the diet ranging from 4.5 – 6.8 g/kg (Table 5.5), there was a remarkably consistent IP composition across treatments. IP_6 levels did appear do diminish in the mega dosed phytase diet though (diet J), and IP_5 levels were higher in both diets H and J, which contained the highest phytase levels.

5.3.2. Growth performance

Growth rates were good, with average live weights in excess of 15 kg by three weeks post-weaning. Though increasing dietary dgP from 5.8 g/kg to 7.8 g/kg had no effect on growth performance from weaning to three weeks post (Table 5.7), the addition of dietary phytase did significantly affect a number of growth performance parameters (Table 5.8).

Table 5.7: Comparison between 5.8 and 7.8 g/kg dietary digestible phosphorus on weaner pig growth performance from weaning (4 weeks of age) to 3 weeks post

Digestible phosphorous (g/kg)	5.8	7.8	S.E.D.	P - VALUE
Mean start weight (kg)	8.6	8.6	0.20	NS
†Mean weight at day 7 (kg)	9.3	9.4	0.15	NS
†Mean weight at day 14 (kg)	11.6	11.5	0.42	NS
†Mean weight at day 21 (kg)	15.4	15.2	0.70	NS
Overall DF (g)	389	384	36.7	NS
Overall DLWG (g)	323	315	33.1	NS
Overall FCR	1.21	1.22	0.071	NS

† Means adjusted for covariate; S.E.D. = standard error of the difference of means

Overall, the exogenous addition of phytase increased DLWG ($p=0.03$) compared to the no added phytase control (diet A), leading to significantly increased body weights of pigs at the end of the first week post-weaning ($p=0.004$) by 0.5 kg and by ~1 kg in the second ($p=0.006$) and third ($p=0.03$) weeks. These were strongly phytase dose dependent in the first two weeks post-weaning ($p<0.01$) and also tended to be a dose response in the third week (Table 5.6). The effect of phytase on bodyweight corresponded to a linear increase ($p<0.05$) in period one DF and deviated responses in DLWG ($p=0.01$), resulting in deviated responses in FCR ($p=0.008$). This pattern was consistent through to the end of week two, where increasing dietary phytase also improved bodyweight ($p=0.006$) and DLWG ($p=0.026$) but not DF, resulting in more than one point of inflection for the FCR model ($CD<0.001$). The first of these points were due to a sharp increase in performance from the control diet (diet A) to when a low dose of phytase was included (diet C). Performance then dipped back to control levels over the range 500 – 1000 FTU/kg phytase, before increasing with the super doses (2000 – 8000) and falling back a little at the mega dose.

Table 5.8: The effect of increasing level of exogenous dietary 6-phytase on the growth performance of newly weaned commercial pigs

Phytase activity (FTU/kg)	<50	125	500	1000	2000	8000	32000	S.E.D.	p-values			
Log_{10+25} of phytase activity	1.4	2.1	2.7	3.0	3.3	3.9	4.5		Phytase	CL	CQ	CD
Mean weaning weight (kg)	8.6	8.6	8.6	8.5	8.6	8.6	8.7	0.20	0.990	0.888	0.609	0.969
Mean weight at day 7 (kg)	9.3	10.0	9.6	9.5	10.0	9.8	9.8	0.20	0.005	0.062	0.276	0.005
Mean weight at day 14 (kg)	11.6	12.8	12.0	11.9	12.9	13.1	12.4	0.42	0.006	0.017	0.242	0.014
Mean weight at day 21 (kg)	15.4	16.9	15.9	15.9	17.1	17.3	16.5	0.71	0.077	0.068	0.365	0.106
Average DF (g)												
Day 0 – 7	158	208	197	175	224	198	204	18.7	0.030	0.045	0.144	0.069
Day 7 – 14	352	394	426	392	444	443	392	46.1	0.411	0.179	0.118	0.764
Day 14 – 21	657	701	749	701	764	772	755	65.9	0.550	0.072	0.492	0.895
All growth periods	389	434	457	423	477	471	450	37.7	0.276	0.055	0.200	0.692
Average DLWG (g)												
Day 0 – 7	102	199	146	136	196	173	164	26.0	0.008	0.063	0.087	0.014
Day 7 – 14	324	392	343	352	422	469	374	42.5	0.026	0.032	0.352	0.047
Day 14 – 21	542	591	554	563	597	601	575	52.3	0.889	0.438	0.680	0.831
All growth periods	323	394	348	350	405	414	371	34.2	0.100	0.079	0.302	0.145
FCR												
Day 0 – 7	1.49	1.06	1.40	1.36	1.17	1.19	1.28	0.115	0.011	0.095	0.210	0.008
Day 7 – 14	1.09	1.02	1.17	1.12	1.06	0.94	1.06	0.044	<.001	0.087	0.305	<.001
Day 14 – 21	1.23	1.19	1.35	1.15	1.31	1.29	1.31	0.100	0.413	0.293	0.949	0.296
All growth periods	1.21	1.11	1.27	1.22	1.19	1.14	1.21	0.070	0.315	0.988	0.941	0.143

CL= Contrast for linear, CQ=Contrast for quadratic, CD=Contrast for deviations; S.E.D. = standard error of the difference of means

5.3.3. Mineral digestibility

Calculated dgP, based on the concentration of Ti added to the feed, was lower compared to formulated levels (Table 5.9), assuming 40 % digestibility. This was especially the case in the lower phytase supplemented diets.

Table 5.9: Formulated and analysed digestible P and Ca content (g/kg diet as fed) of weaner pig experimental diets (trial SM2)

Diet	A	B	C	D	E	G	H	J
Formulated digestible P	5.8	7.8	5.8	5.8	5.8	5.8	5.8	5.8
Calculated digestible P	4.1	4.3	3.7	4.8	5.5	5.5	5.0	5.5
Calculated digestible Ca	2.1	1.6	1.5	2.8	4.5	4.1	3.6	3.7

Levels of Ti measured in the feed samples varied more than expected (Table 5.10), resulting in artificially inflated/deflated digestibility coefficients. In the case of P, excluding the most severely affected treatment (diet D) from the statistical analysis did not significantly change the shape of the curve from that generated using a mean average dietary Ti value to normalise all coefficients (Table 5.10).

Table 5.10: Quantification of titanium by ICP-MS analysis of ^{49}Ti in pig experimental diets for trial SM2

Diet	A	B	C	D	E	G	H	J
Ti (g/kg)	1.24	0.94	1.27	0.82	1.08	1.01	0.97	1.16

Normalised ATTD of the minerals were mostly complex, except for P which exhibited a linear ($p=0.01$) increase and Zn which exhibited a liner ($p<0.001$) decrease in response to increasing phytase (Table 5.11). While the ANOVA model for phytase probability for P is given as 0.08 in Table 5.11, there is one low value in diet G that is ~1.98 SD from its treatment group mean. Removing this outlier reduces the model F probability to <0.05 for phytase effect, linear and quadratic contrasts for P. There was no phytase effect on the digestibility of Ca, Mg and Mo.

Table 5.11: The effect of increasing level of exogenous dietary 6-phytase on mineral apparent total tract digestibility in weaned pigs

Phytase activity (FTU/kg)	<50	125	500	1000	2000	8000	32000	S.E.D.	p-values			
Log_{10+25} of phytase activity	1.4	2.1	2.7	3	3.3	3.9	4.5		Phytase	CL	CQ	CD
P	0.37	0.41	0.50	0.50	0.51	0.50	0.49	0.06	0.08	0.01	0.06	0.93
Ca	0.28	0.35	0.42	0.42	0.36	0.35	0.35	0.07	0.31	0.31	0.05	0.72
K	0.75	0.78	0.66	0.76	0.79	0.76	0.77	0.031	0.00	0.48	0.34	<.001
Mg	-0.10	-0.01	-0.16	0.00	-0.05	-0.11	-0.14	0.077	0.21	0.46	0.27	0.15
Fe	-0.17	-0.22	-0.45	-0.25	-0.19	-0.25	-0.33	0.077	0.01	0.17	0.32	0.01
Cu	0.14	0.31	0.05	0.10	0.11	-0.05	0.03	0.062	<.001	<.001	0.60	<.001
Zn	0.03	0.08	-0.11	-0.05	-0.05	-0.16	-0.15	0.074	0.02	<.001	0.98	0.25
Co	0.05	0.05	-0.18	0.08	0.05	-0.10	-0.10	0.073	0.00	0.03	0.86	0.00
Mo	0.70	0.70	0.56	0.62	0.72	0.68	0.56	0.084	0.216	0.2	0.921	0.155

CL= Contrast for linear; CQ=Contrast for quadratic; CD=Contrast for deviations; S.E.D. is max - min

Digestibility of P in the control diet (diet A) was similar to the expected 40 % at 37 % (Table 5.11). The addition of phytase to this diet tended to improve P ATTD (p=0.08) in a linear and quadratic increase to around 50 % ATTD. This was not the case for Ca, though there was a tendency for a quadratic relationship (p=0.05), with the un-supplemented diet containing 28 % digestible Ca, increasing to ~35 – 42 % in diets containing phytase. Dietary phytase inclusion also increased the ash content of both the third (p=0.02) and fourth (p=0.02) metacarpals over the control diet but in a complex way (Figure 5.1). When the control diet was considered as a 0 FTU/kg dose, then there were only tendencies for a quadratic relationship of phytase on the 3rd metacarpal (p=0.09) (ANOVA model p=0.06) and linear (p=0.08)/deviations (p=0.08) on the 4th metacarpal (ANOVA model p=0.04).

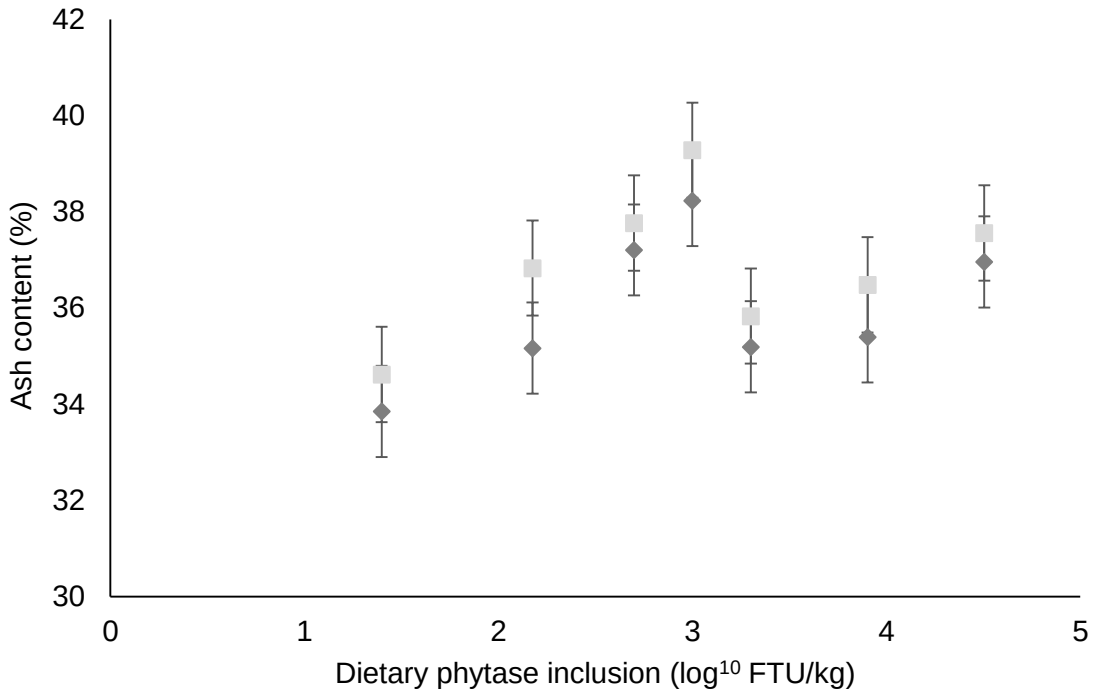

Figure 5.1: The effect of dietary phytase dose on the ash content of the third and fourth metacarpals (light grey square and dark grey diamond respectively) of pigs three weeks post weaning. Means presented with common standard error of mean.

The increased bone mineralisation relating to increasing dietary phytase content (Figure 5.1) did not affect bone shear strength (Table 5.12) or bone mineral composition (Table 5.13) but there was a significant quadratic increase on compression strength of the tibia and femur due to phytase (only measured to 2000 FTU/kg) (Figure 5.2).

Table 5.12: The effect of increasing level of exogenous dietary 6-phytase on bone characteristics of pigs three weeks post-weaning

Phytase activity (FTU/kg)	<50	125	500	1000	2000	8000	32000	S.E.D.	p-values			
Log_{10+25} of phytase activity	1.4	2.1	2.7	3	3.3	3.9	4.5		Phytase	CL	CQ	CD
Length of 3rd metacarpal (mm)	46.7	46.2	47.0	46.7	46.3	47.5	47.8	1.199	0.80	0.21	0.43	0.93
Length of 4th metacarpal (mm)	44.3	44.5	45.3	44.0	44.2	45.7	44.2	1.099	0.66	0.81	0.70	0.43
Area (mm^2) of 3rd metacarpal	153	158	154	156	167	148	158	14.92	0.93	0.89	0.71	0.79
Area (mm^2) of 4th metacarpal	136	140	139	147	156	154	134	15.17	0.68	0.58	0.22	0.71
Breaking strength (N) of 3rd metacarpal§	805	853	769	700	747	707	1005	163.20	0.55	0.59	0.13	0.68
Breaking strength (N) of 4th metacarpal*	669	482	766	616	611	694	658	130.50	0.50	0.60	0.88	0.30
Shear stress (N/mm^2) of 3rd metacarpal	5.30	5.56	5.16	4.80	4.56	4.87	6.39	1.162	0.77	0.70	0.20	0.83
Shear stress (N/mm^2) of 4th metacarpal	5.13	3.55	5.76	4.40	4.08	4.52	5.00	1.048	0.46	0.95	0.54	0.27

CL= Contrast for linear; CQ=Contrast for quadratic; CD=Contrast for deviation; *Freeze dried prior to testing; §Tested wet

Table 5.13: The effect of increasing level of exogenous dietary 6-phytase on the bone mineral concentration of pigs three weeks post-weaning

Phytase activity (FTU/kg)	<50	125	500	1000	2000	8000	32000	S.E.D.	p-values			
Log_{10+25} of phytase activity	1.4	2.1	2.7	3.0	3.3	3.9	4.5		Phytase	CL	CQ	CD
Mg (g/kg)	6.95	6.99	7.41	7.23	7.44	7.18	7.27	0.140	0.12	0.06	0.09	0.38
P (g/kg)	200	200	202	201	204	204	203	2.276	0.71	0.15	0.69	0.83
K (g/kg)	1.72	1.89	1.79	1.78	2.08	1.62	1.75	0.143	0.42	0.78	0.29	0.31
Ca (g/kg)	391	391	394	391	394	397	396	3.670	0.88	0.25	0.89	0.92
Fe (mg/kg)	185	206	214	200	201	210	204	15.490	0.91	0.47	0.47	0.92
Co (mg/kg)	0.33	0.32	0.35	0.38	0.35	0.35	0.33	0.023	0.64	0.68	0.20	0.66
Cu (mg/kg)	14.7	13.3	9.9	17.5	28.1	22.2	18.2	10.570	0.92	0.49	0.87	0.84
Zn (mg/kg)	258	250	271	258	249	251	257	8.410	0.59	0.72	0.87	0.36
Mo (mg/kg)	0.78	0.78	0.7	0.69	0.66	0.78	0.69	0.064	0.69	0.38	0.49	0.63

CL= Contrast for linear; CQ=Contrast for quadratic; CD=Contrast for deviation

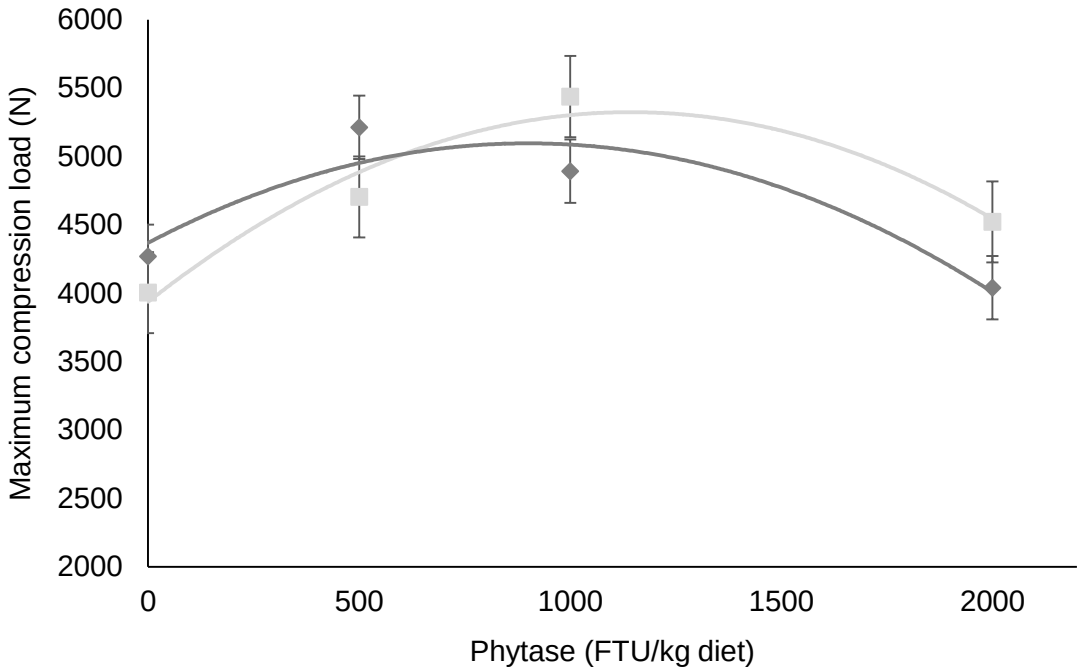

Figure 5.2: The effect of dietary phytase dose on the compression strength of the oven dried tibia and femur (light grey square and dark grey diamond respectively) of pigs three weeks post weaning. Means presented with common SEM.

Increasing dietary phytase up to 2000 FTU/kg significantly affected the compression strength of both the tibia (p=0.03) and femur (p=0.006) in a quadratic manor (Figure 5.2). As expected, blood plasma levels of Ca and P were strongly positively correlated (p<0.001), with a moderate positive correlation for P and Mg (p=0.002), P and Fe (p<0.001) and P and K (p<0.001) (Table 5.14). There was, however, no significant phytase (FTU/kg) correlation for any measured plasma mineral except Mo (p=0.03) (Table 5.14).

