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Running head: Effect of phytase and iron in the weaner pig

Super-dosing phytase improves the growth performance of weaner pigs fed a low iron diet

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ABSTRACT: This experiment was conducted to test the hypothesis that a super-dose of phytase would improve the performance of weaner pigs fed an Fe-deficient diet, through an increase in phytate bound-Fe bioavailability. A total of 234 pigs (initial BW 7.6 ± SE 0.16 kg) were weaned at ~28 d of age and blocked into mixed sex pens of 4 or 5 balancing for weight, sex and litter of origin. Treatments were arranged as a 3 x 2 factorial with 3 levels of phytase (0, 500 or 2,500 FTU/kg feed) and 2 levels of Fe (50 [low; L-Fe] or 300 [high; H-Fe] mg/kg feed as $FeSO_4$) supplemented to an Fe-deficient wheat-SBM based diet. Diets were offered to 8 replicate pens for 20 d post-weaning. At the end of the experiment, 1 pig per pen was euthanized for the collection of blood, plasma, liver and ileal digesta. Pigs fed the L-Fe diet had a lower ADFI ($P < 0.01$) than those fed the H-Fe diet. Phytase at 2,500 FTU/kg improved the ADG of L-Fe fed pigs to a level comparable with the H-Fe fed pigs; however, its effect was diminished when added to the H-Fe diet, resulting in a significant Fe x phytase interaction for ADG ($P < 0.05$). Reducing the dietary Fe level resulted in reductions in haemoglobin ($P < 0.10$), haematocrit (Hct; $P < 0.001$), plasma Fe and ferritin ($P < 0.05$), and liver Fe

concentration ($P < 0.001$). Moreover, plasma transferrin was higher ($P < 0.05$) in L- Fe fed pigs. Supplementary phytase at 2,500 FTU/kg tended to increase Hct concentration ($P < 0.10$) and numerically increased plasma Fe and ferritin but had no influence on other measured indices of Fe status. There was an Fe x phytase interaction for ileal $InsP_6$ degradation ($P < 0.05$), which mirrored that observed for ADG: phytase increased $InsP_6$ hydrolysis to a greater extent when added to the L-Fe than the H-Fe diet (68.2 vs 30.8%). These data demonstrate that high doses of phytase can effectively improve the performance of weaner pigs fed an Fe-deficient diet. The benefit of using high doses of phytase in diets rich in Fe is seemingly reduced, likely as a result of the ability of Fe to degrade phytase efficacy.

Keywords: growth; inositol phosphate; iron; pig; phytase; weaner

1. Introduction

Iron deficiency is the most common mammalian micro-mineral deficiency worldwide and is considered a serious issue in intensive pig production systems (Cook et al., 1994). Young pigs are most at risk of developing Fe deficiency anaemia owing to their rapid growth rate, low hepatic Fe reserves at birth and the inherently low-Fe content of sow milk (Venn et al., 1947). It is, therefore, common practise for farmers to administer a prophylactic i.m injection of Fe (200 mg) to young pigs within the first 3 d of life (Ullrey et al., 1959) in order to maintain normal haemoglobin (Hb) levels through to weaning. However, recent research suggests that such measures may not be sufficient to prevent Fe deficiency, particularly with larger pigs (Jolliff and Mahan, 2011; Perri et al. 2016). Increasing dietary Fe levels to address this issue has risks as pathogens such as E. coli are limited in their growth by limitations in Fe concentrations as recently summarized by Kim et al., 2018.

47 Post-weaning, pigs are fed predominantly plant-based diets which typically contain
48 sufficient Fe to satisfy the requirements of the pig. However, the availability of Fe in plant
49 ingredients is largely unknown and variable (Kornegay, 1972). One of the primary
50 determinants of Fe availability in grain based diets is phytate concentration (Bohn et al., 2008).
51 Phytate is a ubiquitous component of pig diets and is commonly described as an anti-nutrient
52 due to its inhibitory effects on cationic mineral absorption (Tang et al., 2006). At
53 physiologically relevant pH ranges (2 to 7), phytate ($InsP_6$) and most lower inositol phospahte
54 esters ($InsP_x$; inositol x-phosphate) such as $InsP_{3-5}$ readily chelate with Fe to form insoluble
55 phytate-Fe complexes (Bretti et al., 2012; Yu et al., 2012). Consequently, small degrees of
56 phytate degradation that result in the formation of $InsP_{3-5}$, such as those commonly achieved
57 with standard phytase doses (500 FTU/kg), are not expected to yield substantial improvements
58 in Fe bioavailability. Therefore, this study set out to determine if a super-dose of phytase,
59 which would be expected to degrade even the lower esters of phytate, could improve the
60 performance of weaner pigs fed an Fe-deficient diet through improved Fe bioavailability.

61

62 **2. Materials and methods**

63 This study was conducted at the University of Leeds Pig Research Centre. Ethical approval
64 for the protocol was granted by the University of Leeds Animal Welfare and Ethical Review
65 Body.