Table 5.14: The associations (correlation coefficents) between blood plasma mineral concentrations in weaned pigs

Mineral	Correlation coefficients										
Ca	0.02										
Co	-0.14	-0.08									
Cu	-0.10	0.04	0.38								
Fe	0.15	0.52	-0.15	-0.05							
K	-0.02	0.68	0.07	0.20	0.56						
Mg	0.04	0.40	0.34	0.28	0.27	0.52					
Mn	-0.12	-0.38	0.52	0.35	-0.19	-0.15	0.20				
Mo	0.33	0.08	-0.13	0.01	0.18	0.05	0.22	-0.02			
P	-0.04	0.80	-0.20	-0.03	0.51	0.57	0.47	-0.34	0.17		
Ti	-0.09	-0.08	0.13	0.23	-0.15	-0.15	-0.11	0.40	0.10	-0.17	
Zn	-0.13	-0.08	-0.06	-0.09	0.17	0.12	0.01	0.08	0.01	0.02	-0.13
	FTU/kg	Ca	Co	Cu	Fe	K	Mg	Mn	Mo	P	Ti

Highlighted values (yellow) are significantly correlated (p<0.05)

5.3.4. Inositol phosphate breakdown

The concentration of IP esters ($_{6-3}$) and inositol in the ileal digesta of pigs was similar when fed the basal diet containing either 5.8 g/kg dgP or 7.8 g/kg dgP (Table 5.15). Dietary phytase supplementation did, however, influence ileal IP concentration (Table 5.16).

Table 5.15: Comparison of the inositol phosphate product concentration (log_{10+1} nmol/g DM) in weaner pig ileal digesta in response to increasing digestible phosphorus from 5.8 g/kg to 7.8 g/kg

Digestible phosphorous (g/kg)	5.8	7.8	SED	P - VALUE
Phytic acid (IP_6)	4.34 (24487)	4.24 (19602)	0.198	NS
IP_5	3.43 (2968)	3.39 (2948)	0.176	NS
IP_4	3.46 (3108)	3.48 (3796)	0.134	NS
IP_3	2.42 (286)	2.29 (206)	0.106	NS
Inositol	2.79 (895)	2.74 (635)	0.192	NS

Untransformed means presented in brackets (nmol/g DM)

The graded increase in exogenous phytase level resulted in a significant ($p<0.001$) linear decrease ($p<0.001$) in ileal digesta phytic acid and IP_5 concentrations and a corresponding linear increase ($p<0.001$) in inositol concentration (Table 5.16). This resulted in the phytic acid to inositol ratio reducing from 28:1 down to 0.7:1. The digestibility of IP_6 in the ileal digesta increased significantly ($p=0.002$) and linearly ($p<0.001$) as dietary phytase level increased. While phytase did not have a significant effect on ileal IP_3 concentration, it did significantly ($p<0.001$) affect the IP_4 concentration in a complex way (CD=0.03) with linear ($p<0.001$) and quadratic ($p<0.001$) components. The complex relationship between increasing phytase and the ATTD of certain minerals such as iron and copper may depend on the concentration of lower inositol phosphates in the intestinal lumen. There is a strong inverse quadratic relationship between IP_3 and Fe but this is dependent on treatment group (interaction $p=0.008$) (Figure 5.3).

Table 5.16: The effect of increasing exogenous dietary 6-phytase level on the breakdown and digestibility of phytic acid (log_{10+25}) in weaner pig ileal digesta (log_{10+1} nmol/g DM)

Phytase activity (FTU/kg)	<50	125	500	1000	2000	8000	32000	S.E.D.	p - Value	CL	CQ	CD
Log_{10+25} of phytase activity	1.4	2.1	2.7	3.0	3.3	3.9	4.5					
Phytic acid (IP_6)	4.4(24487)	4.0(13864)	3.9(10081)	3.8(7769)	4.0(11638)	3.4(4060)	3.3(3047)	0.20	<0.001	<0.001	0.579	0.450
IP_5	3.4(2968)	3.1(1595)	3.1(1749)	2.8(596)	2.8(865)	2.6(454)	2.7(584)	0.17	<0.001	<0.001	0.190	0.302
IP_4	3.5(3108)	3.6(3836)	3.8(7841)	3.4(2861)	3.5(3228)	3.3(1884)	3.1(1440)	0.13	<0.001	<0.001	0.001	0.029
IP_3	2.4(286)	2.4(261)	2.3(230)	2.5(297)	2.4(246)	2.4(250)	2.5(338)	0.11	0.849	0.790	0.441	0.747
Inositol	2.8(895)	3.2(2710)	3.3(2344)	3.4(2527)	3.5(4202)	3.4(3140)	3.6(4311)	0.20	0.009	<0.001	0.160	0.943
Digestibility	0.14	0.59	0.65	0.68	0.64	0.88	0.84	0.162	0.002	<0.001	0.105	0.576

CL= Contrast for linear, CQ=Contrast for quadratic, CD=Contrast for deviations, ‡Values transformed (log_{10+1}) with untransformed means in parentheses

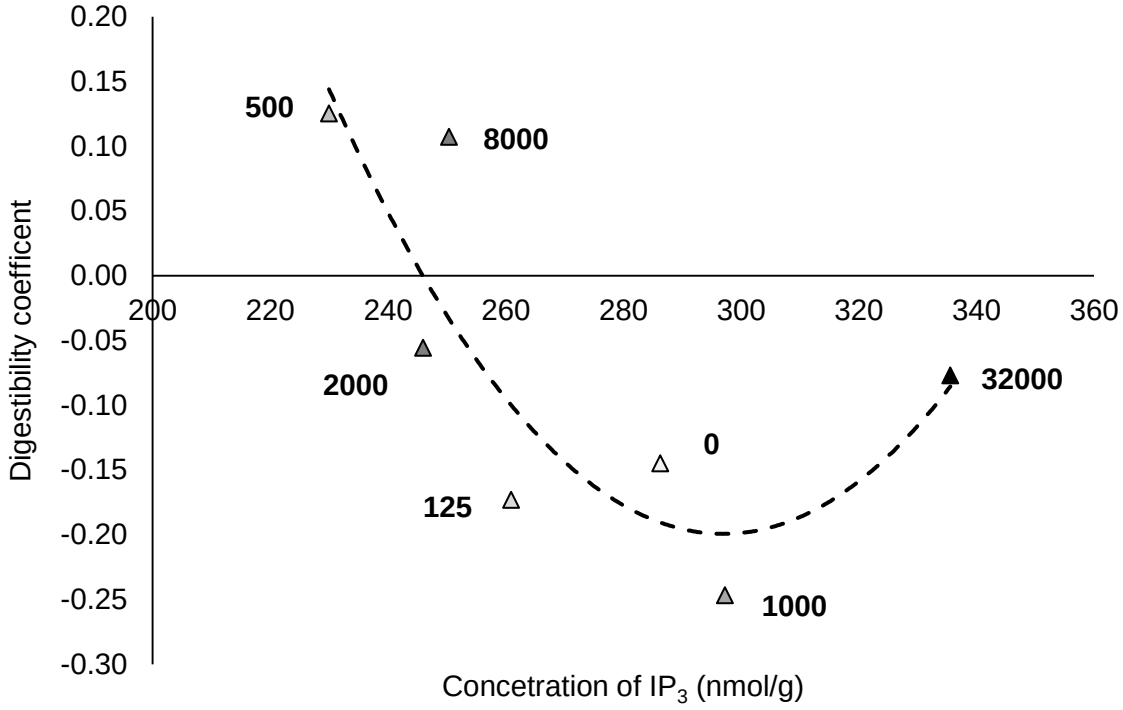

Figure 5.3: The polynomial relationship (p=0.01) between the mean concentration of IP_3 in pig ileal digesta and the mean apparent total tract digestibility coefficent of Fe at graded levels of dietary exogenous phytase ranging from 0 – 32000 FTU/kg of diet (as indicated by the lables).

Although there was no effect of phytase supplementation on IP_3 ileal digesta concentration (Table 5.16), there was a negative polynomial relationship (p=0.01) between the IP_3 ileal digesta concentration and the ATTD of iron when analysed by phytase group (Figure 5.3). The partial correlation of phytase group average IP_3 concentration and mineral ATTD was always negatively correlated and particularly strong for Mo (r=-0.88, p=0.009) and K (r=-0.79, p=0.03) but moderate for all other minerals tested (r>-0.40, p>0.05). Conversely, IP_4 concentration was always positively correlated, with strong coefficents for Mn (r=0.81, p=0.03) and Zn (r=0.80, p=0.03).

5.3.5. Gut transporters and alkaline phosphatase transporter expression

Normalised mRNA copy numbers of SGLT1, ZIP4 and ALPI transcripts did not differ significantly due to phytase treatment in either the duodenum or the ileum but increasing dietary phytase did significantly influence ALPI expression in the jejunum (p=0.02) both linearly (p=0.01) and quadratically (p=0.01) (Figure 5.4).

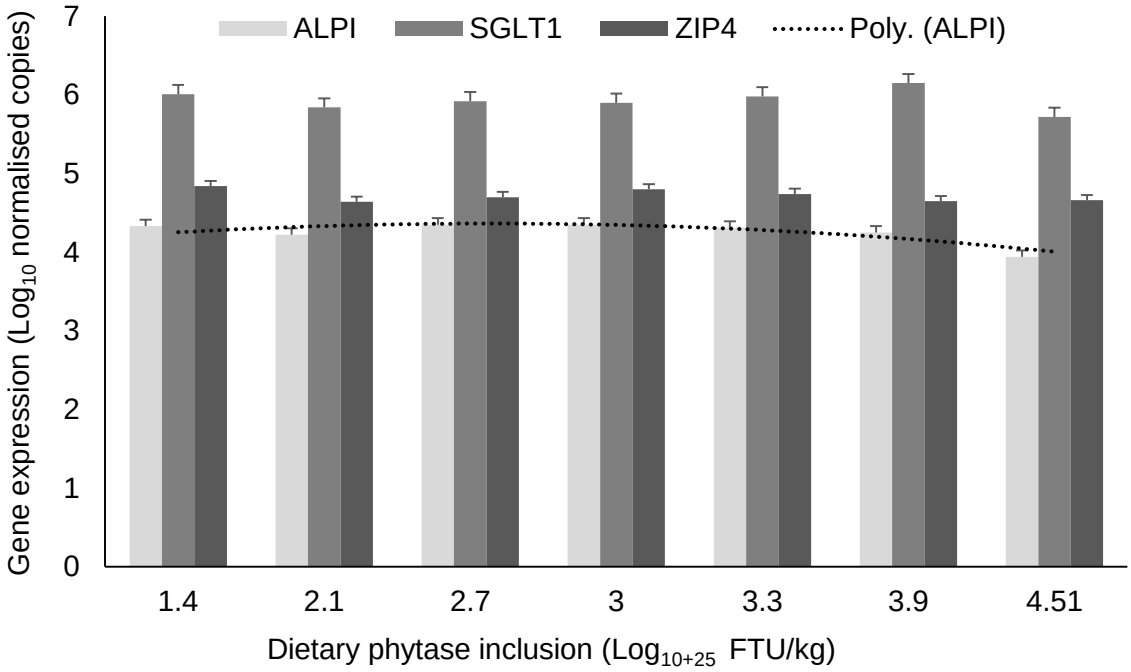

Figure 5.4: The effect of exogenous dietary phytase on jejunal expression of sodium-glucose linked transporter 1 (SGLT1), zinc transporter (ZIP4) and intestinal alkaline phosphatase (ALPI) gene expression in pigs three weeks post weaning. Means presented with common Log_{10} SEM bars.

The levels of mRNA transcripts detected per standardised reaction were good, with the numerically highest expression of ALPI, SGLT1 and ZIP4 all found in the jejunum (Table 5.17). Despite the relatively high CV% of the normalised copy numbers, these were all reduced to ≤7.2% after the log_{10} transformation. Around 10 % of the samples had a second RT run, with a rate of only 10 % exceeding 0.7 cycles difference (2 for ALPI and 3 in ZIP4).

Table 5.17: Summary of ALPI, SGLT1 and ZIP4 gene expression in the duodenum, jejunum and ileum sections of pig intestines three weeks post-weaning in 10 µl RT-qPCR reactions

Intestinal section	Gene	Mean copy no.	Median copy no.	Standard Deviation	CV%
Duodenum		12764	12780	4526	35
Jejunum	ALPI	20376	19717	10847	53
Ileum		17219	16499	8749	51
Duodenum		227658	167107	165049	73
Jejunum	SGLT1	1069188	790001	954512	89
Ileum		565034	510919	309988	55
Duodenum		36966	35198	11133	30
Jejunum	ZIP4	64010	49935	39608	62
Ileum		39913	37022	18930	47

Mean and median copy numbers are normalised to GSR and GPI reference genes.

5.3.6. Effect of phosphorus and phytase level on gut histomorphology

While there were no significant differences in growth performance and ileal digesta IP concentration between pigs fed the basal diet containing 5.8 g/kg dgP and those fed the higher rate of 7.8 g/kg dgP, there tended to be a reduction in total villus height (p=0.07) and an increase in the average mucosal gland length (p=0.07) in those fed the higher dgP concentration. This also resulted in a significant reduction in villi to mucosal gland length ratio (p=0.002) (Table 5.18).

Table 5.18: Comparison between 5.8 and 7.8 g/kg digestible phosphorous on jejunum histomorphology of the weaner pig

Digestible phosphorous (g/kg)	5.8	7.8	S.E.D.	P - VALUE
Total villi/crypt length (µm)	841	747	51.2	0.073
Mucosal gland length (µm)	161	216	28.6	0.066
Villi height : gland length	5.88	3.89	0.608	0.002

A reduction in total villi/crypt length was not observed in relation to phytase dose supplementation (Table 5.19) but increasing phytase level did tend to reduce villi to mucosal gland length ratio (p=0.08) in a linear (p=0.007) fashion (Table 5.19). Sialic acid concentration in the ileal digesta (mean concentration 2.6 ± 0.87 µmol/g DM) was not influenced by phytase but did tend (p=0.06) to be weakly correlated (0.29) to the ratio of villi to mucosal gland length.

Table 5.19: The effect of 6-phytase on the histomorphology of the weaner pig jejunum at three weeks post weaning

Phytase activity (FTU/kg)	<50	125	500	1000	2000	8000	32000	S.E.D.	p-Value	CL	CQ	CD
Log_{10+25} of phytase activity	1.4	2.1	2.7	3.0	3.3	3.9	4.5					
Total villi/crypt length (µm)	841	751	765	820	779	772	774	48.3	0.514	0.348	0.486	0.430
Mucosal gland length (µm)	161	189	187	229	196	211	229	29.0	0.232	0.020	0.646	0.660
Villi height : gland length	5.88	4.36	4.54	4.12	4.39	4.11	3.99	0.624	0.076	0.007	0.135	0.693

CL= Contrast for linear, CQ=Contrast for quadratic, CD=Contrast for deviation

5.3.7. Effect of phytase on gut health

Levels of cytokine mRNA transcripts per reaction were very low or undetectable in many cases. This resulted in data with many missing values, which was analysed by unbalanced ANOVA. IL-17A expression in the ileum tended to be influenced by phytase (p=0.08) and so was further tested with orthogonal polynomial contrasts. The addition of increasing phytase significantly affected the levels of IL-17A mRNA transcripts (p=0.03) with multiple points of inflection (CD=0.01) over the log_{10+25} phytase dose range tested (Figure 5.5).

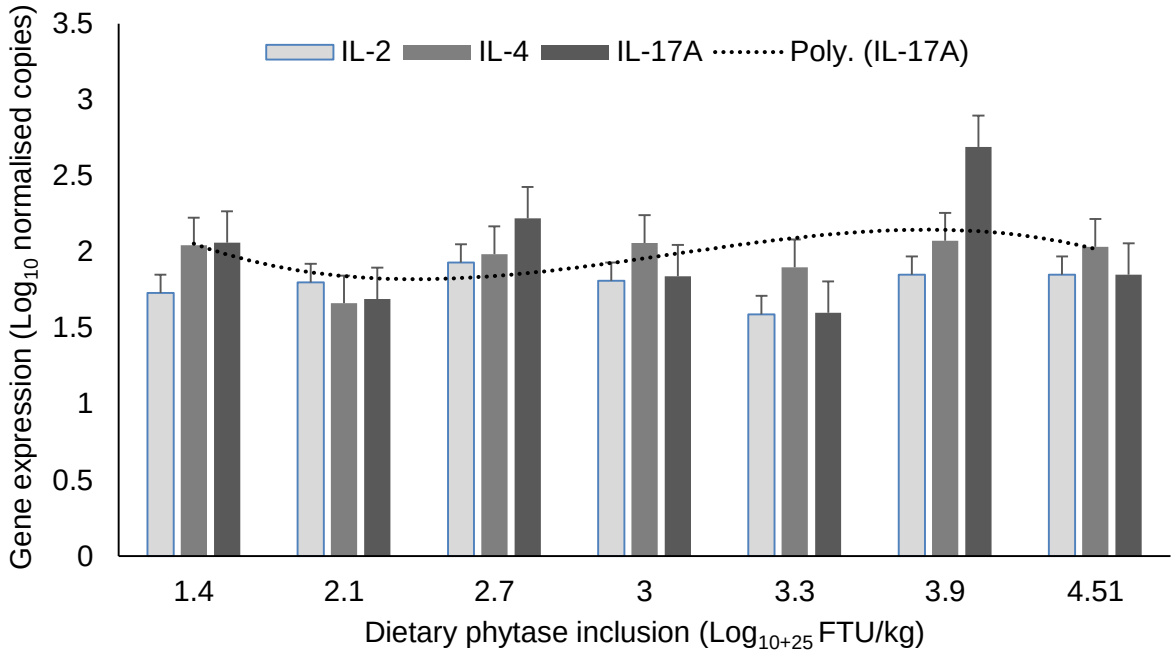

Figure 5.5: The effect of exogenous dietary phytase on ileal expression of interleukin 2 (IL-2), interleukin 4 (IL-4) and interleukin 17A (IL-17A) gene expression in pigs three weeks post weaning. Means presented with common Log_{10} SEM bars.

The expression of all other cytokines (except ileal IL-17A) were either non-significant for a phytase treatment effect by unbalanced ANOVA or did not meet the assumptions of the ANOVA and were deemed unreliable for inferential analysis. Around 10 % of the samples had a second RT run, with a rate of 27 % exceeding 0.7 cycles difference (3 for IL-2, 6 for IL-4 and 4 for IL-17A). While the CV % in Table 5.20 is relatively high for IL-17A, the log transformed data had a CV of 25 %.

Table 5.20: Summary of IL-2, IL-4 and IL-17A cytokine gene expression in the duodenum, jejunum and ileum sections of pig intestines three weeks post-weaning, in 10 µl RT-qPCR reactions

Intestinal section	Gene	Median copy numbers	IQR 25	IQR 75	CV%
Duodenum	IL-2	54	34	80	84
Jejunum		28	23	45	87
Ileum		51	39	92	79
Duodenum	IL-4	19	11	26	90
Jejunum		43	20	118	211
Ileum		65	43	182	173
Duodenum	IL-17A	42	24	79	103
Jejunum		60	37	363	279
Ileum		93	37	161	363

Median copy numbers per reaction are normalised to GSR and GPI reference genes.

There was a slight polarisation of 2.9:1 towards a Th1 balance (IL-2 : IL-4) in the duodenum but no evidence of any bias towards either a Th1 or Th2 response in either of the other intestinal sections (Table 5.20).

5.4. Discussion

Phytase was clearly shown to be effective at breaking down phytic acid into lower inositol phosphates and pure inositol by the time feed had reached the terminal ileum (Table 5.16). This is in agreement with the results of Kemme *et al.* (2006) who showed a 65 % disappearance of phytic acid in ileal digesta when calculated relative to that fed, compared to the 68 % reduction in phytic acid found in this study (dietary phytase activity 959 vs 1060 FTU/kg respectively). The analysed levels of IP_{3-6} were similar to those reported by Laird *et al*. (2016), where ileal IP_6 concentration without phytase supplementation was between 21000 – 25000 nmol/g DM and reduced thereafter due to phytase inclusion. For IP_4 concentration however, in the study of Laird *et al*. (2016), the levels were similar to those in Table 5.16 up until a dose of 8000 FTU/kg phytase, when IP_4 concentration reduced dramatically. This was not seen in the present study, nor was the similar effect on IP_3 concentration observed. While IP_3 concentrations were not affected by phytase in this experiment, it was IP_2 ileal concentrations in Laird *et al*.'s study where phytase had no effect. This suggests that even a third generation phytase fed at super doses struggles to dephosphorylate the lower IP esters in pig diets.

It is important that there is a rapid dephosphorylation of IP_6 to IP_2 in order prevent pepsin inhibition (Yu *et al.,* 2012) and thus reduce the risk of phytate-mineral-protein complex indigestibility in the stomach. While in this chapter it was not possible to show the dynamics of phytic acid destruction in the stomach and along the small intestines, the inositol phosphate quantification pattern in the ileal digesta did closely matched that of Kemme *et al.* (2006). Assuming therefore that phytic acid destruction in the duodenum also matched their findings, it can be speculated that super doses of phytase ensure this rapid destruction of IP_6, thus reducing the risk of reduced protein digestibility. This may in turn account for some of the differences observed in growth.

5.4.1. Growth performance

The results of this chapter confirm the observation of chapter four, that there was no effect from increasing dietary dgP level on the growth performance of pigs under these experimental conditions (Tables 4.7 and 5.7). All subsequent effects seen on growth performance are therefore likely to be 'extra phosphoric' in nature and due to the supplementation of phytase. The addition of the ultra-low dose (125 FTU/kg) of phytase compared to the control, resulted in a 44 % reduction of IP_6 in the gut lumen (Table 5.16), representing a big reduction in a potent ANF. This may help to explain why the addition of phytase at any level resulted in the pronounced biological growth response over the unsupplemented group seen in Table 5.8. The main effects of phytase on the performance of the weaner pigs was observed to be in the first two weeks post-weaning (Table 5.8). This suggests that the inclusion of phytase helped to ensure nutrient intakes were sufficient, thus maintaining growth through this period (supported by the significantly increased feed intake in period 1). Pigs on the 8000 FTU/kg super dose were on average 1.9 kg heavier at day 21 post-weaning than those not fed a phytase supplemented diet. Phytase at low or super levels may therefore be useful in mitigating the post-weaning growth check by promoting nutrient intake in the first week post-weaning (Table 5.8).

Veum *et al.,* (2006) conducted a similar dose response study using a predecessor second generation *Escherichia coli* derived 6-phytase enzyme expressed in *Pichia pastoris*. They reported a significant linear and quadratic response in pig growth performance over the range 0 – 12500 FTU/kg but did not test for higher order polynomials. If the third order contrasts are excluded from this data, the results would agree, as in this experiment the lowest dose (125 FTU/kg) is largely responsible for the significant deviations. This is unlikely, however, to have a strong enough influence on the polynomial fitted line to significantly affect the shape of the curve. In addition, here diets fed exceeded BSAS standards (Whittemore *et al.,* 2003) for dgP inclusion, while the responses quantified by

Veum *et al.* (2006) were from pigs fed P inadequate diets. This may account for the stronger response they observed at the latter stage of their experiment, as pigs on their positive control diet without phytase performed significantly better that those on their 0 FTU/kg added phytase control. This suggests that some of the response that they attributed to the effects of phytase may actually be due to the improvement in P status. In this current study, no such additional benefits in performance from the high P control over the 0 FTU/kg added phytase dose for any response criteria were observed. This may infer that the benefits measured from pigs fed the phytase supplemented diets were not related to the release of P, and were maybe due to the reduction of the anti-nutritional influence of phytate.

5.4.2. Mineral and inositol phosphate digestibility

Faecal mineral digestibility was measured as ATTD rather than STTD, since it was shown in chapter four that diets containing excessive P, such as the experimental diets used in this study, do not allow for accurate estimations of endogenous P losses. Titanium dioxide was chosen as a suitable in feed marker due to favourable reviews by Jagger *et al*. (1992) over chromic oxide and previous successful use in the experiment of chapter four. Analysis was carried out as in chapter four using ICP-MS to quantify ^{49}Ti, due to known interferences of Ar with the more common ^{48}Ti isotope. The accuracy of Ti quantification was verified with a certified EU reference sample (BCR® - 129 hay powder) and measured 3.9 mg/kg compared to the 3.8 mg/kg certified value, indicating reliable results. Samples of feed that had been sub-sampled from 25 kg bags throughout the trial were homogenised and analysed for Ti, which was found to be particularly low in diet D (Table 5.10). This low value is likely due to the sampling, since the manufacturer's records show the inclusion of TiO_2 had been accurate and consistent at the point of manufacture. If the individual diet Ti levels from the samples are used to calculate digestibility, then the values for diet D in particular is artificially inflated, defying the point of using polynomial contrasts to assess dose response. Using the average dietary Ti concentration of 1.08 g/kg as a common marker value for all diets formulated to 5.8 g/kg dgP, corrected for this discrepancy and allowed the dose response of phytase to be assessed.

The relationship between the level of phytase and the ATTD of minerals such as Fe and Cu is very complex (Table 5.11). Each one is known to be absorbed and secreted in a particular manner and are generally influenced by the form fed, the age and physiological state of the animal and the presence of dietary antagonists and agonists. This presents particular challenges in assessing their bioavailability, as discussed in chapter four. It is, however, appropriate (and the industry norm) to use indigestible markers to quantify ATTD (or STTD) of P and Ca (NRC, 2012; González-Venga and Stein, 2014) due to the relatively high dietary

levels. As expected, phytase increased the ATTD of P in the unsupplemented diet from 37 % to ~50 % in diets containing above 500 FTU/kg phytase (Table 5.11). While the statistical evidence for the phytase effect for P is given as 0.08 in Table 5.11, there is one low value in diet G that is ~1.98 SD from its treatment group mean. Removing this outlier reduces the model F probability to <0.05 for phytase effect, linear and quadratic contrasts. This confirms that phytase up to 500 FTU/kg is adequate for achieving the traditional goal of enhancing ATT phosphorus digestibility. There are, however, advantages to increasing phytase supplementation to 8000 FTU/kg, since only 65 % of phytic acid had been dephosphorylated at the terminal ileum with a 500 FTU/kg dose but this increased to 88 % at the super level (Table 5.16).

Both copper and zinc are poorly absorbed in the pig with a balance study suggesting retention levels of 7.2 % of intake for Cu and 19 % of intake for Zn (Adeola, 1995). High levels of dietary P and Ca, such as in this study, would likely reduce the bioavailability of these minerals further (Atkinson *et al*., 1993). Where ATTD of these minerals are reported in the literature, phytase supplementation has inconsistent effects on digestibility. For example, Rutherfurd *et al*. (2014) showed that apparent digestibility of Zn and other trace minerals varies by the GI site measured but phytase supplementation up to 2215 FTU/kg did not influence Zn digestibility, or that of any other trace elements measured, in low P corn diets. In contrast, at 500 FTU/kg phytase supplementation, Bikker *et al*. (2012) showed increased digestibility from similar levels reported in this chapter up to ~10 %. Results from this chapter show that while there was an initial increase in Zn ATTD to 8 % with the lowest level of phytase, all higher doses (including 500 FTU/kg) resulted in negative digestibility up to 16 % (Table 5.11). This may be due to hindgut processes (e.g. metabolism processes and modulation from the microbiota (Metzler-Zebeli *et al*., 2010) and homeostatic regulation) rather than small intestinal absorption, since there was no change in ZIP4 mRNA expression in any section of the small intestine (Figure 5.4) in this study.

There does, however, appear to be some high correlations between the concentrations of lower inositol phosphate esters (especially IP_3 and IP_4) in the ileal digesta and ATT mineral digestibility, depending on the level of phytase fed (Figure 5.3). Given that there was no phytase effect on the ileal digesta concentration of IP_3 and a significant but complex response for IP_4 (Table 5.16), it is likely that it is the concentrations of these lower esters that is important for the interaction with minerals (Bedford and Walk, 2016), rather than phytase *per se* in this IP range. This is supported by a number of studies that report strong interaction with lower inositol phosphates and metal ions *in vitro* solutions (Persson *et al*., 1998) and even in human intestinal cell lines (Han *et al*., 1994). So even though IP_6, which is a very strong mineral chelator (Champagne, 1988), has been significantly reduced in the

digesta, the inhibitory mechanism of the lower inositol phosphates may still pose a problem for absorption of some metal ions (including Fe, Zn and Cu). The influence of phytase on these lower inositol phosphate products in the gut is thus less than that for IP_{5-6}, which are readily catalysed by phytase. This reduction in efficiency is likely to be multi-factorial, arising from reduction in phytase activity in the lower small intestines, the pH optimum of the phytase and dietary factors such as the concentration of minerals and the ratio of Ca:P (Dersjant-Li et al., 2014).

5.4.3. Nutrient transport

Dietary inclusion of 2 % phytic acid has been associated with a range of negative effects in young pigs, including histomorphological changes (Woyengo *et al*., 2011), nutrient digestibility and absorption modulation (Woyengo and Nyachoti, 2013) and a reduction in growth performance (Woyengo *et al*., 2012). It has also been shown to reduce the mRNA expression of SGLT1 in all three sections of the small intestines by between 1.1 and 5.4 fold, depending on site measured (Woyengo *et al*., 2011). In PA unsupplemented diets however, there is often no effect of dietary phytase supplementation on either the mRNA expression (Vigors *et al*., 2014) or SGLT1 protein levels (Woyengo *et al*., 2012). This is confirmed in this study, where there were no phytase effects on the mRNA expression levels at any site in the small intestine, despite good expression levels being detected (Table 5.17). Supplementary phytase was shown to influence SGLT1 when in combination with high levels of dietary PA (Woyengo *et al*., 2011) but the results were inconsistent with the hypothesis that phytase should help restore expression levels throughout the small intestines to when no PA is present in the diet. Based on this evidence, at background levels of PA (0.5 %) found in weaner pig feeds (Table 5.5), it is unlikely that phytase would be effective at increasing glucose transport via the Na co-transporter and therefore not capable of significantly influencing net energy by this route.

The expression of intestinal alkaline phosphatase was significantly modulated by phytase supplementation in the jejunum section only (Figure 5.4). Doses of phytase up to 2000 FTU/kg did not appear to negatively impact ALPI expression but there was a decline in ALPI expression at mega doses. Alkaline phosphatase plays a number of diverse roles in the intestine (Lallès, 2010) but is especially useful in the release of P from lower inositol phosphates (KEGG, 2015) and a reduction in the expression levels at high phytase doses may slightly reduce the effectiveness of IP_1 hydrolysis at the D-2 position. There did not appear to be any major impacts on phytate destruction though, as indicated by the linear response in Table 5.16. It was expected that there would be a significant phytase effect on the ZIP4 transporter given that phytate and lower inositol phosphates chelates Zn very

strongly (Champagne, 1988; Persson *et al*., 1998) and the reduction in phytate should increase the availability of Zn for absorption. This was not found to be the case in the small intestine suggesting that Zn absorption is tightly regulated. The naturally low Zn absorption levels reported by Adeola (1995), may indicate that there is a limit on intestinal zinc transport, though this could be breed dependent since there is a known mutation in the porcine Zip4-like zinc transporter used in the current study that results in differences in Zn absorption ability (Siebert *et al*., 2013). It is possible that phytase may be more effective at influencing Zn absorption in certain breeds but this has not been well characterised.

5.4.4. Bone structure and mineralisation

Bone ash content in the third metacarpal is known to be strongly associated (R^2=0.91) with the Ca content of the diet (Kääntee, 1983), of which the level was held static in the current study. Regardless, bone ash increased significantly with phytase dose from ~34 % in the third and fourth metacarpals from the unsupplemented diet, to a maximum response at 1000 FTU/kg dietary phytase of ~39 %, and a decline to ~36 % at higher phytase doses (Figure 5.1). These levels are consistent with other studies; for example Yáñez *et al*. (2013) reported bone ash contents of 36 % in phytase untreated third metacarpals, increasing to 38 % with 500 FTU/kg phytase. Ash content of metatarsals, in similar age pigs, have also been shown to increase to ~31 % with 2000 FTU/kg supplemental phytase, compared to ~27.5 % in 0 phytase control (Pagano *et al*., 2007). The quadratic response may be related to the ATTD of Ca which, while not significantly affected by phytase dose *per se*, exhibited a quadratic tendency peaking at 1000 FTU/kg phytase and then reducing at higher phytase inclusion levels (p=0.05, Table 5.11). Despite this increase in ash content, the mineral composition of the ash remained consistent regardless of phytase addition (Table 5.13) with a mean Ca:P of 1.95 (SD=0.008). This was very similar to the data on metatarsal minerals (with up to 2000 FTU/kg phytase) published by Pagano *et al*. (2007), who also did not find significant phytase effects on Ca, P, Mg, Fe or Zn composition.

Increased mineralisation does not always mean improved structural integrity (NRC, 2012), as noted in this chapter with the increase of bone ash but no resulting change in shear strength (Table 5.12). This may partly be down to the way in which bone strength is assessed and reported. The last major review on the topic was published by Crenshaw *et al*. (1981) and noted that many authors were incorrectly confusing bone bending moment (a measure of force required before structural failure, adjusting for bone length) with bone strength (force per unit of area). While the former allows some adjustment for different bone lengths, only the latter takes into account different shapes of bone. Although these techniques have been well evaluated and reported, there continues to be inconsistencies

in reported methods with a database search (Web of Science with the terms: bone, strength, pig*, swine) from 2010 to 2016 returning 69 hits, of which 13 had used bone strength as a response criterion to nutrition. Of these, only two had investigated techniques other than 3-point bending. In this chapter, bone strength is reported as a modified version of the shear strength proposed by Combs *et al*. (1991), where shear strength is defined as maximum shear force (N) divided by the total area of the bone at the shear site. This gives a measure independent of the length of the bone.

Bone is comprised of a complex biphasic polymer containing collagen and mineral crystals. The use of shear or flexure (3-point bending) techniques may not therefore be sufficient to describe the mechanical properties adequately for use in nutrition. Nikodem *et al*. (2012) proposed a range of mechanical tests on pig femur bones to address this problem using different sources of Ca and P. These included compression strength and assessments of hardness. Given that phytase increases the bone mineralisation (Table 5.11), compression testing is likely to be a more sensitive method for identifying differences due to mineralisation, as there is less emphasis on shearing collagen fibres. Indeed, Figure 5.2 confirms this and shows that phytase quadratically increased bone compression strength up to the 2000 FTU dose tested. The peak bone ash content corresponds to the peak compression strength at a phytase level of 1000 FTU/kg, which was not detectable with the assessment of shear strength alone.

5.4.5. Intestinal health and histomorphology

Intestinal histomorphology in relation to dietary phytase supplementation has not been well characterised in the pig. Woyengo *et al.* (2011) did however find that the addition of dietary phytic acid reduced jejunal crypt depth while having no effect on villus height or villi – crypt ratio. In the current study there is a tendency ($p=0.076$) that dietary phytase addition linearly reduced jejunal villi – mucosal gland ratio, possibly because of the linear increase in mucosal gland length (although the effect of phytase was not statistically significant, $p=0.232$, there was a significant linear contrast at $p=0.02$), and a lack of any effect on villus height. The increase in mucosal glands should result in more secretions into the gut lumen, which was assessed through the measurement of sialic acid in digesta (data not shown). There did indeed tend to be a weak positive correlation ($r=0.29$, $p=0.06$) between jejunal villi to mucosal gland ratio and the concentration of sialic acid as measured in ileal digesta. This may have been more in response to the slight Th1 polarisation noted in the upper section of the intestine which suggests some gut inflammation, of which mucin production would be an innate response. In general though, the cytokine levels calculated per reaction

reported in Table 5.20 were very low despite the good quality RNA (checked for purity by nanodrop and integrity by Bioanalyzer) and PCR reaction conditions. This data was normalised to GPI and GSR which had previously been identified as a suitable combination of reference genes through a pilot geNorm analysis (see section 3.6.2).Taking both studies together, it is likely that phytase may be alleviating the negative effect of phytic acid on crypt and mucosal gland length, rather than any influence of phytase causing extended villi. Further work may therefore be needed to ascertain the effects of phytic acid and phytase on intestinal histomorphology. This may include assessment of the expression of a number of mucin genes to confirm secretions are elevated and assessment of B cells and antibody levels to exclude inflammation as the cause.