66 2.1. Animals and housing

67 Two hundred and thirty-four cross bred pigs [(Large White x Landrace) x MAXGRO] were
68 weaned onto the 20 d trial at 28 (±4) d of age (initial BW ± SE 7.6 ± 0.16 kg). Prior to the
69 commencement of the study, all pigs received an i.m. injection of 200 mg of Fe as gleptoferron
70 at 1 to 3 d of age, according to standard operating procedures. However, the following

precautions were taken throughout the suckling phase to ensure low body Fe reserves at the initiation of the experiment: pigs had no access to supplementary feed, sow faeces was cleared twice daily, and pigs were separated from the sow as she was fed to deny piglets access to overspill. Pigs were blocked into mixed sexed pens balancing for weight, sex and litter of origin. Replicates had comparable numbers of pigs (4/5) per pen, with each pen within a replicate containing the same number of pigs. Pens within a replicate were randomly assigned to 1 of the 6 dietary treatments to create 8 replicates per treatment. Pigs were housed in a weaner-grower facility consisting of 8 identical rooms each comprising 16 fully slatted floored pens (135 x 155 cm). Room temperature was initially maintained at 29 $\pm$ 2 °C and then gradually reduced to 22 $\pm$ 2 °C over the course of the experiment. Pens were equipped with a multi-space trough feeder, two nipple drinkers and a ball on chain as a source of environmental enrichment. Feed and water were provided ad libitum throughout the study. The mean analyzed Fe concentration of the drinking water throughout the experiment was 63 µg/l, and thus Fe contribution from water intake was considered negligible

2.2. Experimental Design and Treatments

All diets were based on wheat-soybean meal and fed in pellet form (Table 1). With the exception of Fe, all diets were formulated to meet or exceed the nutritional requirements for pigs of 10 to 30 kg for all nutrients (BSAS, 2003). Dietary treatments were arranged as a 2 x 3 factorial, with two levels of supplemental Fe (50 [L-Fe] or 300 mg/kg [H-Fe]) and 3 levels of supplemental phytase [0, 500 and 2,500 phytase units (FTU)/kg]. Iron was added to the basal diet in the form of the highly available ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$). Levels of supplemental Fe were selected to create a marginally deficient (L-Fe) and a high Fe (H-Fe) diet according to the BSAS Nutrient Requirement Standards (2003). To achieve this, a low-Fe

vitamin and mineral mix was used, which provided Fe at a rate of ~20% of typical commercial premixes. The phytase used was a modified 6-phytase derived from E.coli (Quantum Blue: AB Vista, Marlborough, UK). One FTU is defined as the amount of enzyme activity needed to liberate 1 µmol of P per min from an excess of Na-phytate at 37 °C and pH 5.5.

The basal diet was prepared as a single batch at Target Feeds Ltd (Shropshire, UK) with the vitamin and mineral mix added at a level to provide 50 mg Fe/kg feed as fed. This mix was split into two and $FeSO_4$ was added to one lot at 250 mg/kg to create the H-Fe (300 mg of supplemental Fe/kg) treatment. The two lots were equally divided into three and phytase was added as appropriate to create the six dietary treatments. Titanium dioxide (TiO_2) was added to all diets at a rate of 5 g/kg feed as an inert dietary marker.

2.3. Measurements and Sampling

Pigs were weighed individually on d 0, 7, 14 and 20 to determine pen ADG. Troughs and feed refusals were weighed every 24 h for the determination of pen ADFI. ADG and ADFI measurements were used to calculate the average weekly G:F per pen. The health status of the animals was monitored daily throughout the experiment. Drinking water samples were collected weekly and stored at -20 °C pending Fe quantitation analysis. Experimental diets were sampled weekly, stored at -20 °C and pooled prior to chemical analysis. At the end of the 20 d experiment, 48 mixed sex pigs (1 per pen; mean BW $\pm$ SE 12.38 $\pm$ 0.62 kg) were euthanized for the collection of portal and peripheral blood, ileal digesta, duodenal mucosa, bone and liver samples. Pigs were killed via captive bolt penetration following sedation with an i.m. injection of Azaperone (4 mg/kg BW; Stresnil, Elanco, Hampshire, UK). Those selected for sampling had a BW that closely matched that of the pen average, and where possible, selected pigs within a replicate were littermates.

Following euthanasia, portal and peripheral blood samples (~7 mL) were drawn from the portal and the jugular vein respectively directly into 10 mL heparinised Vacutainers (BD, Oxford, UK) and immediately stored on ice. A 250 μL subsample of the whole heparinized peripheral blood was collected and stored at -80 °C pending Hb analysis. Within 30 min of collection, remaining blood samples were centrifuged at 1,500 x g for 15 min at 4 °C and the plasma fraction stored at -80 °C. Digesta were collected from the terminal ileum (approximately 60 cm anterior from the ileocecal valve to the ileocecal valve), immediately snap-frozen on dry ice to prevent post-collection $InsP_x$ degradation. The third metatarsal (M3) was obtained from the right foot and stored at -20 °C in a sealed zip-lock bag to prevent desiccation. The whole liver was excised, weighed, and a subsample (~ 3 cm x 3 cm) harvested from the left-lobe. Ice-cold PBS was used to remove blood contamination from the liver sample prior to storage at -80 °C. Mucosal scrapings were collected from the duodenum (~60 cm distal to the pyloric sphincter) for subsequent nutrient transporter gene expression analysis. To do this, a section of the intestine (~10 cm) was excised, cut longitudinally and the mucosa gently flushed with ice-cold PBS. Mucosal scrapings (~100 mg) were collected into 1 mL of Trizol (Thermo Fisher Scientific) using a sterile polyethylene spoon and immediately snap frozen in liquid N_2.

2.4. Laboratory Analysis

Freeze dried digesta and feed were ground to pass a 1 mm sieve prior to all analyses. Feeds were analyzed for phytase activity, inositol phosphate esters ($InsP_{2-6}$), MYO, Fe, Ca, P and Ti concentrations. Digesta were analyzed for $InsP_{2-6}$, MYO and Ti concentrations. Phytase analysis was performed by ESC (Ystrad Mynach, Wales, UK) according to the internal manufacturers assay for Quantum Blue (validated Standard Analytical Method, SAM020; AB

143 Vista). Calcium, P and Fe were analyzed by inductively coupled plasma optical emission
144 spectroscopy (ICP-OES). In brief, duplicate samples were dry ashed in a muffle furnace at 550
145 °C for 16 hr, digested in 10 mL of 5 M HCl, filtered, and the resulting filtrate analyzed on a
146 Thermo iCAP 7400 (Thermo Scientific). Inositol bis- to hexa- phosphate and MYO were
147 analyzed by high-performance ion chromatography (HPIC) and HPLC respectively, as
148 described in Laird et al. (2016). Titanium was analyzed according to the colorimetric method
149 of Short et al. (1996).

150 Haematocrit concentration of whole blood was assayed within 30 min of collection
151 according to the microhaematocrit method (INACG, 1985). Whole blood Hb was measured
152 colorimetrically using Drabkin's Reagent (#D5941; Sigma Chemical Co.) according to the
153 manufacturer's instructions. Plasma Fe was determined by ICP-OES following
154 deproteinization with dilute HNO_3. Portal and peripheral plasma were analyzed for MYO by
155 HPLC after deproteinization with acetonitrile. Peripheral plasma transferrin (Elabscience
156 Biotechnology Co. Ltd., China) and ferritin (Cloud-Clone Corp., China) were measured by
157 sandwich ELISA. The M3 bones were cleaned of adhering connective tissue, defatted in diethyl
158 ether for 72 h and oven dried at 100 °C for the determination of fat-free dry weight (ffdw).
159 Bone ash content was measured following incineration of the fat-free dry bone at 600 °C for
160 24 h. The resulting ash was assayed for Ca, P and Fe content by ICP-OES. Liver Fe was
161 measured using the ash produced after incineration at 600 °C for 24 h.

162 The duodenal mRNA expression profiles of selected nutrient transporter genes were
163 measured by real-time RT-PCR using a CFX-96 Real Time PCR Detection System (Bio-Rad
164 Ltd). Genes selected for expression analysis along with their template sequences or unique
165 assay id are presented in Table 2. Primers were purchased as predesigned PrimePCR Assays
166 (Bio-Rad Ltd.), with the exception of DMT1 and SMIT2 for which primers were designed using
167 NCBI Primer-Blast (Ye et al. 2012). All primers were purchased from Bio-Rad Ltd as pre-

optimized PrimePCR Assays. Total RNA was extracted using the Direct-Zol RNA MiniPrep Kit (Cambridge Biosciences), according to the manufacturer's instructions. The concentration and the purity of the extracted RNA was assessed using a NanoDrop ND1000 spectrophotometer (Thermo Scientific). RNA integrity was assessed visually via inspection of the 28S:18S ratio following agarose gel electrophoresis. Extracted RNA (2 μg) was converted into cDNA using random hexamer primers according to the protocol of the First Strand cDNA Synthesis Kit (Thermo Scientific).

PCR assays were performed in a total reaction volume of 20 μL comprising 4 μL of cDNA, 0.5 μL of each primer, 10 μL of SsoAdvanced universal SYBR Green (Bio-Rad Laboratories Ltd.) and 5 μL of nuclease-free water. PCR cycling was as follows: 95 °C for 2 min followed by forty cycles of 95 °C for 5 sec, 60 °C for 30 sec. All samples were run in triplicate, and primer specificity was confirmed by melt curve analysis. Relative mRNA abundance were normalised to the geometric mean of the reference genes ACTB, HMBS and HPRT1. Gene expression data are presented as calibrated normalised relative quantities, which were generated using the qbasePLUS package (Biogazelle).

2.5. Statistical Analysis

Data were analyzed as a randomized complete block design with a 3 x 2 factorial arrangement of treatments using the GLM procedure of SPSS Statistics (version 22.0, Chicago IL, US). The pen mean served as the experimental unit for all performance analyses, whereas the individual pig served as the experimental unit for all other analyses. The model included the effects of Fe, phytase and their associated interaction as fixed effects, and block as a random effect. Non-significant interactions were removed from the model and the main effects were analyzed individually. A Pearson's Product-Moment Correlation test was conducted to test for

192 associations between plasma MYO and weaner pig performance. Differences were classed as
193 significant if $P < 0.05$. Significantly different means were separated using the Tukey's HSD
194 test. Data are presented as least square means $\pm$ SEM.

195

196 **3. Results**

197 The analyzed nutrient composition, phytase activity and $InsP_{2-6}$ content of the experimental
198 diets is presented in Table 1. Phytase supplemented diets contained 568 and 2,520 FTU/kg
199 feed, which was close to targeted levels. Iron levels were higher than anticipated but attempts
200 to create a low-Fe and a high-Fe diet were achieved: L-Fe diets contained 105 mg Fe/kg feed,
201 which is marginally Fe deficient (~20 mg/kg below recommendation), and H-Fe diets
202 contained 320 mg Fe/kg which is considered much more than adequate (BSAS, 2003).