5.5. Conclusions

In conclusion, the addition of a third generation *E. coli* derived phytase (expressed in *Trichoderma reesei*) fed to newly weaned pigs in this study was effective at phytate hydrolysis, resulting in an 84 % digestion of ileal digesta phytic acid concentration at the highest dose of 32000 FTU/kg. Super dosing (>2000 FTU/kg) of this phytase significantly improved pig weight gains in the first two weeks post-weaning over unsupplemented pigs, resulting in pigs that were 11 % heavier at the end of the three week trial period. Pigs fed diets containing the mega dose of 32000 FTU/kg did not show any better growth performance than those fed the super dose of 8000 FTU/kg, suggesting a plateau had been reached between these ranges. It is likely that the observed effects are not related to any additional release of phosphorous from the hydrolysis of phytic acid, as there were no significant effects on growth performance or phytic acid breakdown patterns detected between the 0 FTU/kg added phytase dose and a high digestible phosphorus control. Phytase supplementation of 500 FTU/kg was sufficient to increase ATTD of P from 37 % to 50 % but there were no further benefits beyond this phytase level, though increasing the dose to 8000 FTU/kg resulted in maximum phytate digestibility. Maximum bone mineralisation and subsequent compression strength was met by the supplementation of a super dose of 1000 FTU/kg dietary phytase. Recommendations for phytase supplementation in weaner pig diets based on these results would be feeding a super dose of 8000 FTU/kg for the first two weeks post-weaning to maximise growth performance and the hydrolysis of phytate in the small intestines; reducing to 1000 FTU/kg in the third week post-weaning to facilitate bone mineralisation.

CHAPTER SIX

Interaction of dietary phosphorus, phytase and pharmaceutical ZnO on growth performance and health status of the weaner pig

6.0. Introduction

The addition of zinc in animal feeds has been under scrutiny from the EU for some time due to the potential environmental consequences of soil and water contamination arising from animal waste (SCAN, 2003). Although physiological requirements for zinc in pigs is relatively low (estimated to be 100 mg/kg of diet (90% DM) for piglets, decreasing to 80 mg/kg post-weaning and even lower in growing pigs (50 mg/kg) (NRC, 2012)), supplementary levels of up to 250 mg/kg of complete feed have been authorised by the EU since 1970 (Council Directive (EEC) No 70/524/EEC). This limit was reviewed and reduced to 150 mg/kg of feed in 2003 (Commission Regulation (EC) No 1334/2003) and the definitions updated in 2016 (Commission Implementing Regulation (EU) No 2016/1095), in line with reported typical industry practices (FEEDAP, 2014). Despite this, ZnO supplementation of between 2000 and 6000 mg/kg were noted as reportedly having therapeutic effects by the Scientific Committee for Animal Nutrition as far back as 2003, although they were not wholly convinced (SCAN, 2003). In 2006 though, the EU ban on antibiotic growth promoters (Commission Regulation (EC) No 1831/2003) came into effect, so high levels of ZnO as a pharmaceutical compound in pig diets remained wide-spread to try and mitigate post-weaning diarrhoea (PWD) in the absence of routine antibiotic medicated feed. Case studies reviewed by the European Commission indicated that ZnO inclusion at 2500 mg/kg of feed are still commonly used in at least Belgium and Denmark (EC, 2015). Other countries such as France and Germany, however, take a different view and do not allow the use of ZnO as a Veterinary Medicinal Product (VMP) for pigs, even though it is authorised in the EU (FVE, 2014).

In the UK, ZnO is marketed for use in pigs by two companies: DSM Nutritional Products (UK) Ltd and Provimi Ltd. It is authorised as a VMP under the trade names PigZin Premix (Vm 19108/4000) and ZincoTec® (Vm 03941/4000) to combat PWD (Mounsey, 2016). While it is generally recognised as clinically effective against PWD (FVE, 2014) and an effective growth promoter (Sales, 2013), it is noted by the VMD (Veterinary Medicines Directorate) that the precise action of ZnO in the treatment and control of diarrhea in pigs has not been fully determined (VMD, 2000). Reducing the particle size of ZnO to below 100 nm, the so-called nano-scale, has however been shown to enhance the antimicrobial properties of the compound, due to the larger surface area available for surface reactions causing damage to the bacterial cell membrane (Liu *et al*., 2009; Sirelkhatim *et al*., 2015). In this form, ZnO

nanoparticles (ZnO-NP) have potent antibacterial activity against *Campylobacter jejuni* (0.05 mg/ml) but are less effective (8 – 16 fold lower activity) against *Escherichia coli* O157:H7 and *Salmonella enterica Serovar enteritidis* (Xei *et al*., 2011). The level for complete inhibition of *Escherichia coli* O157:H7 reported by Xei *et al*. (2011) of 0.80 mg/ml is similar to that previous reported by Liu *et al*. (2009) of 0.98 mg/ml ZnO-NP, suggesting the compound is an effective antibacterial agent, at least *in vitro*.

Experiments in pigs have also shown that high doses of regular pharmaceutical grade ZnO (3000 mg/kg feed) can modulate intestinal bacterial communities (Vahjen *et al*., 2010) and in particular is effective at reducing post-weaning colonisation of enterobacteria for up to eight days post-weaning, before levels of bacteria in low zinc diets naturally equalise (Vahjen *et al*., 2016). Similar results have been reported by Jensen-Waern *et al*. (1998) with a significantly lower number of enterococci excreted at three days post-weaning only in 2500 mg/kg feed ZnO supplemented pigs but no overall difference over the course of the 63 day study between control and ZnO groups. There is also direct evidence of improved growth rates following an Enterotoxigenic *Escherichia coli* Serotype K88 (ETEC-K88) challenge on outdoor reared pigs supplemented with 3100 mg/kg ZnO verses a non-supplemented control (but no difference between indoor reared pigs under the same treatments) and reduced intestinal inflammation (Sargeant *et al*., 2010). Zinc oxide also has confirmed efficacy both *in vitro* and *in vivo* against *Campylobacter coli* at concentrations of 0.21 mg/ml and in feed at 3100 mg/kg ZnO (Bratz *et al*., 2013), though it shows no action against viruses such as PPRSV (Chai *et al*., 2014). In line with this data, the normal recommended use of pharmaceutical ZnO inclusion is on a 14 day program post-weaning (FEEDAP, 2014; FVE, 2014).

Despite the effectiveness of pharmaceutical doses of ZnO noted in the majority of studies, there are reports of ineffectiveness to reduce the numbers of Enterobacteriaceae and *Clostridia* in both digesta and faecal samples taken post-weaning (Li *et al*., 2001). Also, not every form of zinc shows therapeutic action. For example, Šperling *et al*. (2014) did not find any action of zinc chelate at 250 ml/1000 ml water against a challenge of *B. hyodysenteriae* to which an antibiotic (valnemulin) was effective. In addition, there are risks with continued use of ZnO, such as concerns over antimicrobial resistance and environmental issues. Recent work by Slifierz *et al*. (2015) demonstrated that methicillin-resistant *Staphylococcus aureus* (MRSA) can be quite tolerant to therapeutic ZnO in post-weaning pigs, with increases in prevalence and persistence in ZnO treated groups verses low zinc controls. This may lead to reservoirs of antimicrobial resistant bacteria on farm, posing a risk to public health. Furthermore, pharmacological ZnO supplementation may interact with dietary minerals such as impacting phosphorus digestibility, so that consideration must be given to

increasing dietary phosphorus or including a phytase enzyme in ZnO medicated feeds (Walk *et al*., 2015).

Phytic acid ($IP_{6)}$ is of particular concern in the context of supplemental zinc because it is a reactive molecule, normally found as a salt (phytate) chelated to divalent cations, including magnesium and especially, zinc. Exogenous phytase activity to hydrolyse phytate needs to primarily occur in the stomach, due to the solubility of phytate at low pH (Schlemmer *et al*., 2001). If the activity is not enough, phytate complexes may precipitate in the small intestines and lock up nutrients. In fact, Zn dependent phytase has been reported to be localised to the brush boarder membrane of the duodenum of the rat (Davies and Flett, 1978), suggesting this is an important site for phytase and Zn interactions. It is well known that phytase addition is effective at releasing chelated zinc from phytate and can therefore be used to reduce supplemental zinc inclusion levels at normal phytase doses (Revy *et al*., 2006; Schlegal *et al*., 2013). At therapeutic levels however, there is evidence that 1500 mg/kg ZnO or zinc chloride can reduce the efficacy of phytase at releasing phytate-P by decreasing the availability of phytate for hydrolysis by phytase (Augspurger *et al*., 2004). High levels of ZnO have also been demonstrated to modulate blood serum calcium and phosphorus levels, indicating there may be Ca-Zn-P precipitation (Walk *et al*., 2013), as well as reducing P, K and Na apparent total tract digestibility (Walk *et al*., 2015). Phytase has also been shown to significantly interact with pharmaceutical levels of ZnO in the intestinal mucosa to increase the relative abundance of metallothionein (MT) mRNA, especially at levels of 2000 mg/kg diet ZnO (Martínez *et al*., 2004). This may in part suggest that phytase can modulate the quantities of zinc entering the blood. Routine metaphylactic use of ZnO in weaner pigs may therefore not be advisable without due consideration to phytase supplementation strategies.

Recent research has suggested that super doses of phytase enzymes may be alternatives to high levels of zinc supplementation post-weaning in healthy pigs due to its growth promoting effects (Walk *et al*., 2013). This was certainly shown to be the case in chapter five, where extra phosphoric growth rates were 92 % higher in the first week post-weaning with a 2000 FTU/kg of feed dose verses a non-phytase supplemented diet (Table 5.8). There would be a clear benefit of substituting routine therapeutic ZnO use in weaner pig feeds with super doses of phytase from both an environmental and antimicrobial resistance where there is no clinical reasoning for medicating the feed. Given the reported interactions between pharmaceutical ZnO and phytase supplementation, further research is needed to investigate the role of phytase super dosing in conjunction with ZnO medicated feeds.

6.1. Objective

The aim of this chapter is to assess the role of super dosing phytase in conjunction with feeding pharmaceutical ZnO to weaned pigs under non-challenge conditions by assessment of growth performance and systemic/intestinal health. Furthermore, this chapter will investigate the interaction of high levels of ZnO on the requirement for dietary digestible phosphorus post-weaning, for optimising growth performance. The general hypotheses under test in this chapter are:

1. Increasing dgP from 2.8 g/kg to 5.8 g/kg will not significantly increase growth performance above that of those fed the lower rate (confirming the results of chapter four).
2. Increasing phytase supplementation from 0 FTU/kg to 2500 FTU/kg will significantly increase the growth performance of weaned pigs up to three weeks post-weaning (confirming the results of chapter five).
3. There will be no significant interaction between the dose of supplemental phytase and the level of dgP in the diet, since increasing dgP above 2.8 g/kg diet does not lead to a growth response.
4. The inclusion of pharmaceutical ZnO will not significantly improve pig growth performance under non-challenge conditions.
5. There will be a significant interaction between ZnO inclusion and phytase inclusion on pig growth performance, since ZnO has been shown to reduce phytase efficacy.
6. There will be a significant interaction between ZnO inclusion and dgP level on pig growth performance, since ZnO has been shown to precipitate with Ca/P, forming insoluble complexes.
7. There will be a significant dose response to increasing dgP level in ZnO medicated diets on pig growth performance parameters, since ZnO has been shown to precipitate with Ca/P, forming insoluble complexes.
8. There will be no significant effects of any treatment or combination of treatments on systemic and gut health/immune markers (tested as a null hypothesis).

6.2. Materials and methods

General materials and methods relating to this section can be found in chapter three. All experimental procedures were approved by Harper Adams University Research Ethics Committee and in compliance with the Animals (Scientific Procedures) Act 1986. Approval for using Quantum Blue phytase in this trial was granted by the Food Standards Agency (FSA). Two experiments were carried out and reported in this chapter.

6.2.2. Treatments and trial designs

The first experiment (designated trial SM3) comprised of a 2 x 2 x 2 factorial design to test the main effects of digestible phosphorus (dgP) at 2.8 g/kg of feed (low) and 5.8 g/kg of feed (high), effect of phytase at either 0 FTU/kg feed or 2500 FTU/kg feed (super dose) and the effect of pharmaceutical ZnO at either 0 mg/kg or 3100 mg/kg feed (medicated) on growth performance, and to allow assessment of the interactions (Table 6.2). The two levels of dgP selected represented the lowest dose from chapter four (to which there was no further increase in growth performance when this level was increased) and the mid-range level of 5.8 g/kg feed dgP, which was also the level in the basal diet for chapter five. To test the hypothesis that in the presence of high levels of phytase and ZnO there would be a dose response to increasing dgP (unlike chapter four when neither were supplied), two further treatment groups were included in the design, comprising of 3.8 g/kg dgP of feed (with inclusion of 2500 FTU/kg phytase and 3100 mg/kg ZnO) and 4.8 dgP g/kg of feed (with inclusion of 2500 FTU/kg phytase and 3100 mg/kg ZnO). This provided an additional dose response design with graded levels of dgP (2.8, 3.8, 4.8 and 5.8 g/kg feed) in the presence of 2500 FTU/kg phytase and 3100 mg/kg ZnO (Table 6.3).

The main effect of phytase should confirm the data in chapter five, where improvements in growth rates for the first two weeks post-weaning should be evident with super dosing. If confirmed, this would indicate that Quantum Blue phytase is an efficacious zootechnical growth promoter when added at 2500 FTU/kg diet for the first 14 days post-weaning. Dietary inclusion of pharmaceutical ZnO should also have growth promoting ability at similar or higher levels to super dosed phytase. This may not be the case in healthy pigs not suffering from PWD (which should be the norm) but the hypothesis that there should not be any adverse effects for ZnO supplementation will be tested. In particular, testing for interactions with phosphorus and phytase is of interest in this experiment due to the emerging evidence of complex precipitations. Further assessment of super dosing phytase and ZnO was assessed in the second experiment.

The second trial (designated trial SM5) was a 2 x 2 factorial design based on the 5.8 g/kg dgP diet with and without phytase (0 or 2500 FTU/kg) and with and without pharmaceutical ZnO (0 or 3100 mg/kg). The aim of this trial was to assess gut health and immune response. Digestible phosphorus in the diets was provided from dicalcium phosphate to the formulated levels required. The phytase used in both experiments was a third generation *Escherichia coli* derived 6-phytase enzyme (EC 3.1.3.26) expressed in *Trichoderma reesei* (Quantum Blue, AB Vista Feed Ingredients, Marlborough, UK), as used in chapter five. Zinc oxide was

supplied as PigZin Premix (Vm 19108/4000) at a dose rate of 3100 mg/kg complete feed, to provide 2500 mg/kg zinc.

6.2.1. Animals

In experiment one (SM3), 400 commercial type pigs (JSR 800 sire line and PIC 337 sire line genotypes) from two farrowing batches were sourced from the Harper Adams University high health status herd at weaning (~26 days of age) and balanced 50:50 across treatments for genotype, sex and selection weight. Animals were housed in pens of five pigs under commercial indoor conditions. Each pen represents one experimental unit (replicate). Statistical power was assessed prior to the experiment based on the procedure of Berndtson (1991), to detect a 10 % difference, with 80 % power, at the 95 % level of probability. Treatments were assigned to pens using a random number generator in Excel 2013 (Microsoft Corporation), to provide a total of eight replicates (pen of five pigs) per treatment group.

In experiment two (SM5), 64 commercial type pigs (JSR Genpacker 90 X JSR 900) were weaned at 26 days of age and vaccinated against *Mycoplasma hyopneumoniae* (M+PAC™, Intervet UK Ldt, Milton Keynes, UK) and porcine circovirus virus type 2 (Ingelvac CircoFLEX®, Boehringer Ingelheim Vetmedica GmbH, Germany). Pigs were weighed one day prior to weaning to establish single sex pens of four pigs (mean weight 8.3±0.1 kg), equally split between two buildings (see section 3.1.5.). Treatments were assigned to pens using a random number generator in Excel 2013 (Microsoft Corporation), to provide a total of four replicates (pen of four pigs) per treatment group (two replicate pens per building).

6.2.3. Diets

In experiment one (SM3), pigs were fed one of ten treatment diets (Table 6.1) for 22 days. All diets were isoenergetic and isonitrogenous. In the second experiment (SM5), pigs were fed diets E – J only, for 23 days. All diets were based on the formulation used in experiments SM1 and SM2, including 3 g/kg TiO_2 as an inert marker. Diets were analysed for their nutrient content by Sciantec Analytical (Stockbridge Technology Centre, Cawood, North Yorkshire, YO8 3SD) and phytic acid / phytase was determined as specified in section 3.2.

Table 6.1: Composition of raw ingredients (g/kg) for all experimental pig diets fed in experiments SM3 and SM5

Ingredients	A	B	C	D	E	G	H	J	K	L
Micronized barley	76	76	76	76	76	76	76	76	76	76
Raw whole wheat meal	173	173	173	173	159	159	159	159	169	164
Micronised wheat meal	173	173	169	168	159	159	155	154	163	159
Micronised maize	25	25	25	25	25	25	25	25	25	25
Supertherm oats	50	50	50	50	50	50	50	50	50	50
Herring meal	31	31	31	31	31	31	31	31	31	31
Hypro soya	100	100	100	100	100	100	100	100	100	100
Full fat soyabean	122	122	122	122	122	122	122	122	122	122
Premix[1]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Skimmed milk powder	25	25	25	25	25	25	25	25	25	25
Delactosed whey	25	25	25	25	25	25	25	25	25	25
Whey powder	123	123	123	123	123	123	123	123	123	123
Sugar/sucrose	13	13	13	13	13	13	13	13	13	13
L-lysine HCL	5.1	5.1	5.1	5.1	5.2	5.2	5.2	5.2	5.2	5.2
Dl-methionine	2.6	2.6	2.6	2.6	2.7	2.7	2.7	2.7	2.6	2.6
L-threonine	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
L-tryptophan	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
L-valine	1.2	1.2	1.2	1.2	1.3	1.3	1.3	1.3	1.2	1.3
Crina 693[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin E	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Quantum blue phytase	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.5	0.5
Sucram®[3]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Benzoic acid (VevoVitall)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Pigzin (zinc oxide bp)	0.0	0.0	3.1	3.1	0.0	0.0	3.1	3.1	3.1	3.1
Limestone flour	5.2	5.2	5.2	5.2	2.6	2.6	2.6	2.6	4.3	3.4
Dicalcium phosphate 18	0.0	0.0	0.0	0.0	21.8	21.8	21.8	21.8	7.3	14.5
Soya oil	32.8	33.0	34.2	34.4	41.5	41.7	42.9	43.1	37.3	40.2

[1]Premix detailed in chapter three; [2]Flavouring made from essential oils manufactured by DSM Nutritional Products (UK) Ltd; [3]Sweetener manufactured by Pancosma SA

6.2.4. Statistical analysis

Data handling and calculations were performed in Excel 2013 (Microsoft Corporation) and statistical analysis was performed using GenStat 16th edition (VSN International Ltd) statistical software. Summary statistics were used to check data for outliers (<> 3 SD) and normality. Statistical significance was accepted at the 95% level and trends were reported at the 90 % level. Experiment one (SM3) was analysed as a randomised block (batch) 2 x 2 x 2 factorial design by ANOVA for treatments A – J (Table 6.2), consisting of eight replicates (pens of five animals) per treatment group.