203 The effect of Fe and phytase supplementation on weaner pig growth performance is
204 presented in Table 3. There were no interactive effects between supplementary Fe and phytase
205 for ADFI or G:F ($P > 0.10$) throughout this study, whereas an interaction was observed for
206 ADG ($P < 0.05$). Weaners fed the L-Fe diet had a lower ADFI than those fed the H-Fe diet (P
207 < 0.01). Supplementary phytase tended to increase ADFI in a dose dependent manner ($P <$
208 0.10). Dietary Fe concentration had no significant influence on weaner G:F ratio, whereas
209 phytase improved it with increasing dose ($P < 0.01$). In diets devoid of added phytase, weaners
210 fed the L-Fe had a 23% lower ADG than H-Fe fed pigs ($P < 0.05$). Phytase at 2,500 FTU/kg
211 substantially improved weaner pig ADG when added to the L-Fe diet (34%; $P < 0.05$), to levels
212 comparable with H-Fe fed pigs; however, its effects were reduced (7%; $P > 0.05$) when added
213 to the H-Fe diet resulting in an Fe x phytase interaction ($P < 0.05$). The greatest BW was
214 achieved by piglets fed the H-Fe diet with 2,500 FTU/kg (13% greater than L-Fe 0 FTU/kg fed
215 pigs; $P < 0.05$). The standard phytase dose of 500 FTU/kg had no effect on weaner pig ADG

when added to the H-Fe diet, but numerically increased ADG (16%; $P > 0.05$), to a level not dissimilar from those receiving the 2,500 FTU/kg dose when added to the L-Fe diet.

The effect of Fe and phytase supplementation on weaner pig haematological status is summarised in Table 4. Pigs fed the H-Fe diet had a higher Hct ($P < 0.001$) and tended to have a high Hb concentration ($P = 0.051$) when compared with L-Fe fed pigs. Circulating levels of transferrin were higher in pigs fed the L-Fe diet than the H-Fe diet (45.02 vs 38.04 mg/mL; $P < 0.05$), whereas ferritin and Fe levels in the plasma increased in response to the 250 mg/kg increase in supplemental Fe content. Phytase had no significant effect on any of the blood response criteria measured, although the high phytase dose, when added to the L-Fe diet, numerically improved Hb, plasma ferritin and plasma Fe concentration to a level comparable with H-Fe fed pigs (data not shown).

Increasing supplementary Fe from 50 to 300 mg/kg resulted in 10% increase in bone Fe content ($P < 0.05$), but had no effect on bone ash, Ca or P concentrations (Table 5). In contrast, supplementary phytase increased bone ash ($P < 0.05$), Ca ($P < 0.05$) and P ($P < 0.01$) concentration when added at 2,500 FTU/kg, but, despite a large numerical increase, the effect on bone Fe was not significant. Total liver Fe content and liver Fe concentration were 83% ($P < 0.001$) and 68% ($P < 0.001$) higher respectively in pigs receiving the H-Fe treatment than the L-Fe treatment (Table 5). No liver Fe differences were observed between any of the phytase treatments. Gene expression of measured duodenal nutrient transporters were not influenced by phytase treatment in the present experiment (Table 5). However, weaners fed the L-Fe diet exhibited a 2.3 fold ($P < 0.01$) increase in duodenal DMT1 mRNA expression when compared with the H-Fe fed pigs.

The effect of Fe and phytase supplementation on ileal $InsP_6$ hydrolysis, $InsP_{2-6}$ and MYO concentration is presented in Table 6. Phytate hydrolysis occurring within the

gastrointestinal tract by the terminal ileum was influenced by an Fe x phytase interaction, similar to that observed for ADG ($P < 0.05$). In diets with supplementary phytase, dietary Fe concentration had no influence on ileal $InsP_6$ degradation; however, in diets devoid of added phytase, those receiving the H-Fe diet degraded 34% more phytate than those on the L-Fe diet ($P < 0.05$). Ileal $InsP_5$ concentration was influenced by an interaction similar to that described for $InsP_6$ degradability ($P < 0.01$). Dietary Fe concentration had no influence on ileal concentrations of $InsP_{2-4}$ or MYO. The standard dose of phytase numerically increased $InsP_4$ and $InsP_3$ content, whereas the 2,500 FTU/kg treatment reduced concentrations of these inositol phosphate esters ($P < 0.01$). Supplementary phytase at 2,500 FTU/kg increased ileal MYO concentration from 266 to 673 nmol/mg Ti ($P < 0.001$).

Dietary Fe concentration had no influence on plasma MYO concentration in the portal or peripheral blood (Table 7). Phytase, however, increased MYO in both the portal and peripheral plasma when supplemented at 2,500 FTU/kg ($P < 0.01$). Peripheral plasma MYO concentrations were approximately 1.7 to 1.8 fold higher than portal MYO concentrations ($P < 0.001$). Correlation analyses between plasma MYO and performance revealed a direct relationship between portal MYO concentration and ADG ($r = 0.365$; $P < 0.05$), as shown in Figure 1. There was no correlation between peripheral MYO and ADG ($r = 0.211$; $P = 0.160$) in the present experiment (data not presented).

4. Discussion

4.1. Growth performance

Pigs fed the low-Fe diet throughout the experiment had a lower ADFI and ADG compared with those fed the high-Fe diet. Studies looking into the effects of supplemental Fe on pig performance are surprisingly scarce; however, these results are in agreement with those of

Jolliff and Mahan (2011) who, using pigs of similar genetics, also found that young pigs are indeed responsive to dietary Fe concentration. In their study, weaned pigs (17 d) were offered a diet (intrinsic Fe = 200 mg/kg) supplemented with 0, 80 or 160 mg Fe/kg as $FeSO_4$ for 35 d. The authors reported a linear increase in ADG and d 21-35 ADFI in response to increasing Fe concentration. Similarly, Rincker et al. (2004) supplemented pig diets (3 phase dietary regime; intrinsic Fe 189, 224 and 98 mg/kg) with 0, 25, 50, 100 or 150 mg Fe/kg after weaning for 35 d and found linear improvements in ADG between d 7 and 21. However, in their study supplemental Fe had no effect on ADG for the whole experimental period. It is possible that in their longer study, the basal diet provided sufficient Fe in the latter stages of the trial due to a natural increase in feed intake. The NRC (2012) Fe requirement for weaned pigs stands at 80 mg/kg, a value derived from studies by Pickett et al. (1960) conducted over half a century ago. Although the NRC Fe recommendation was not tested in either of these studies, the observed improvements in performance to increasing supplemental Fe beyond 100 mg/kg may indicate that this recommendation requires re-evaluation for modern weaner pigs.

The results presented herein demonstrate that a high dose of phytase can improve the performance of young pigs fed a marginally Fe deficient diet. Weaner pig ADG was influenced by an interaction between supplemental Fe and phytase concentration, with phytase, particularly at the higher dose, having a greater effect when added to the low-Fe diet. It was originally speculated that the phytase induced performance benefit would be mediated through improved phytate bound-Fe availability. However, despite numerical increases in Hb, Hct, ferritin and plasma Fe with the high phytase dose, phytase had no significant influence on any of the measured indices of Fe status, suggesting that the observed performance benefit was occurring mainly through alternative mechanisms. It is noteworthy, however, that the observed interaction between supplemental Fe and phytase for ADG mirrors that observed for $InsP_6$ degradation (discussed below). Phytate is a potent anti-nutrient with the capacity to restrict

mineral (Tang et al., 2006; Schlegel et al., 2010), AA and energy availability (Liao et al., 2005), stimulate endogenous secretions and losses (Woyengo and Nyachoti, 2013; Dersjant-Li et al., 2015) and interfere with gastric proteolysis (Woyengo et al., 2010). Therefore, improvements in ADG may be attributed to the degree of phytate hydrolysis occurring within the gastro-intestinal tract, and the associated anti-nutrient ameliorative effect. Studies looking into the effects of phytase in combination with dietary Fe supply are limiting, thus, to the best of the authors knowledge, this study is the first to show that the magnitude of the phytase response is dependent on dietary Fe concentration.

4.1. Iron status

Up to 80% of the total Fe in the pig is associated with the Hb protein (Perri et al., 2015), thus both Hb and Hct provide an accurate measure of the total active Fe within the body (Crichton, 2006). It is well documented that Hb and Hct content in young pigs are sensitive to changes in dietary Fe supply (Miller et al., 1981; Rincker et al, 2004). In the current study, there was a significant 3.6% reduction in Hct and a tendency for a 1 g/dL reduction in Hb in low-Fe fed pigs. Despite the decline in Hb content with the low-Fe treatment, the degree of dietary Fe restriction was not severe enough to cause anaemia, as Hb concentrations remained within the 'adequate' reference range published by Thorn (2010). Moreover, pigs offered the high-Fe diet had a higher concentration of plasma Fe. Although this response is commonly observed in young pigs (Yu et al. 2000; Rincker et al., 2004; Hansen et al., 2009), plasma Fe measurements should be interpreted with caution as circulating Fe is sensitive to a range of factors, such as chronic infection, hypothyroidism and renal disease (Kaneko, 1980).

The use of Hct and Hb indices alone to assess an individual's Fe status has been criticised for failure to detect the early signs of Fe deficiency, such as a depletion in iron storage reserves (Smith et al., 1984). Depletion of hepatic Fe reserves is one of the first biochemical

changes associated with Fe insufficiency, and thus liver Fe provides a more sensitive assessment of Fe status than Hb and Hct measurements. Total liver Fe in the high-Fe fed pigs was in line with reported values for young pigs fed adequate-Fe diets (Hansen et al., 2009; Fang et al., 2013). Feeding the low-Fe resulted in a 40% reduction in liver Fe, indicating that the Fe content of ~100 mg/kg in this diet was not sufficient to prevent the onset of Fe-storage deficiency. Similar findings were reported by Furugouri (1972), and more recently Yu et al. (2000), both of who found linear reductions in liver Fe and ferritin concentrations in response to graded reductions in dietary Fe.