Table 6.2: Factorial experimental design arrangement for experiment one (SM3), with digestible phosphorus (dgP g/kg of diet) at two levels, Quantum Blue phytase at two levels and PigZin Premix at two levels

	Diet A	Diet B	Diet C	Diet D	Diet E	Diet G	Diet H	Diet J
DgP	2.8	2.8	2.8	2.8	5.8	5.8	5.8	5.8
Phytase[1]	-	+	-	+	-	+	-	+
ZnO[2]	-	-	+	+	-	-	+	+

[1]phytase at either 0 FTU/kg diet (-) or 2500 FTU/kg diet (+); [2]PigZin Premix at either 0 mg/kg diet (-) or 3100 mg/kg diet (+)

Orthogonal polynomial contrasts blocked by batch were then used to test for linear and quadratic response to increasing dgP dose in the presence of phytase and ZnO (Table 6.3). This was followed by least mean squares regression blocked by batch to ascertain the parameters of the response. Eight replicates per treatment were used in the analysis.

Table 6.3: Dose response experimental design parameters for experiment one (SM3), with digestible phosphorus (dgP) at four levels, Quantum Blue phytase at 2500 FTU/kg diet and PigZin Premix at 3100 mg/kg feed

	Diet D	Diet K	Diet L	Diet J
DgP (g/kg)	2.8	3.8	4.8	5.8
Phytase	+	+	+	+
ZnO	+	+	+	+

In experiment two (SM5), growth performance and blood data was analysed as a 2 x 2 factorial design in GenStat 16th Edition (VSN international Ltd). Significance was accepted at the 95 % level and trends reported at the 90 % level. For growth performance data, the pen of four pigs was considered the experimental unit. For haematology and cytokine analysis, the average values of the pair of two pigs from the same pen was considered the experimental unit.

6.2.5. Growth performance

In experiment one, the only parameters for assessing the effects of the treatment groups was considered to be growth production performance. Individual pigs were weighed at weaning, day seven, 14 and 22 post-weaning, while feed intake was measured on a whole pen basis at each of these time points. Feed conversion ratio (FCR), daily liveweight gain (DLWG) and daily feed disappearance (DF) were calculated for each of these three periods respectively. Period one was regarded as an acclimatisation period and the results interpreted appropriately. Growth performance was also assessed in experiment two but haematological and intestinal health parameters were also measured.

6.2.6. Haematology

Assessment of systemic health status was conducted through measurements of total white blood cell count (WBC) and subsequent differential quantification of basophils, eosinophils, neutrophils, monocytes and lymphocytes. In experiment two only, blood was collected from 08:00 – 09:00 by jugular venepuncture at day 12 and 13 post-weaning into a 4.0 ml K_2EDTA coated BD Vaccutainer® from two mean weight pigs per treatment pen. The treatment order of blood sampling was randomised but balanced so that two pens per treatment were sampled on each day. Whole blood analysis, including WBC differentiation, was performed in technical duplicates using an $MS4_5$ impedance counter blood analyser (Melet Schloesing Laboratories, Osny, France), validated with a normal level control sample (Woodley Equipment Company Ltd, Lancashire, UK) (see section 3.4.1 for further details).

6.2.7. Collection of intestinal samples and cDNA preparation

Over days 22, 23 and 24 post-weaning, two mean weight pigs from each pen were euthanized by captive bolt followed by destruction of the brain stem. Following a vertical midline incision, the small intestines were clamped at the junction of the stomach/duodenum and the terminal ileum/colon, before being removed and carefully spread out on a clean surface. Plastic cable ties were used to divide the intestines into three equal sections representing the duodenum, jejunum and ileum. A 2 CM sample was cut from each section and immediately stabilised in RNA*later*® (Sigma-Aldrich, St. Louis, Missouri, USA), prior to storage at -80 °C for five months. RNA from ~20 mg of whole intestinal samples from the duodenum and ileum were extracted, purified and reverse transcribed according to the procedure detailed in section 3.6.3.

6.2.8. Determination of reference genes and SYBR green assays

Reference genes for normalisation of data were selected using the geNorm™ method described in section 3.6.1. Immune status of the intestines was assessed by measuring the expression levels of key pro-inflammatory cytokines in the duodenum and ileum section of the small intestine using RT-qPCR (see section 3.6.3). The cytokines chosen differed from those selected in chapter five, since IL-2, IL-4 and IL-17A were designed to indicate the response pattern (Th1, Th2 or Th17,) whereas IFN-γ, IL-6 and TNF were selected in this chapter as markers of inflammation only. Challenge from bacteria and viruses to the intestine should elicit a pro-inflammatory response through the action of cytokines, such as the three selected in this chapter. Elevated levels would therefore indicate poor gut health and would allow assessment of the dietary treatments, in particular the effectiveness of pharmaceutical ZnO and super doses of phytase. For the gene expression assays, eight biological replicates per treatment group were analysed in duplicate technical replicates at

the qPCR stage. These were run under the same conditions as specified in the geNorm procedure with the exception that each 20 µl reaction was loaded with 25 ng of cDNA. Two reference genes (GSR and GPI) and three genes of interest (IFNG, IL-6 and TNF) were run on individual plates, each containing a complete set of samples and controls (Table 6.4).

Table 6.4: Assay details for qPCR primers designed to detect IFNG, IL-6 and TNF mRNA transcripts from pig (Sus Scrofa) intestinal tissue in experiment SM5

Gene symbol	Gene	Primer sequence	Product length	Accession number
IFNG	Interferon-gamma	S – AAGAAATAACGATCCTAAAGGACTAT A – TGTCACTCTCCTCTTTCCAATT	108	NM_213948
IL6	Interleukin 6	S – CTATGAACTCCCTCCACAAG A – GCAGTAGCCATCACCAGAAG	76	NM_214399
TNF	Tumour necrosis factor	S – TCCCCTGTCCATCCCTTTATTA A – TTCTAAGTGTTGCTGTTGTTGTT	130	NM_214022

S = Sense primer, A = Anti-sense primer (Source: PrimerDesign Ltd, 2014)

In all cases, specificity of the target was confirmed by a unified peak in the melt curve at the expected amplicon temperature. The presence of gDNA was excluded by no template controls (NTC), and RT-negative controls. geNorm™ analysis was performed using the procedure of Hellemans *et al*. (2007) in qBase+ software (Biogazelle NV, Belgium). For the SYBR assays, raw fluorescence data without baseline correction was exported from CFX manager (Bio-Rad, Hemel Hempstead, UK) into LinRegPCR (version 2014.2), where Cq values were set by amplicon group and PCR efficiencies calculated using a four point window-of-linearity (Ramakers et al, 2003; Ruijter et al, 2009). Calculated Cq values were converted into normalised (against GPI and GSR) relative quantities (NRQ) to the control group, in qBase+ (Hellemans *et al*, 2007), adjusting for PCR amplicon group efficiency. Technical replicates were excluded from data analysis if they were in excess of 0.7 cycles apart. NRQ were compared to the control group by comparison contrasts in GenStat (16th Edition).

6.3. Results – experiment one

All live pigs in the first trial were assessed by a qualified veterinary surgeon on completion of the trial period and showed no signs of adverse long term effects from treatments. Four pigs were removed due to injuries (mostly shoulder problems) or poor growth during the course of the trial and one pig died of a suspected heart attack. All data was adjusted based on the number of pig days completed.

6.3.1. Diet analysis

Feed sample analysis (Table 6.5) confirmed that phytase and zinc were added to the diets correctly. Total phosphorus measured was as expected with a step change of ~1 g/kg but levels of phytic acid were approximately twice those reported in chapter five. The ratio of Ca to tP was slightly higher than formulated at between 1.21 and 1.44 (verses 1.14 to 1.16), but still within tolerance.

Table 6.5: Analysis of phytic acid, phytate-P, P, Ca (g/kg as fed basis), phytase activity (FTU/kg), zinc (mg/kg) and macro nutrients (%) of experimental diets fed to pigs in trials SM3 and SM5

Diet	A	B	C	D	E	G	H	J	K	L
Measured phytic acid	12.8	12.5	12.3	11.9	13.3	13.1	13.4	13.4	12.4	12.8
Calculated phytate P	3.6	3.5	3.5	3.4	3.8	3.7	3.8	3.8	3.5	3.6
Formulated total Ca	5.7	5.7	5.7	5.7	10.3	10.3	10.3	10.3	7.2	8.8
Measured total Ca	7.2	7.1	7.2	7.1	11.1	10.2	10.9	10.8	8.2	8.7
Formulated total P	5.0	5.0	5.0	5.0	8.9	8.9	8.9	8.9	6.3	7.6
Measured total P	5.0	5.3	5.4	5.3	8.4	8.1	8.9	8.9	6.3	7.0
Formulated Ca:tP	1.14	1.14	1.14	1.14	1.16	1.16	1.16	1.16	1.15	1.15
Calculated Ca:tP	1.44	1.34	1.33	1.34	1.32	1.26	1.22	1.21	1.30	1.24
Measured Zn	140	137	3213	2852	147	130	3093	3401	3058	2801
Expected Zn[1]	24	24	2524	2524	24	24	2524	2524	2524	2524
Measured phytase	0	2030	0	2600	0	2560	85	3210	2900	2830
Expected phytase	0	2500	0	2500	0	2500	0	2500	2500	2500
Crude Protein, %	18.5	18.9	19.0	18.8	18.8	18.2	18.5	18.5	18.8	18.9
Crude fibre, %	2.0	2.3	1.9	1.9	1.8	2.1	2	2.3	2.1	1.9
Moisture, %	8.8	9.0	8.8	8.7	8.6	8.5	8.6	8.8	8.9	8.5

[1]Not including native Zn from feed ingredients

6.3.2. Effects of increasing dgP on performance

Growth rates were good and comparable with those in chapters four and five, with overall DLWG between 300 and 400 g. In the first two weeks post-weaning there were no significant effects on pig performance observed from increasing dietary phosphorous in the presence of exogenous phytase and ZnO. Three weeks post-weaning however, increasing dietary tP linearly (p=0.004) improved DF (p=0.012) with a quadratic tendency (p=0.091) (Table 6.6). DLWG also increased (p=0.001) in a linear (p=0.002) and quadratic (p=0.01) fashion, while FCR was not effected by tP dose (Table 6.6).

Table 6.6: The effects of increasing dietary phosphorus on week three post-weaning pig growth performance

tP (g/kg feed)	5.3	6.3	7.0	8.9	S.E.D.	p-value	Linear	Quadratic
DLWG (g)	405	466	535	511	31.0	0.001	0.002	0.010
DF (g)	606	666	713	718	35.1	0.012	0.004	0.091
FCR	1.54	1.44	1.34	1.41	0.092	0.221	0.206	0.109

The quadratic equations for the lines were best fitted as polynomial models for each batch individually. They show that both DLWG and DF could be explained by the level of tP ($p<0.001$ & $p=0.002$ respectively), accounting for 47.4 % and 41.5 % of the variation respectively (adjusted r^2). These effects were strong enough that despite no detectable dose response in the first two weeks post-weaning, over the whole three weeks, increasing dietary phosphorus in the presence of exogenous phytase and zinc increased overall DLWG ($p=0.017$) in a linear ($p=0.019$) and quadratic ($p=0.036$) fashion (Figure 6.1).

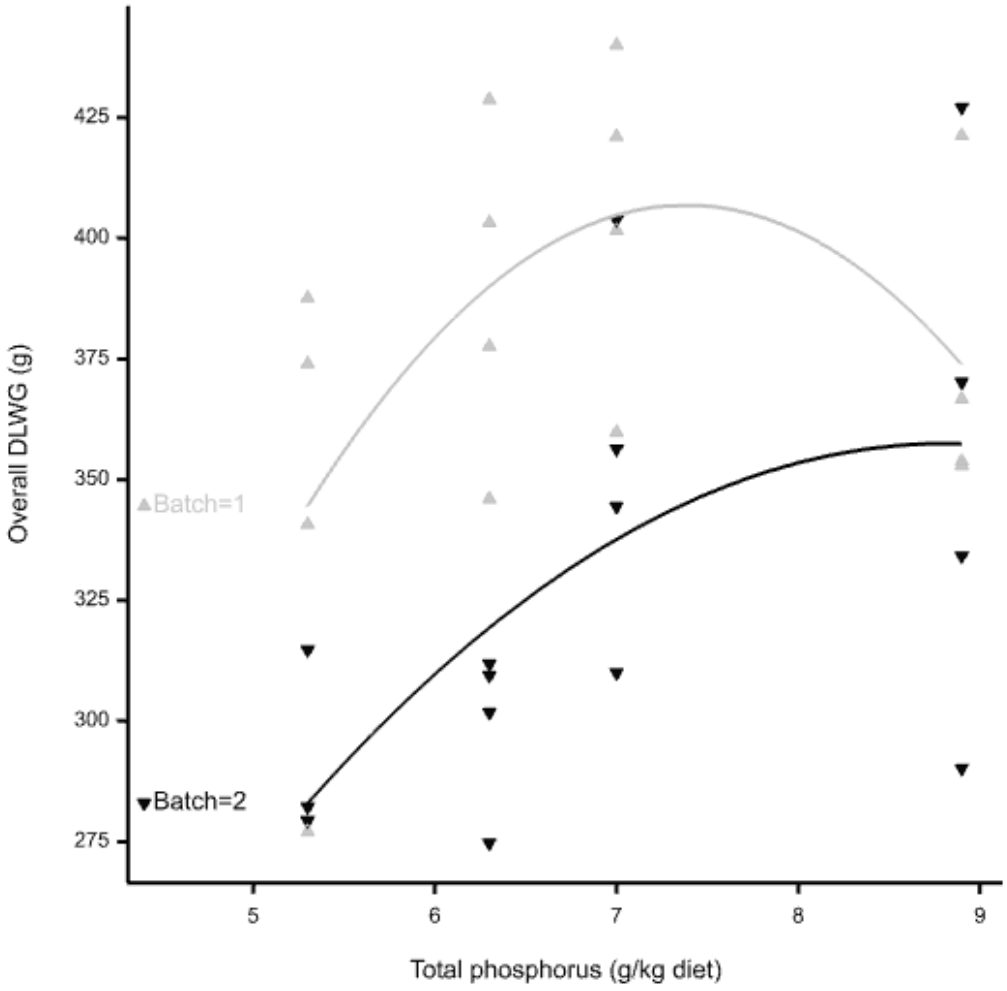

Figure 6.1: The effects of increasing dietary phosphorus in diets containing 2500 FTU/kg phytase and 3100 mg/kg pharmaceutical ZnO on overall weaner pig performance from weaning to three weeks post, split by batch. Data points represent mean averages of pens of five pigs. Standard error = 37.6.

There was a clear batch effect but fitting individual lines to each batch only marginally improved the adjusted r^2 from 43.8 % to 45.2 % compared with fitting parallel lines (Figure 6.1). Both batches show that with inclusion of both a super dose of phytase and therapeutic levels of ZnO, overall DLWG demonstrated a quadratic response to the level of dietary tP ($p<0.001$).

6.3.3. The effects of phosphorus, phytase and zinc on performance

Results of the factorial analysis are shown in Tables 6.7, 6.8, 6.9 and 6.10. Increasing dietary dgP from 2.8 to 5.8 g/kg improved both overall DLWG ($p<0.001$) and FCR ($p<0.001$) but only tended to increase DF ($p=0.065$) (Table 6.7). The addition of phytase was only efficacious on DLWG ($p=0.04$) and FCR ($p=0.02$) in the low dgP diet, as indicated by a dgP x phytase interaction (Table 6.7). Pharmaceutical ZnO depressed both overall DLWG ($p=0.002$) and FCR ($p=0.045$) as well as a tendency to reduce DF ($p=0.096$), when assessed over the entirety of the three weeks. Performance results taken seven days post-weaning (Table 6.8) show that ZnO suppressed both DF ($p<0.001$) and DLWG ($p=0.016$) early on in the trial. If phytase and ZnO were fed in combination, there was an interactive effect on FCR ($p=0.026$) and this also tended to occur with DLWG ($p=0.08$), with no additives giving the worst FCR and both on their own giving the best FCR.

Fourteen days post-weaning, pigs were still responsive to the higher level of dgP, showing a 0.05 point lower FCR ($p=0.05$) and tending to have a higher DLWG ($p=0.088$) (Table 6.9). Those pigs super dosed with phytase showed significantly enhanced DLWG ($p=0.032$) by ~20 g and improved FCR ($p=0.019$) by the end of two weeks post-weaning (Table 6.9). By the end of three weeks post-weaning (Table 6.10), DF was increased at the higher level of dgP ($p=0.015$) by ~50 g but phytase was still only effective in the low dgP diet only (dgP x phytase, p-value=0.039). Pharmaceutical ZnO inclusion however, tended to have a negative effect by the third week post-weaning on DF ($p=0.088$) but there was a strong interaction observed between level of dgP and ZnO ($p=0.006$), where those fed the low dgP diet supplemented with ZnO consumed 92 g/day less feed and grew over 100 g/day less (dgP x ZnO, p-value= 0.018) than those on the low dgP without ZnO. FCR was lower at the higher dgP inclusion rate compared to the lower dgP diet ($p<0.009$) independent of ZnO and the addition of ZnO independently resulted in an increased FCR ($p=0.034$).