In the body, unbound Fe is considered cytotoxic owing to its ability to catalyse the production of cell damaging reactive free radicals via the Fenton reaction (Jenkins and Kramer, 1988). Consequently, most body Fe is found associated with proteins. Two proteins central in Fe homeostasis include ferritin and transferrin, the principle Fe storage and transport proteins respectively. Serum ferritin is directly proportional to Fe stores and therefore considered a sensitive measure of Fe status (Smith et al. 1984). Transferrin, a liver derived glycoprotein found mainly in the plasma, functions to transport Fe around the body as Fe^{3+} (Gkouvatsos et al., 2012). In contrast to ferritin, transferrin expression is inversely proportional to Fe status (McKnight et al., 1980). As expected, feeding a low-Fe diet increased plasma transferrin and reduced plasma ferritin. Similar findings were reported by Rincker et al. (2004) who found linear reductions in plasma transferrin, from 44.66 to 39.08 g/l, in response to increasing supplemental Fe from 0 to 150 mg/kg. These findings demonstrate that during periods of Fe insufficiency, young pigs respond by upregulating transferrin expression to mobilise Fe from storage to erythropoietic tissue, in order to prevent reductions in Hb synthesis

The body has a very limited capacity to excrete Fe, therefore, Fe homeostasis is predominantly regulated at the level of intestinal absorption (Hallberg and Hulthen, 2000). The majority of Fe absorption occurs at the proximal intestines via the apical proton-coupled

transporter divalent-metal transporter (DMT1), which absorbs Fe in the ferrous (Fe^{2+}) state (De Domenico et al., 2008). The DMT1 mRNA expression findings of the current experiment are consistent with those of others (Hansen et al., 2010; Fang et al., 2013; Espinoza et al., 2014) and show that pigs respond to Fe inadequacy by upregulating duodenal DMT1 expression in an effort to maximise Fe uptake from the gastro-intestinal tract.

4.3. Ileal phytate hydrolysis and MYO generation

Inositol phosphates were measured in the ileal digesta to test the hypothesis that high-doses of phytase improve Fe bioavailability through greater phytate hydrolysis. Interestingly, an unexpected interaction was observed between supplemental Fe and phytase on ileal phytate degradation. This interaction can be attributed to greater $InsP_6$ and $InsP_5$ hydrolysis occurring in the high-Fe fed pigs than the low-Fe fed pigs but only for diets devoid of supplemental phytase. Possible explanations for this effect remain unclear. It is, however, assumed that phytate hydrolysis occurring in pigs fed heat-treated diets originates primarily from the animals' endogenous mucosal phosphatases (Hu et al., 1996). Therefore, it is possible that Fe is acting as a necessary cofactor for mucosal phosphatases in pigs. If Fe is a necessary cofactor for endogenous phytases then it is possible that in the low-Fe fed pigs, available Fe was used preferentially for Hb synthesis, and as such, the activity of Fe-dependent enzymes diminished. This result supports the notion of Maenz (2001) who suggested that animal phytase enzymes likely require mineral cofactors for optimal activity.

The inhibitory properties of phytate on Fe absorption in monogastrics has been known for decades (Davies and Nightingale, 1975). It has been suggested that degradation to at least $InsP_2$ is necessary to alleviate the inhibitory properties of phytate on Fe digestion, as intermediate hydrolysis products $InsP_5$, $InsP_4$ and $InsP_3$ retain 73, 35 and 30% of the binding potential of $InsP_6$ to Fe^{3+} respectively (Yu et al., 2012). In the current experiment, the 500

FTU/kg phytase dose numerically increased ileal $InsP_4$ and $InsP_3$ concentrations, whereas the higher dose reduced them to levels below that of non-supplemented diets. Similar findings were reported by Holloway et al. (2016). Despite the more extensive phytate hydrolysis, the high phytase dose had little impact on the recorded measures of Fe status, and therefore these results do not support the original hypothesis. In contrary, Stahl et al. (1999) demonstrated that phytase dosed at 1,200 FTU/kg increased the availability of Fe for Hb repletion in anaemic weaner pigs. The use of anemic pigs in their study may explain the discord between these studies, as it is known that dietary Fe utilization is influenced by Fe status. In the current study, however, there were indications of phytase mediated improvements in Fe bioavailability: phytase tended to increase Hct, numerically reduced plasma transferrin and numerically increased Hb, plasma Fe, ferritin and bone Fe. Although none of these responses were significant at the $\alpha = 0.05$ level, drawing the conclusion that phytase had no effect on Fe bioavailability does not seem fully justified and warrants further attention.

High doses of phytase have been shown to improve pig performance, beyond that expected due to improvements in P nutrition (Santos et al., 2014). The underlying mechanism for these 'extra-phosphoric' effects remain unclear; however, there is a growing consensus that a combination of the alleviation of the anti-nutritional effects of phytate, and improved MYO bioavailability (Cowieson et al., 2011) are important. Evidence to support improved MYO bioavailability with high phytase dosing, particularly in the pig, is limited. However, this effect is clearly demonstrated in the current study with substantial increases in ileal and plasma MYO in response to 2,500 FTU/kg. Interestingly, plasma MYO in the non-supplemented and low phytase dose treatments were similar, which may be related to the bottleneck in phytate degradation observed beyond $InsP_{3\text{-}4}$ at this dose, and thus explain why performance benefits are not observed with lower phytase doses. Comparable increases in ileal MYO in response to 2,000 FTU/kg of the same enzyme were observed by Kühn et al. (2016) in both grower and

finisher pigs. These findings demonstrate that high levels of phytase stimulate complete phytate dephosphorylation within the pig gut. The observed increase in plasma inositol in the present study confirms that the liberated MYO is available for absorption and systemic distribution.