Table 6.7: The effects of low (2.8 g/kg dgP) and high (5.8 g/kg dgP) dietary phosphorus, phytase (0 or 2500 FTU/kg) & pharmaceutical ZnO (0 or 3100 mg/kg) on overall pig performance up to three weeks post-weaning

Treatment factor	DF (g)	DLWG (g)	FCR
Digestible phosphorus			
Low	439.68	336.24	1.32
High	461.77	375.79	1.23
Phytase			
+ve	455.59	361.56	1.27
-ve	445.87	350.47	1.28
Zinc Oxide			
+ve	440.78	341.47	1.30
-ve	460.67	370.56	1.25
Pooled S.E.D. main effects	11.731	9.132	0.025
DgP vs Phytase			
Low DgP, no phytase	432.96	321.09	1.36
Low DgP + Phytase	446.40	351.40	1.28
High DgP, no phytase	458.77	379.85	1.21
High Dgp + phytase	464.77	371.72	1.25
DgP vs ZnO			
Low DgP, no ZnO	457.16	357.07	1.29
Low DgP + ZnO	422.20	315.42	1.35
High DgP, no ZnO	464.19	384.05	1.21
High DgP + ZnO	459.35	367.52	1.25
Phytase vs ZnO			
No phytase, no ZnO	448.91	358.72	1.26
No phytase + ZnO	442.82	342.23	1.30
Phytase, no ZnO	472.44	382.40	1.23
Phytase + ZnO	438.73	340.71	1.30
Pooled S.E.D. 2-way interactions	16.590	12.914	0.035
DgP vs Phytase vs ZnO			
Low DgP	436.77	328.23	1.34
Low DgP + Phytase	477.55	385.91	1.24
Low DgP + ZnO	429.14	313.96	1.37
Low DgP + Phytase + ZnO	415.26	316.88	1.32
High DgP	461.05	389.20	1.18
High DgP + Phytase	467.33	378.90	1.23
High DgP + ZnO	456.50	370.50	1.23
High DgP + Phytase + ZnO	462.20	364.55	1.27
Pooled S.E.D. 3-way interactions	23.462	18.263	0.050
Probabilities of differences			
DgP	0.065	<0.001	<0.001
Phytase	0.411	0.230	0.506
ZnO	0.096	0.002	0.045
DgP X Phytase	0.752	0.040	0.020
DgP X ZnO	0.205	0.175	0.791
Phytase X ZnO	0.244	0.173	0.674
DgP X Phytase X ZnO	0.254	0.111	0.508

Table 6.8: The effects of low (2.8 g/kg dgP) and high (5.8 g/kg dgP) dietary phosphorus, phytase (0 or 2500 FTU/kg) & pharmaceutical ZnO (0 or 3100 mg/kg) on pig performance in the first week post-weaning

Treatment factor	DF (g)	DLWG (g)	FCR
Digestible phosphorus			
Low	197.55	154.70	1.36
High	204.29	160.71	1.33
Phytase			
+ve	199.21	158.00	1.33
-ve	202.62	157.40	1.37
Zinc Oxide			
+ve	180.07	142.69	1.32
-ve	221.77	172.71	1.37
Pooled S.E.D. main effects	9.706	12.120	0.055
DgP vs Phytase			
Low DgP, no phytase	196.64	149.29	1.44
Low DgP + Phytase	198.46	160.11	1.29
High DgP, no phytase	208.61	165.52	1.30
High Dgp + phytase	199.96	155.89	1.36
DgP vs ZnO			
Low DgP, no ZnO	213.25	160.79	1.45
Low DgP + ZnO	181.86	148.61	1.28
High DgP, no ZnO	230.29	184.64	1.29
High DgP + ZnO	178.29	136.77	1.37
Phytase vs ZnO			
No phytase, no ZnO	216.79	161.61	1.49
No phytase + ZnO	188.46	153.20	1.26
Phytase, no ZnO	226.75	183.82	1.26
Phytase + ZnO	171.68	132.18	1.39
Pooled S.E.D. 2-way interactions	13.727	17.140	0.078
DgP vs Phytase vs ZnO			
Low DgP	205.14	139.71	1.67
Low DgP + Phytase	221.36	181.86	1.23
Low DgP + ZnO	188.14	158.86	1.21
Low DgP + Phytase + ZnO	175.57	138.36	1.34
High DgP	228.43	183.50	1.30
High DgP + Phytase	232.14	185.79	1.29
High DgP + ZnO	188.79	147.54	1.31
High DgP + Phytase + ZnO	167.79	126.00	1.44
Pooled S.E.D. 3-way interactions	19.413	24.239	0.110
Probabilities of differences			
DgP	0.491	0.622	0.686
Phytase	0.727	0.961	0.547
ZnO	<0.001	0.016	0.538
DgP X Phytase	0.592	0.403	0.174
DgP X ZnO	0.293	0.147	0.112
Phytase X ZnO	0.174	0.080	0.026
DgP X Phytase X ZnO	0.917	0.427	0.172

Table 6.9: The effects of low (2.8 g/kg dgP) and high (5.8 g/kg dgP) dietary phosphorus, phytase (0 or 2500 FTU/kg) & pharmaceutical ZnO (0 or 3100 mg/kg) on pig performance in the second week post-weaning

Treatment factor	DF (g)	DLWG (g)	FCR
Digestible phosphorus			
Low	443.1	384.6	1.16
High	448.2	408.5	1.11
Phytase			
+ve	449.3	411.7	1.10
-ve	442.0	381.4	1.17
Zinc Oxide			
+ve	455.0	401.2	1.14
-ve	436.3	392.0	1.13
Pooled S.E.D. main effects	14.74	13.77	0.029
DgP vs Phytase			
Low DgP, no phytase	441.9	366.1	1.22
Low DgP + Phytase	444.2	403.1	1.11
High DgP, no phytase	442.1	396.8	1.12
High Dgp + phytase	454.4	420.3	1.09
DgP vs ZnO			
Low DgP, no ZnO	428.3	383.5	1.13
Low DgP + ZnO	457.9	385.7	1.20
High DgP, no ZnO	444.3	400.4	1.12
High DgP + ZnO	452.2	416.6	1.09
Phytase vs ZnO			
No phytase, no ZnO	426.9	376.5	1.15
No phytase + ZnO	457.0	386.4	1.19
Phytase, no ZnO	445.6	407.5	1.11
Phytase + ZnO	453.1	415.9	1.09
Pooled S.E.D. 2-way interactions	20.85	19.47	0.040
DgP vs Phytase vs ZnO			
Low DgP	409.5	356.8	1.16
Low DgP + Phytase	447.0	410.3	1.10
Low DgP + ZnO	474.2	375.4	1.27
Low DgP + Phytase + ZnO	441.5	396.0	1.12
High DgP	444.4	396.1	1.13
High DgP + Phytase	444.2	404.7	1.11
High DgP + ZnO	439.8	397.4	1.11
High DgP + Phytase + ZnO	464.6	435.9	1.07
Pooled S.E.D. 3-way interactions	29.48	27.54	0.057
Probabilities of differences			
DgP	0.726	0.088	0.051
Phytase	0.619	0.032	0.019
ZnO	0.208	0.508	0.545
DgP X Phytase	0.737	0.625	0.189
DgP X ZnO	0.466	0.612	0.098
Phytase X ZnO	0.477	0.957	0.307
DgP X Phytase X ZnO	0.112	0.259	0.614

Table 6.10: The effects of low (2.8 g/kg dgP) and high (5.8 g/kg dgP) dietary phosphorus, phytase (0 or 2500 FTU/kg) & pharmaceutical ZnO (0 or 3100 mg/kg) on pig performance in the third week post-weaning

Treatment factor	DF (g)	DLWG (g)	FCR
Digestible phosphorus			
Low	650.12	453.2	1.46
High	700.36	536.6	1.32
Phytase			
+ve	687.82	497.3	1.41
-ve	662.66	492.5	1.37
Zinc Oxide			
+ve	657.95	463.6	1.45
-ve	692.53	526.2	1.33
Pooled S.E.D. main effects	19.938	17.32	0.052
DgP vs Phytase			
Low DgP, no phytase	633.04	432.4	1.47
Low DgP + Phytase	667.20	473.9	1.45
High DgP, no phytase	692.28	552.6	1.27
High Dgp + phytase	708.44	520.7	1.37
DgP vs ZnO			
Low DgP, no ZnO	695.88	505.7	1.39
Low DgP + ZnO	604.37	400.7	1.53
High DgP, no ZnO	689.19	546.8	1.27
High DgP + ZnO	711.53	526.5	1.37
Phytase vs ZnO			
No phytase, no ZnO	671.25	515.7	1.32
No phytase + ZnO	654.07	469.4	1.42
Phytase, no ZnO	713.81	536.8	1.35
Phytase + ZnO	661.83	457.8	1.47
Pooled S.E.D. 2-way interactions	28.197	24.49	0.074
DgP vs Phytase vs ZnO			
Low DgP	663.31	468.2	1.43
Low DgP + Phytase	728.44	543.1	1.36
Low DgP + ZnO	602.77	396.7	1.52
Low DgP + Phytase + ZnO	605.97	404.7	1.54
High DgP	679.19	563.1	1.21
High DgP + Phytase	699.19	530.5	1.33
High DgP + ZnO	705.38	542.1	1.32
High DgP + Phytase + ZnO	717.69	510.9	1.41
Pooled S.E.D. 3-way interactions	39.876	34.64	0.104
Probabilities of differences			
DgP	0.015	<0.001	0.009
Phytase	0.212	0.784	0.434
ZnO	0.088	<0.001	0.034
DgP X Phytase	0.653	0.039	0.234
DgP X ZnO	0.006	0.018	0.712
Phytase X ZnO	0.387	0.348	0.788
DgP X Phytase X ZnO	0.499	0.328	0.579

6.4. Results – experiment two

All pigs completed the second trial, however, two pigs were treated for lameness during the first week of the experiment with a three day course of Betamox (Vm 02000/4071) and Metacam (Vm EU/2/97/004/041-042). Following post-mortem examination of pigs sacrificed for gene expression analysis, one pig was found to have a bladder infection and was subsequently excluded from the gene expression analysis.

6.4.1. Growth performance

All treatment groups were 8.6 SD±0.14 kg at the start of the trial and did not differ significantly in BW by three weeks post-weaning (Table 6.11). Dietary ZnO reduced FCR in period one (p=0.05) and tended to increase DF in period three (p=0.07). Phytase reduced FCR in period two only (p=0.002). Daily liveweight gain in the second trial using the JSR 900 sire was noticeably better than in the first experiment which used PIC 337 and JSR 800 sires.

Table 6.11: The effects of super dosing phytase (0 or 2500 FTU/kg) & pharmaceutical ZnO (0 or 3100 mg/kg) on pig growth performance in diets containing 5.8 g/kg digestible phosphorus over three weeks post-weaning. Replicates are mean averages for pens of five pigs.

Treatment	Phytase[1]		Zinc[2]		S.E.D. (12 d.f.)	p-values		
Level	0	2500	0	3100		P[3]	Z[4]	P X Z[5]
Weaning BW (kg)	8.6	8.6	8.6	8.6	0.08	0.54	0.78	0.80
Day 1 – 7								
DLWG (g)	244	244	233	254	24.0	1.00	0.40	0.55
DF (g)	295	296	295	296	20.0	0.95	0.94	0.51
FCR	1.22	1.24	1.29	1.17	0.055	0.79	0.05	0.59
Day 7 – 14								
DLWG (g)	475	521	467	529	35.2	0.22	0.10	0.87
DF (g)	559	578	533	604	40.5	0.64	0.11	0.70
FCR	1.18	1.11	1.14	1.15	0.018	0.002	0.80	0.23
Day 14 – 23								
DLWG (g)	553	595	554	594	43.5	0.36	0.38	0.36
DF (g)	764	824	761	828	33.8	0.10	0.07	0.89
FCR	1.41	1.40	1.39	1.47	0.080	0.88	0.82	0.15
Day 1 – 23								
DLWG (g)	424	453	418	459	23.6	0.24	0.11	0.78
DF (g)	539	566	529	576	26.0	0.32	0.10	0.67
FCR	1.28	1.25	1.27	1.26	0.033	0.45	0.73	0.15
Final BW (kg)	17.5	18.1	17.4	18.2	0.50	0.21	0.12	0.75

[1]FTU/kg; [2]mg/kg; [3]Phytase main effect; [4]Zinc main effect; [5]Phytase/ZnO interaction

Individual pigs killed at the end of the three weeks were analysed by treatment group to assess representativeness. No significant effects were observed on growth rates of individual pigs during any period and they appeared representative of the pen average parameters (Table 6.12). Feed intake and FCR could not be calculated on an individual pig basis.

Table 6.12: The effects of super dosing phytase (0 or 2500 FTU/kg) & pharmaceutical ZnO (0 or 3100 mg/kg) on pig growth performance in diets containing 5.8 g/kg digestible phosphorus over a three week post-weaning. Replicates are individual pigs.

Treatment	Phytase[1]		Zinc[2]		S.E.D. (28 d.f.)	p-values		
Level	0	2500	0	3100		P[3]	Z[4]	P X Z[5]
Weaning BW (kg)	8.5	8.4	8.4	8.5	0.13	0.58	0.59	0.76
DLWG Day 1 – 7 (g)	231	252	245	237	28.3	0.46	0.78	0.10
DLWG Day 7 – 14 (g)	452	496	455	493	42.2	0.32	0.37	0.98
DLWG Day 14 – 23 (g)	552	599	565	586	46.7	0.32	0.65	0.21
Overal DLWG (g)	411	449	421	439	28.4	0.20	0.54	0.91
Final BW (kg)	17.1	17.8	17.2	17.7	0.61	0.25	0.48	0.86

[1]FTU/kg; [2]mg/kg; [3]Phytase main effect; [4]Zinc main effect; [5]Phytase/ZnO interaction

6.4.2. Haematology

Overall, there were no significant treatment effects on the number of white blood cells/ml (Table 6.13). These were slightly elevated (numeric only) above the normal range in the no phytase/no ZnO control group (23.42 million/ml), whereby the reference range is 11 – 22 million/ml (Kahn *et al*., 2005) but all other treatment groups were within range (Table 6.13). There also tended to be an interactive effect of phytase and ZnO in combination, whereby the proportion of lymphocytes and neutrophils within the WBC population were increased ($p=0.091$) and decreased ($p=0.097$) respectively (Figure 6.2). All other average cell populations reported in Figure 6.2 were close to normal reference values for healthy pigs in all treatment groups.

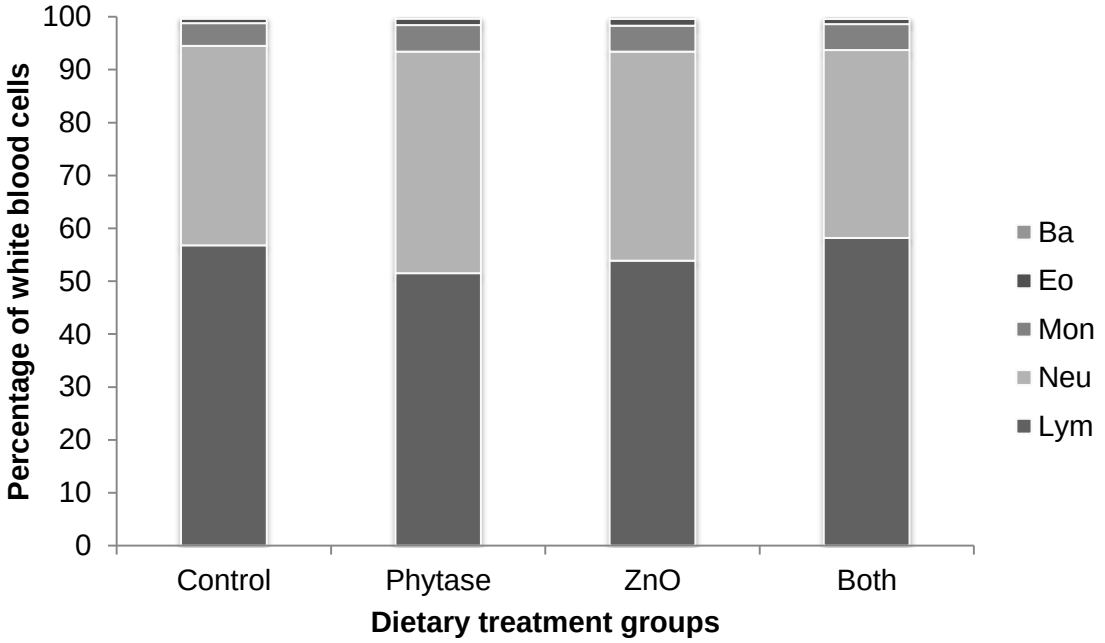

Figure 6.2: proportions of white blood cells in whole blood taken from pigs 12 and 13 days post-weaning, fed a control diet or supplemented with pharmaceutical ZnO at 3100 mg/kg, phytase at 2500 FTU/kg diet or a combination of both. Ba=basophils, Eo=eosinophils, Mon=monocytes, Neu=neutrophils, Lym=lymphocytes.

While Hb and Hct did not significantly differ individually, the calculated MCV revealed a significant interation (p=0.04), whereby levels were lower when diets contained neither the ZnO nor phytase (either singularly or in combination) (Table 6.13). When expressed on a per cell basis (MCHC), only a main effect of ZnO was observed (p=0.05), with a 2.7% decrease for diets medicated with ZnO (Table 6.13).

Table 6.13: Haematological parameters of whole blood taken from pigs 12 and 13 days post-weaning, fed a control diet or supplemented with pharmaceutical ZnO at 3100 mg/kg, phytase at 2500 FTU/kg diet or a combination of both.

Treatment factor	WBC (M/mm^2)	Lym (M/mm^2)	Mon (M/mm^2)	Neu (M/mm^2)	Ba (M/mm^2)	Eo (M/mm^2)	RBC (M/mm^2)	Hb	Hct	MCV	MCHC
Phytase											
+ve	20.7	11.44	1.008	7.9	0.082	0.22	7.0	12.57	39.0	55.89	32.18
-ve	23.4	13.00	1.057	9.04	0.090	0.25	6.5	11.59	35.7	54.93	32.43
Zinc Oxide											
+ve	21.4	12.00	1.038	8.05	0.086	0.24	6.5	11.52	36.2	55.97	31.85
-ve	22.7	12.44	1.028	8.89	0.087	0.23	7.0	12.63	38.6	54.85	32.76
Pooled S.E.D. main effects	1.722	1.232	0.084	0.814	0.0134	0.032	0.494	0.984	2.77	1.142	0.419
Phytase X ZnO											
No phytase, no ZnO	25.03	14.24	1.059	9.43	0.089	0.22	6.55	11.55	34.9	53.02	33.14
No phytase + ZnO	21.82	11.76	1.055	8.64	0.091	0.27	6.45	11.62	36.6	56.84	31.72
Phytase, no ZnO	20.31	10.65	0.997	8.35	0.084	0.25	7.47	13.72	42.3	56.69	32.38
Phytase + ZnO	20.99	12.24	1.02	7.45	0.080	0.20	6.47	11.42	35.8	55.09	31.98
Pooled S.E.D. 2-way interactions	2.435	1.742	0.1187	1.151	0.0189	0.046	0.698	1.392	3.91	1.615	0.593
Probabilities of differences											
Phytase	0.13	0.23	0.58	0.19	0.55	0.48	0.36	0.34	0.25	0.42	0.56
ZnO	0.48	0.73	0.91	0.32	0.93	0.89	0.29	0.28	0.39	0.35	0.05
Phytase X ZnO	0.28	0.12	0.88	0.95	0.81	0.14	0.38	0.25	0.16	0.04	0.25
CV%	15.6	20.2	16.3	19.2	31.0	27.7	14.7	16.3	14.8	4.1	2.6

WBC=White blood cells; Lym=lymphocytes; Mon=monocytes; Neu=neutrophils; Ba=basophils; Eo=eosinophils; RBC=Red blood cells; Hb=haemoglobin; Hct=haematocrit; MCV= Mean Corpuscular Hemoglobin; MCHC= Mean Corpuscular Hemoglobin per Cell

6.4.3. Intestinal gene expression

The assay for IL-6 failed repeatedly to give acceptable performance with melt curves suggesting a problem with the primers and so results are not reported. Expression of IFNG was up-regulated in the duodenum when zinc and phytase were fed in combination (p=0.006) with one outlier in the control group removed, also remaining significant (p=0.047) with the outlier included in the analysis (Figure 6.3).

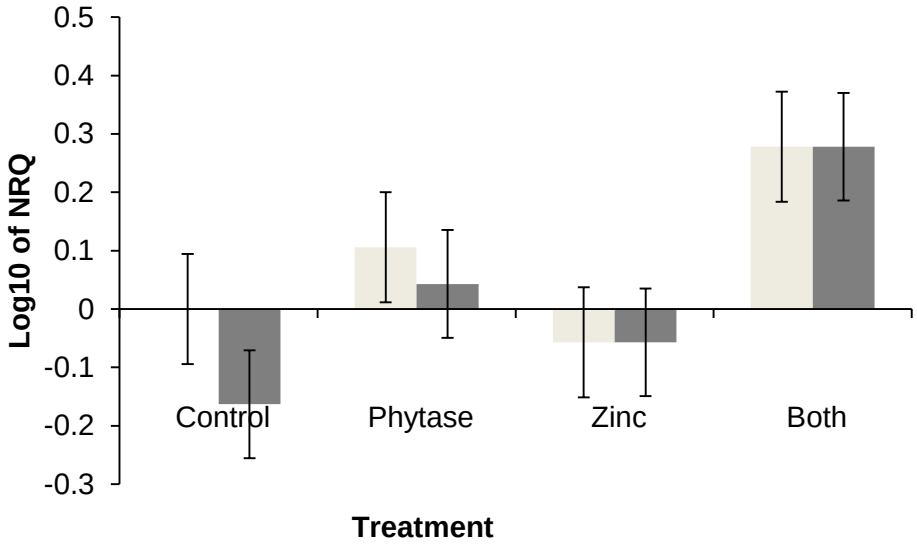

Figure 6.3: Duodenal expression of IFN-γ (relative to the control treatment group) in pigs three weeks post weaning; Light shaded bars are means including one outlier in the control group. Dark shaded bars are means excluding one outlier in the control group. Error bars represent the common standard error of the mean.

There were no significant differences detected in TNF expression (Figure 6.4).

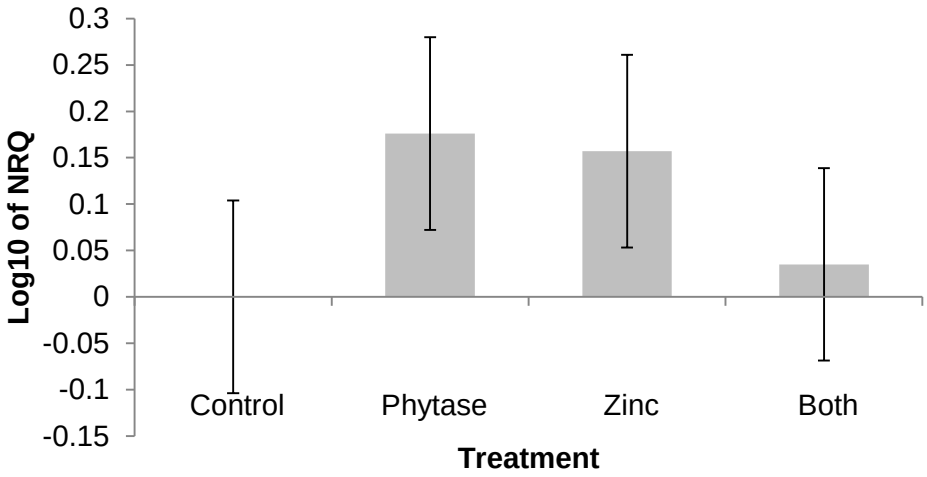

Figure 6.4: Duodenal expression of TNF (relative to the control treatment group) in pigs three weeks post-weaning. Error bars represent the common standard error of the mean.

Both the IFN-γ and TNF assay in the ileum failed the QC, with the DNase treatment at the RNA extraction stage not removing all gDNA contamination. Post a second DNase treatment directly on to the extracted RNA, the controls (NTC) still failed to pass the QC, amplifying within 5 cycles of the main data (Figure 6.5).

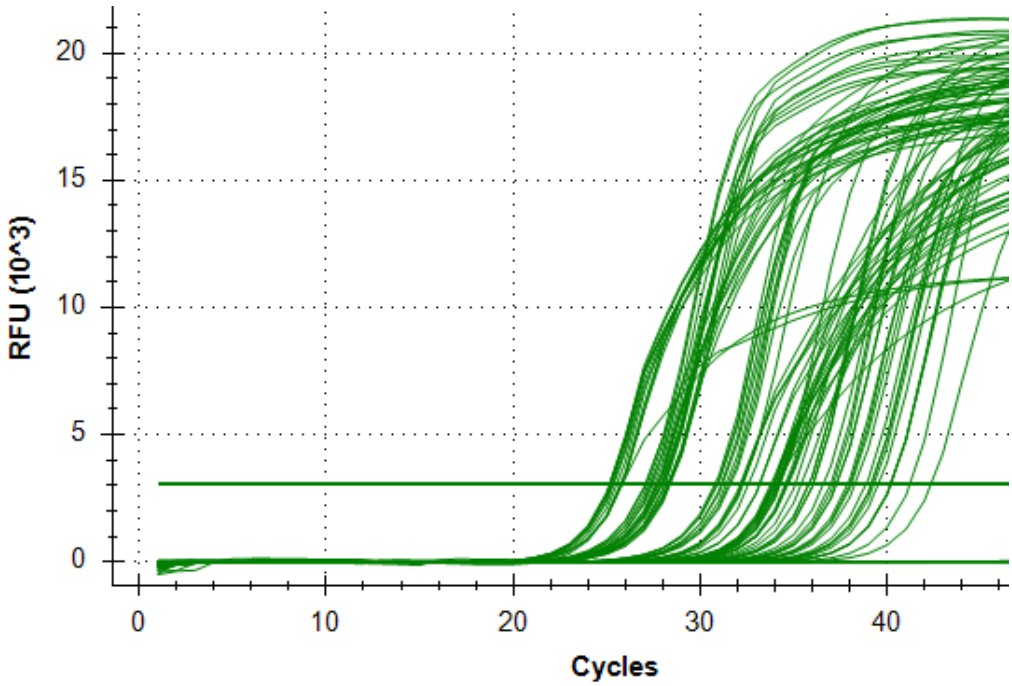

Figure 6.5: RT-qPCR real-time amplification plots for IFNG, TNF and IL-6 from ileal tissue samples and controls (NTC, NC) with a failed DNase treatment during RNA extraction, taken from pigs three weeks post-weaning.

6.5. Discussion

Analysis of the diets confirmed that the levels of all additives were close to those expected (Table 6.5), validating the experiments. Levels of zinc in the groups not supplemented with pharmaceutical ZnO were just below 150 mg/kg, which is partly from the 110 mg of ZnSO4 in the premix (24.2 mg elemental zinc) and also from natural zinc contained in feed ingredients. Morbidity occurred on four occasions in experiment one and twice in experiment two. These largely consisted of shoulder injuries due to mixing at weaning. All cases were treated as reported in the relevant results sections and where the animals were removed from the trial, performance data was adjusted based on the number of 'pig days' the animal had completed on trial. For sudden injuries, this is unlikely to have any noticeable impacts on pen growth performance or feed intake but if an animal was chronically ill for several days prior to removal, then it may not have been eating for 24 hours before being removed. One pig died suddenly of a suspected heart attack. A breakdown in herd health occurred between the two experiments and so subsequently, all animals on experiment two were vaccinated against *Mycoplasma hyopneumoniae* and porcine circovirus type 2 at weaning.

6.5.1. Response to dietary phosphorus level

In chapter four, dgP was included at levels from just below requirement at 2.8 g/kg dgP (Whittemore *et al*., 2003) up to an excess of 8.8 g/kg dgP. There was no dose response over this range, except for a reduction in DF at the highest level (Table 4.7), when tested in phytase and ZnO free diets. This was expected since clear growth response in phosphorus deficient diets are often reported but not when phosphorus needs are met, typically at between 3.3 and 3.5 g/kg dgP (Ekpe *et al*., 2002). In contrast, a significant quadratic response to increasing levels of dgP over the range 2.8 g.kg to 5.8 g/kg was seen in this chapter on DLWG and DF, in the third week post-weaning (Table 6.6). This was the predicted response to the hypothesis that there may be a zinc-phosphorus interaction in the small intestine, possibly due to precipitation. The result of such a condition would then require the animal to consume more phosphorus to compensate for that which is unavailable. In this case, there were parallel increases in DF and DLWG but no significant improvements in FCR (Table 6.6), suggesting that higher levels of dgP encouraged feed intake, which in turn, increased DLWG. Voluntary feed intake is a complex mechanism but negative and positive feedback stemming from a nutrient imbalance tends to be at play when feed intake is modulated in this way (Kyriazakis and Whittemore, 2006).

Interactions between the level of dgP and ZnO inclusion reveal that while there are no interferences in the first two weeks post-weaning, inclusion of pharmaceutical ZnO significantly reduces DLWG and DF in the third week post-weaning, dramatically in low dgP diets (Table 6.10). This is in agreement with the results of the dose response assessment, whereby any further growth response to dgP has already plateaued at 5.8 g/kg dgP (Figure 6.1), so that growth response due to not meeting demand for P is not noticeable in the 5.8 g/kg dgP group. The ratio of P to Zn in these diets increased from 1.86 in diet D, to 2.06 in diet K, 2.50 in diet L and 2.62 in diet J (as measured), having a very strong positive correlation ($r=0.93$, $p<0.05$) with DLWG in period three. Walk *et al*. (2013) detected significant reductions in serum P and increases in serum zinc with graded levels of ZnO at a similar growth period. While the molar ratio of zinc to phytic acid remained constant at around 2.5:1 (below the 3.5 - 4.1 precipitation range reported at pH 7.0 by Champagne and Fisher (1990)), taken together, there is likely to be phosphorus inhibition by high ratios of dietary zinc. This is enough to manifest as reductions in growth performance.

6.5.2. Growth response to phytase

Similar to chapter five, super dosing phytase was shown to be efficacious at improving growth performance and reducing FCR in the first two weeks post-weaning only, in both experiment SM3 (Table 6.9) and FCR only in experiment SM5 (Table 6.11). These are in

good agreement with data published by Walk *et al*. (2013), where super dosing phytase only increased DLWG and DF in the first three weeks post-weaning. In experiment SM3 it was also possible to show that in addition to the independent super dosing effect early on post-weaning, phosphorus level becomes more important in the third week post-weaning. This was demonstrated by the addition of phytase in the low dgP diet significantly improving DF but not in the dgP excessive diet in the third week post-weaning (Table 6.10). The implications of this may be that there should be some inclusion of phytase in low dgP diets, to ensure animals do not become deficient but that no performance boost should be expected in dgP adequate diets.

In the first week post-weaning, the addition of pharmaceutical ZnO in the diet resulted in a ZnO x phytase interaction that tended ($p=0.08$) to reduce DLWG (Table 6.8). Augspurger *et al*. (2004) reported a 2:1 Zn:PA molar ratio (close to the 2.5:1 in these experimental diets) in a similar pig experiment and concluded this would be enough to substantially inhibit the activity of phytase in releasing iP, based on a reduction in bone ash. This is validated by the results of this experiment through the reduction in DLWG and suggests that P should be increased in ZnO medicated weaning pig diets from inorganic sources only, rather than from phytase. There was no evidence of ZnO x phytase interactions after this period.

6.5.3. Growth response to zinc oxide

No clinical symptoms of PWD were noted during routine health inspections. Under these non-challenge conditions, feeding pharmaceutical ZnO at 3.1 g/kg feed over three weeks post-weaning had a negative effect on growth performance, significantly ($p<0.05$) reducing DLWG and FCR by 7.9 % and 3.9 % respectively, and tending ($p<0.10$) to reduce DF by 4.3 % by the end of the trial (Table 6.7). The effect was especially pronounced in the first week post-weaning (Table 6.8) and was compounded when in combination with phytase, increasing FCR (though omitting both was worse than having them in the diet) (Phytase x ZnO, $p<0.05$). Pigs seem to acclimatise to high levels of ZnO in the second week post-weaning, with no difference in growth performance detectable (Table 6.9) but very pronounced negative effects of ZnO inclusion in the third week post-weaning (Table 6.10). This is to be expected since it is well documented that post 14 days, high levels of zinc may become toxic (FEEDAP, 2014). The reduction in DF was not expected though since several studies feeding high levels of zinc have reported increases in feed intake and growth linked with increases in hormones that regulate these traits, including Insulin-like growth factor – 1 (IGF-1) and ghrelin (Li *et al*., 2006; Yin *et al*., 2009). There is evidence, however, of ZnO being dose dependent, with Walk *et al*. (2013) reporting quadratic reductions in DLWG when ZnO was fed at 3500 mg/kg compared to a lower dose of 1750 mg/kg. Richness of

intestinal microbial communities are also dose dependent to ZnO with distinct clustering effects detectable at 50, 150, 250, 1000 and 2500 mg/kg zinc (Pieper *et al*., 2011). These differences may modulate metabolic activity and account for some of the growth effects observed. Typically this could be through the reduction of lactate, due to lower *Lactobacillus* numbers in the small intestines (Starke *et al*., 2014).

In the second experiment (SM5), there was some evidence of ZnO inclusion improving FCR in the first week post-weaning (Table 6.11) but this did not translate into any significant growth improvement over the three week trial. While ZnO inclusion was not detrimental in experiment SM5, compared to experiment SM3, this is likely due to the buffer of phosphorus available in the diet used (5.8 dgP) in the second trial (dgP x ZnO interaction observed in Table 6.10). Growth response to pharmaceutical ZnO in healthy pigs is often inconsistent with reports of little, if any, improved growth during the first few weeks post-weaning (Katouli *et al*.,1999; Piper *et al*., 2012; Walk *et al*., 2013), some response detectable (Jensen-Waern *et al*.,1998) and hints of toxicity after a few weeks (Starke *et al*., 2014). Bigger datasets analysed by meta-regression though have concluded that overall there is likely to be growth benefits from feeding pharmaceutical ZnO (Sales, 2013). These studies were mostly conducted in the USA, where pathogenic challenge and microbiome may differ to the UK. This theory is confirmed with data from challenge studies showing ZnO to be effective at reducing Coliform numbers in the GI tract or at least reducing PWD post ETEC infection, leading to improved growth performance (Slade *et al*., 2011; Stensland *et al*., 2015).

6.5.4. General and intestinal health

Individual pigs for blood sampling and euthanasia were selected to be closest to the mean average weight for the pen replicate. This is reflected by the data in Table 6.12 which confirms they were consistent with the growth rates in Table 6.11. Systemic WBCs are markers of immune system activation and while Zn has not been shown to affect activated neutrophils and IFN-γ producing cells post-weaning (Jenson-Waern *et al*, 1998, Chai *et al*, 2014), little is known about the influence of phytase. The effects of high doses of phytase and ZnO are more pronounced in the first two weeks post-weaning, so blood analysis to assess general health status was conducted at 12 days post-weaning. Analysis revealed no effect on total WBC (Table 6.13), with levels within the normal biological range expected (Kahn *et al*., 2005). There is therefore no clinical relevance of this data, which is to be expected since the pigs were not challenged in this experiment.

In the group fed the combination of additives, circulating lymphocyte percentage tended to be higher than when phytase and ZnO were either not fed or included on their own (Figure

6.2). This group also had significantly higher expression of the pro-inflammatory cytokine interferon-γ in the duodenum compared to the control group, suggesting intestinal inflammation in the anterior section. This was not detectable in the ileum, possibly related to a reduced buffering capacity at the stomach/duodenal junction or differences in the upper small intestine microbiome (Starke *et al*., 2014). The addition of 100 mg/kg CS-Zn and 500 mg/kg ZnO have been shown to rival doses of 3000 mg/kg and 2250 mg/kg ZnO respectively for improved small intestine histophysiology and immunity (Han *et al*., 2014; Hu *et al.*, 2013). In this study, both the control group and the phytase group diets contained 150 mg/kg Zn. This may have limited the detection of differences expected from the high Zn treatment as the basal level of zinc was probably sufficient for optimal gut health and growth. Pharmaceutical ZnO supplementation alone did not significantly affect TNF and IFN-γ mRNA abundance in this study either and expression levels of both cytokines relative to the control were low (<0.5 $\log_{10}$ change), suggesting no major challenge was taking place at day 21 post-weaning. Overexpression of pro-inflammatory cytokines have been implicated in reduced tight junction protein expression (Hu *et al*, 2013), thereby leading to reduce intestinal integrity.