The mechanism of intestinal MYO absorption in mammals remains obscure. To the best of our knowledge no work on MYO transport proteins in the pig intestine has been published. The Na^+ dependent transport protein SMIT2 is thought to mediate all apical uptake of MYO in the rat intestine (Aouameur et al., 2007). Its expression has also been detected in the human intestine (Chen et al., 2010) although its contribution to intestinal MYO uptake is unclear. In this study, we have demonstrated the presence of SMIT2 in the duodenum of young pigs. Interestingly, the observed changes in MYO absorption were not met with changes in SMIT2 mRNA expression. Considering the dearth of information on SMIT2 it is difficult to clarify this finding; however, it is possible that SMIT2 is post-transcriptionally regulated, thus mRNA levels do not accurately reflect protein expression. Moreover, it may be that MYO absorption occurs predominantly in a different region of the intestine or via an alternative transporter.

Myo-inositol is a structural component of inositol phosphates and phosphoinositides and thus has important roles in cell trafficking and signal transduction (Lee and Bedford, 2016). The nutritional significance of dietary MYO is unclear; however, it evidently has an important role in neonatal development, with high levels commonly found in breast milk (Brown et al., 2009) and in the tissue of developing foetuses' and juveniles (Hamilton and Hogan, 1944; Pereira et al., 1990). This is supported by the work of Zyla et al. (2013), who found MYO improved broiler growth efficiency more effectively in the starter phase than in the grower phase. In the present experiment, MYO concentration in the portal plasma was positively correlated with ADG. As MYO concentration in the portal plasma is directly linked to the degree of phytate degradation, this relationship may be due to removal of the anti-nutritional

effects of phytate, the growth promoting properties of MYO, or both. This study confirms the link between MYO and growth performance in the pig, and thus provides support for the commonly postulated view that MYO plays an important role in the performance benefits observed with super-doses of phytase. Similar links between MYO and growth performance have been reported in broilers (Walk et al., 2014).

5. Conclusions

Super-dosing phytase improved the growth performance of young pigs fed a marginally Fe deficient diet. Feeding the low-Fe diet throughout the weaner phase reduced Hct, liver Fe, bone Fe, plasma transferrin, ferritin and Fe and elevated plasma transferrin. Despite these physiological changes, Hb content remained within a physiological 'normal' range, suggesting that during periods of sup-optimal supply, Fe is preferentially shunted from storage to erythropoietic tissue for Hb synthesis. The effectiveness of super-dosing phytase in improving low-Fe fed pig performance seemingly stemmed from greater phytate hydrolysis and improved MYO bioavailability. Although phytase had no significant effect on the recorded indices of Fe status, there were indications of improved Fe availability, particularly in the low-Fe diet. Further research to elucidate the effects of high doses of phytase on Fe bioavailability is warranted.

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581 **Table 1.** Composition and nutrient specifications of the experimental diets (%, as-fed basis)

Supplemental Fe (mg/kg)	50			300		
Phytase (FTU/kg)	0	500	2,500	0	500	2,500
Ingredient						
Wheat	36.41	36.40	36.36	36.28	36.27	36.23
Soybean meal	22.41	22.41	22.41	22.41	22.41	22.41
Micronized barley	15.00	15.00	15.00	15.00	15.00	15.00
Whey powder	6.94	6.94	6.94	6.94	6.94	6.94
Fishmeal	5.50	5.50	5.50	5.50	5.50	5.50
Micronized wheat	5.00	5.00	5.00	5.00	5.00	5.00
Full fat soyabean	3.00	3.00	3.00	3.00	3.00	3.00
Soya oil	2.49	2.49	2.49	2.49	2.49	2.49
Vitamin-mineral premix[2]	1.25	1.25	1.25	1.25	1.25	1.25
Lignosulphonate	0.63	0.63	0.63	0.63	0.63	0.63
Titanium dioxide	0.50	0.50	0.50	0.50	0.50	0.50
Benzoic Acid	0.50	0.50	0.50	0.50	0.50	0.50
Dicalcium phosphate	0.39	0.39	0.39	0.39	0.39	0.39
Salt	0.20	0.20	0.20	0.20	0.20	0.20
L-lysine HCL	0.155	0.155	0.155	0.155	0.155	0.155
$FeSO_4 \cdot 7H_2O$[2]	-	-	-	0.125	0.125	0.125
Phytase[3]	-	0.008	0.040	-	0.008	0.040
Pan-Tek® Robust[4]	0.015	0.015	0.015	0.015	0.015	0.015
Sucram®[5]	0.01	0.01	0.01	0.01	0.01	0.01
Calculated composition						
NE (MJ/kg)	10.2	10.2	10.2	10.2	10.2	10.2
CP	21.9	21.9	21.9	21.9	21.9	21.9
Ca	0.72	0.72	0.72	0.72	0.72	0.72
Total P	0.66	0.66	0.66	0.66	0.66	0.66
Available P	0.40	0.40	0.40	0.40	0.40	0.40
Analyzed composition						
Ca (%)	0.78	0.77	0.78	0.77	0.77	0.76
Fe (mg/kg)	107	105	102	315	329	342
Total P (%)	0.59	0.59	0.60	0.59	0.61	0.61
$InsP_6$ (nmol/g)	9,162	11,053	9,335	9,833	10,896	10,547
$InsP_5$ (nmol/g)	1,740	1,491	1,357	1,822	1,202	1,724
$InsP_4$ (nmol/g)	218	92	296	173	261	192
$InsP_3$ (nmol/g)	251	175	223	279	202	218
$InsP_2$ (nmol/g)	ND[1]	ND	ND	ND	ND	ND
MYO (nmol/g)	538	596	663	630	655	674
Phytase (FTU/kg)	147	592	2,230	116	543	2,810