Many studies rely on only one reference gene (RG) for normalisation of the transcript expression of genes of interest (GOI). Often the gene selected has not been assessed for stability or suitability and is chosen entirely for historical reasons. Normalising GOI expression in this way is prone to error, as using a single RG has been shown to vary greatly in stability depending on tissue type (Ryan *et al*., 2010) and should not be used without validation (Bustin *et al*., 2009). Prior to the GOI expression assays, a geNorm™ experiment was conducted to find appropriate reference genes to normalise the cytokine data (see section 3.6.2.). The results showed that the best combination in duodenum tissue was GPI and GSR, so these were subsequently used to normalise the GOI data. To improve the accuracy of the Cq values, PCR efficiency was estimated using regression analysis on a four-point-window of linearity of the log-linear phase, by amplicon group (Ramakers *et al*., 2003; Ruijter *et al*., 2009). The NRQ presented in Figures 6.3 and 6.4 are therefore likely to be as accurate as currently possible. The addition of no template controls and reverse transcription negatives also assisted in stringent quality control. As a consequence, the IL-6 assay failed the QC by amplifying gDNA (despite a DNase step and a small intron being present, the assay was found to “jump”) and the results were therefore not included in this chapter. The one pig that was found to have a bladder infection upon post-mortem inspection showed highly elevated TNF levels in the intestine and was also excluded from the data analysis.

6.6. Conclusions

In conclusion, the addition of super dosing phytase (2500 FTU/kg feed) was confirmed to be efficacious at improving production performance (8 % increase in DLWG and 0.07 point decrease in FCR) if fed up to 14 days post-weaning. In this study with pigs showing no clinical signs of PWD, medicating feed with pharmaceutical ZnO (3100 mg/kg feed) is contraindicated, as it was shown to significantly reduce production performance in this chapter, especially in diets containing relatively low levels of digestible phosphorous. Where pharmaceutical ZnO is included for seemingly healthy pigs, this should not be in conjunction with super doses of phytase, due to potential inhibition of phytase activity. Higher levels of dgP from inorganic sources of between 1 – 2 g/kg more than normal may be warranted for optimal growth response in ZnO medicated diets. In any case, supplementation with pharmaceutical ZnO should not exceed 14 days post-weaning.

CHAPTER SEVEN

General discussion and conclusions on super dosing phytase and supplementation of pharmaceutical zinc oxide for enhancing the production performance of newly weaned pig

7.0. Introduction

Pharmaceutical grade ZnO is a widely used replacement to antibiotic growth promoters in many EU countries (EC, 2015). There is, however, growing concern about the environmental impact of increased zinc usage in the pig industry (SCAN, 2003), lack of consistent efficacy and possible development of microbial resistance (Slifierz *et al*., 2015) developing. Research into alternative growth promoters and their implications for use in ZnO medicated feeds is therefore of growing interest to the pig industry. A new strategy for using phytase enzymes by super dosing at levels beyond that generally required for improving P digestibility had emerged as a possible growth promoter but direct evidence of 'extra phosphoric' effects in newly weaned pigs was not well documented. The objective of this thesis was therefore to evaluate the efficacy and possible explanatory mechanisms of super dosing phytase at enhancing pig performance post-weaning, beyond that attributable to increased phosphorus digestibility. In addition, the thesis has assessed the suitability for super dosing phytase in ZnO medicated feed.

7.1. Weaning and overall production performance

Formulating diets that are nutritionally adequate and balanced is only one part of optimising pig growth performance. In the typical abrupt weaning systems commonly found in commercial pig production, multifactorial stressors can cause an acute growth check and increased susceptibility to pathogenic challenge (Lallès *et al*., 2004). It is therefore important to consider the management of stress around weaning, in conjunction with appropriate nutritional formulations. With the ban on AMGPs in 2006 (Commission Regulation (EC) No 1831/2003), alternative control measures against pathogenic challenge are more important than ever. Growth performance remained strong throughout the trials detailed in chapters four, five and six, with no clinical symptoms of a weaning associated disease observed in the 1064 pigs studied. The exception being an unrelated breakdown in herd health, due to enzootic pneumonia (EP) during the third experiment (SM3). This was mostly seen in pigs that had been weaned for a number of weeks and vaccination against *Mycoplasma hyopneumoniae* and porcine circovirus type 2 proved effective at controlling the disease. While pre-weaning growth rates were not available for comparison, DLWG in the first week after weaning were consistent at 150 – 160 g/day for the three main experiments. This is

slightly lower than the general herd performance of 193 g/day for this period but this is likely to be down to the formulation of a single phase diet, targeted to be optimal in the second week post-weaning and the removal of any commercial additives, such as xylanase, acids and probiotics, in the experimental diets.

Villus atrophy in the first week post-weaning is normally associated with a reduction of feed intake (Kelly *et al*., 1991; Dong and Pluske, 2007), likely due to the mixing of litters at weaning, focusing attention away from feeding to fighting and establishing a new social hierarchy. This provides opportunistic pathogens such as *Escherichia coli* the chance to proliferate, resulting in scouring 2 – 3 days post-weaning. Creep feed was provided in all of the experiments detailed to help ease the transition from milk to solid feed, thereby encouraging feed intake post-weaning. Any growth check should have therefore been minimised. In addition, it was recognised that while in semi-natural systems piglets would wean at between 11 and 17 weeks after birth (Jensen and Recén, 1989; Bøe, 1991), the experiments detailed in this thesis needed to reflect UK commercial practice. For this reason piglets were abruptly weaned in the fourth week post farrowing, in line with the EU average (AHDB pork, 2015).

7.2. Beyond phosphorus

Responsible use of non-renewable resources like phosphorous and due consideration of the environmental impact through feeding high levels of metals such as zinc and copper are high priorities within the animal feed industry. For this reason, guidelines from BSAS (Whittemore *et al*., 2003) and NRC (NRC, 2012) detailing recommended nutrient levels for pig feed, in addition to legal limits (Commission Regulation (EC) No 1334/2003) and EU scientific reviews (FEEDAP, 2014) are published. The results of chapter four broadly agree with the dgP levels currently recommended by Whittemore *et al*. (2003) and are similar to levels published by Ekpe *et al*. (2002), based on the lack of growth response to increasing dgP above 2.8 g/kg feed (Table 4.7). Once P requirements for maintenance and growth have been met, some additional P may be incorporated into bone stores but any beyond that is excreted in faeces and urine (Fernández, 1995). Supporting the data in chapters four and five, P digestibility does not increase when levels of dietary dgP are increased (Table 4.6), suggesting more P is absorbed as a consequence of the increasing levels in the diet. The excess P is then likely regulated by the kidneys and excreted in urine. In contrast, as the quantity of Ca increases in the diet, so the level of Ca digested reduces, as measured by higher faecal Ca concentrations. This would make sense, as the main route for Ca excretion is via the faeces (Fernández, 1995). Furthermore, when the level of total P is kept

constant (and only modulated by the addition of phytase), then Ca digestibility is largely unaffected (Table 5.11).

7.2.1. Phytate destruction

Phosphorus from inorganic and animal sources is highly digestible (for example dicalcium phosphate = 64 %, monosodium phosphate = 90 %, bone precipitate = 61 % and skimmed milk powder = 90 % (Lenis and Jongbloed, 1999)). In contrast, the organic P found in plants (phytate) is only around 30 % digestible for non-ruminant animals (Lenis and Jongbloed, 1999). In addition to being a poor source of dgP in non-fermented or phytase supplemented diets, there are a number of other reasons for its continued classification as a major ANF. These range from chelating minerals (such as calcium, zinc, magnesium, sodium and iron) into indigestible complexes (Maenz *et al.,* 1999), to increasing endogenous mineral losses (Woyengo and Nyachoti, 2013), binding to protein (Selle *et al.*, 2012) whilst concurrently reducing pepsin activity (Yu *et al.,* 2012) and physically changing the gut histomorphology by reducing crypt depth – probably due to reduced cell proliferation (Woyengo *et al.,* 2011). The effects of this are also confirmed by the results of chapters five and six of this thesis. For example, in chapter five, removing phytate by super dosing phytase >2000 FTU/kg (as confirmed by the 80% digestibility of phytic acid reported in Table 5.16) significantly improved pig growth performance compared to the negative control (Table 5.8). This was found not to be a consequence of increasing the level of dgP (Table 5.7 showed no difference between the base level of 5.8 g/kg dgP and a higher level of 7.8 g/kg dgP), confirming the 'extra phosphoric' effects of phytase super dosing. In both experiments SM2 and SM3, the effects of super dosing phytase (2000 – 8000 FTU/kg of diet) were seen in the first and second week post-weaning (Table 5.8 and 6.9) but phosphorus was more important in the third week post-weaning. This may have been partly due to the diets being nutritionally optimised for pigs at two weeks post-weaning. Super dosing of 2000 FTU/kg as part of a "low phytate nutrition program" was also reportedly to be effective at enhancing growth performance up to 42 days post-weaning (Wilcock and Walk, 2016). Based on the evidence from this thesis and the body of published literature referred to, super dosing (>2000 FTU/kg) for phytate removal should be a standard protocol for weaner pig nutrition.

7.2.2. Phytase efficacy

Phytase acts as the catalyst in the hydrolysis of phytate with the products being free inorganic P, any released cations and water (KEGG, 2012). Low phytate diets resulting from the super dosing of phytase are only possible with phytases that can dephosphorylate phytate down to IP_1 or even pure inositol. The quantum Blue phytase used in this series of

experiments is considered third generation (having enhanced intrinsic thermostability and substrate affinity over second generation products, which in turn have been modified from the first generation “wild type” phytases) and was very effective at phytate destruction, with 65 % digestibility of IP_6 at 500 FTU/kg feed, increasing to over 80 % at super and mega doses (Table 5.16). This is in good agreement with Laird *et al*. (2016) using the same Quantum Blue phytase in pig diets, where ileal IP_6 concentration reduced from 21616 nmol/g DM to 7868 nmol/g DM with a dose of 8000 FTU/kg in his study, compared to a reduction from 24487 nmol/g DM to 4060 nmol/g DM in this present study (Table 5.16). Similarly, the Quantum Blue phytase derived from *Escherichia coli* (expressed in *Trichoderma reesei*) used in this thesis resulted in a comparable digestibility of IP_6, when fed at 1000 FTU/kg, as a preparation of 1500 FTU/kg from *Aspergillus niger* (var. *ficuum*) strain NRRL 3135 (Jongbloed *et al*., 1992) (68% versus 74%). In this case, both 6-phytase and 3-phytase are suitable for use in super dosing strategies despite their differing pH and temperature optimums (Table 2.10). The use of these in combination may thus allow for a wider range of applications in situations where pH is likely to be variable (e.g. due to the buffering effect of feeds). The resulting different species of lower inositol phosphates arising from the preferential hydrolysis of carbon in the *myo*-inositol ring (either starting at the L-6 position or the D-3 position for 6-phytase and 3-phytase respectively) may be important for cellular signalling pathways but this is an area where further research is needed.

7.2.3. Immune modulation and health status

Biomarkers for stress have been fairly well documented (Martínez-Miró *et al*., 2016) but are of limited use unless specifically defined for the hypothesis under test. In chapters five and six, cytokines localised to each section of the small intestine were selected to help describe the health of the gut in general. The patterns were assessed through measurements of the concentration of mRNA transcripts using RT-qPCR. The technique is very sensitive but can be misleading if not carried out or reported correctly (Bustin *et al*., 2009). Reference genes for the normalisation of copy numbers / relative quantities were not taken from the literature but instead formed part of a preliminary experiment to assess the suitability of a range of candidates specific for the tissue samples used. Those selected (GSR and GPI) were used for both experiments reported in chapters five and six (SM2 and SM5) and were validated as acceptable. This is interesting since genes such as ACTB and GAPDH are commonly used as RG but were shown to be a very poor choice in this situation. The range of controls and corrections used to obtain and validate the results of the gene expression analysis ensures robustness of the results, however, low expression levels of the cytokines indicate only baseline expression was occurring in the gut, with levels often below the quantification limit, contributing to a weak contribution of the data to overall interpretation.

Several studies in poultry have suggested phytase may be implicated in enhancing immunity by boosting the immune response to vaccination (Liu *et al.* 2008; Ghahri *et al.* 2012). While in this study it was attempted to isolate and culture white blood cells from pigs to assess cell-mediated immune response, this was unsuccessful in the laboratories (data not reported) but could be done to provide further information on this in future experiments. In general, there is no evidence though from the trials reported in this chapter that in pigs phytase is implicated in any intestinal immune-regulatory pathways, such as those described in section 2.2.3. However, studies at the cellular levels reveal that lower inositol phosphates (such as IP_3) are definitely involved in the regulation of T lymphocyte activation (Porciello *et al*., 2016). This is largely through precursors (such as phosphatidylinositol 4,5-biphosphate), so it is unclear if dietary derived lower IPs can directly modulate this or whether it is exclusively the precursors that have the effect. Further experiments that look at stable isotopes of *myo*-inositol (such as ^{13}C inositol) to differentiate dietary derived IP_x from that of host cellular origin should be conducted to investigate the tissue cellular signalling pathways. The result of this may be a targeted phytase strategy that uses a combination of 3-phytases, 5-phytases and 6-phytases for immune-modulation.

7.4. Phosphorus requirement in ZnO medicated feed

In ZnO medicated diets there was a clear requirement for additional P_i supplementation, as indicated by a quadractic growth response of pigs to increasing levels of dgP (Table 6.6). Under these conditions, phytase generated P_i (which should have been up to 2.38 g/kg of diets based on a phytate P level of 3.5 g/kg (Table 6.5) with a complete dephosphorylation of 68% of IP_6 (Table 5.16) down to free inositol) appeared not to be as effective at increasing growth as supplemental P_i, since providing additional P_i in the diet resulted in further growth performance (Table 6.6). It is possible that 2500 FTU/kg releases only some P_i and although the phytic acid is removed, it may only have been dephosphylated to lower IP_x (Table 5.16). There is evidence that IP_4 and even IP_3 may still have potent ANF properties (Bedford and Walk, 2016), so any super dosing strategy needs to make sure it is able to remove these lower inositol phosphates. This was shown to be a dose ≥8000 FTU/kg in this thesis (Table 5.16). This would only need to be the case in diets containing <5.8 g/kg dgP, since the growth response to increasing dgP had platued by 5.8 g/kg dgP (Table 6.6), confirmed by a phytase x dgP effect, where there was an increase in growth at 2.8 g/kg dgP in phytase supplement diets but not in 5.8 g/kg dgP (suggesting dgP was sufficient for maximal growth at this higher level) (Table 6.7). Optimising the level of P in ZnO medicated feeds from either phytase generated P_i or supplemental P_i is an area for further research.

7.5. General conclusions

This thesis has evaluated the strategies for using phytase enzymes in weaned pig diets, in the context of enhancing production performance beyond that exclusive to increased phosphorus digestibility and with relevance for use in ZnO medicated feeds. The findings suggest that super dosing Quantum Blue phytase at levels of between 2000 – 8000 FTU/kg of feed for the first 14 days post-weaning is efficacious at enhancing growth performance in healthy pigs, probably by a more complete removal of dietary phytate than can be achieved with doses <2000 FTU/kg feed. Depending on the economic cost of phytase, recommendations for phytase supplementation in weaner pig diets based on these results would be feeding a super dose of 8000 FTU/kg for the first two weeks post-weaning to maximise growth performance and the hydrolysis of phytate in the small intestines; reducing to 1000 FTU/kg in the third week post-weaning to facilitate bone mineralisation. If pigs are not likely to be pathogen challenged, pharmaceutical ZnO at high levels (3100 mg/kg feed) should not be used metaphylactically, as it may inadvertently lead to a reduction of growth performance through suppression of feed intake. Where it is used, it should not be fed for more than 14 days post-weaning. There may also be justification for a higher inclusion level of dietary dgP in ZnO medicated diets at a level of 4.8 g/kg feed post-weaning.

7.6. Outcomes and practical recommendations

1. Super dosing of phytase enzymes significantly improved pig growth performance for the first two weeks post-weaning, was effective at dephosphylating phytate down to inositol and lower IP_x, enhanced P digestibility and increased bone mineralisation. Depending on the economic cost of phytase, recommendations for phytase supplementation in weaner pig diets would be super dosing at 8000 FTU/kg of diet for the first two weeks post-weaning to maximise growth performance and the hydrolysis of phytate in the small intestines; reducing to 1000 FTU/kg of diet in the third week post-weaning to facilitate bone mineralisation.

2. Medicating feed with high levels of ZnO (3100 mg/kg feed) reduced feed intake and growth performance in seemingly healthy pigs. Subject to the advice of a veterinarian, if pigs are not likely to be pathogen challenged, pharmaceutical ZnO at high levels (3100 mg/kg feed) should not be used metaphylactically, as it may inadvertently lead to a reduction of growth performance through suppression of feed intake. Where it is used, it should not be fed for more than 14 days post-weaning.

3. In diets not containing ZnO there was no improvement in growth performance when dgP was increased from 2.8 g/kg up to 8.8 g/kg. Conversely, when feed contained ZnO there was a quadratic improvement in growth performance when dgP was increased from 2.8 g/kg up to 5.8 g/kg. There may thus be justification for a higher inclusion level of dietary dgP (from inorganic P) in ZnO medicated diets at a level of 4.8 g/kg feed post-weaning.

7.7. Areas for further research

Several promising areas for further research have been identified in this thesis:

1. The use of multiple phytase enzyme cocktails for optimising inositol phosphate nutrition in the gut. Is it better to have low phytate diets by dephosphorylating phytate down to IP_1 or even pure inositol, or would generating certain lower inositol phosphate species (IP_{4-3}) be of benefit to improve gut health through immune-regulation?

2. What are the chemical interactions between P, Ca and ZnO in the pig and what are the practical implications of this for weaner pig nutrition? Do we need to increase Pi levels to compensate for P lock-up in ZnO medicated diets, or could we generate comparable P from phytate with phytase (how does phytate derived P compaire to inorganic P?) which is as effective as inorganic P? What is the effect of high Zn on phytase activity and can pharmaceutical ZnO be inhibitory to phytase, preventing adequate release of P_i?

3. Would lower doses of pharmaceutical ZnO be more suitable for metaphylactic use in weaner pig diets? What is the efficacy of using lower than the current prescription requirements on targeted microbes and would supplemental P still be required to maintain pig growth performance?

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