582 [1] Vitamin and trace mineral premix provided per kg of diet: 12,500 IU vitamin A, 2,000 IU vitamin D_3, 60 IU
583 vitamin E, 4 mg vitamin K, 4.2 mg thiamine (B_1), 5.6 mg riboflavin (B_2), 5 mg pyridoxine (B_6), 50 µg
584 cyanocobalamin (B_{12}), 20 mg pantothenic acid, 40 mg nicotinic acid, 150 µg biotin, 1 mg folic acid, 50 mg Fe as
585 $FeSO_4$, 160 mg Cu, 2.2 mg I, 62 mg Mn, 0.3 mg Se, 100 mg Zn.
586 [2] $FeSO_4$ was supplemented to both diets via the vitamin-mineral premix to provide 50 mg Fe/kg. Additional Fe
587 was added to the basal diet as $FeSO_4$ at the expense of wheat to create the high-Fe test diet.
588 [3] Phytase was added to the basal diet at the expense of wheat to create the 500 and 2,500 FTU/kg test diets
589 respectively. The phytase enzyme used was Quantum Blue (AB Vista, Marlborough, UK)
590 [4] Flavouring additive supplied by Pancosma S. A. (Le Grand-Saconnex, Switzerland).

[5] Artificial sweetener supplied by Pancosma S. A. (Le Grand-Saconnex, Switzerland).

Table 2. Selected genes for real-time PCR and associated primer characteristics

Gene[1]	PrimePCR Unique Assay ID[2]	Amplicon size (bp)	Efficiency (%)
Reference genes			
ACTB	qSscCED0016579	110	97
HMBS	qSscCID001281	116	104
HPRT1	qSscCID0002342	100	110
Genes of interest			
DMT1	N/A[3]	195	104
SMIT2	N/A[4]	88	97

[1] ACTB = β-actin, HMBS = hydroxymethylbilane synthase, HPRT = hypoxanthine phosphoribosyltransferase 1, DMT1 = divalent metal transporter 1 and SMIT2 = Na/myo-inositol transporter 2

[2] ACTB, HMBS, HPRT and TFRC Primers were purchased as predesigned assays from Bio-Rad Ltd.

[3]DMT1 primer sequence: forward (5'to 3') AAGGTTCCGCGAATTATCCT, reverse (5' to 3') TAGCTTCCGCAAGCCATACT.

[4] SMIT2 primer sequence: forward (5'to 3') GTTTACTCGCCATGACCCCA, reverse (5' to 3') TGGTGTCCCGTTCTGAGAGA

612 **Table 3**. Effect of supplemental phytase and iron on weaner pig growth performance[1]

Fe (mg/kg)[2]	Phytase (FTU/kg)	Start BW (kg)	ADFI (g)	ADG (g)	G:F (g/kg)	End BW (kg)
50	0	7.5	288	189^{a}	691	11.4^{a}
50	500	7.6	287	219^{ab}	760	12.0^{ab}
50	2,500	7.5	308	253^{bc}	828	12.7^{bc}
300	0	7.6	312	247^{bc}	768	12.5^{bc}
300	500	7.6	311	246^{bc}	782	12.5^{bc}
300	2,500	7.6	331	264^{c}	817	12.9^{c}
	SEM	0.1	7.63	9.40	26.6	0.18
Main effects						
Fe	50	7.5	294	220	759	12.0
	300	7.6	318	252	789	12.6
	SEM	0.03	5.31	5.48	15.5	0.10
Phytase	0	7.6	300	218^{a}	729^{a}	11.9^{a}
	500	7.6	299	233^{a}	771^{ab}	12.2^{a}
	2,500	7.5	319	259^{b}	822^{b}	12.8^{b}
	SEM	0.03	6.50	6.55	19.0	0.13
P-value						
Fe		0.116	<0.01	<0.001	0.115	<0.001
Phytase		0.466	0.055	<0.001	<0.01	<0.001
Fe x Phytase		0.965	0.213	<0.05	0.250	<0.05

613 [1] Data are means of 8 replicate pens of 5 or 4 mixed sex pigs.
614 [2] Analyzed total Fe of the 50 mg Fe/kg and 300 mg Fe/kg diets were 105 mg/kg and 320 mg/kg
615 respectively.
616 [a-c] Means within a column that do not share a common superscript are significantly different ($P < 0.05$)
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633 **Table 4.** Effect of supplemental phytase and iron on weaner pig haematological status[1]

	Fe (mg/kg)[2]			Phytase (FTU/kg)				P-value	
	50	300	SEM	0	500	2,500	SEM	Fe	Phytase
Whole blood									
Haemoglobin, g/dL	10.0	11.0	0.38	10.2	10.5	10.9	0.47	0.051	0.495
Haematocrit, %	26.4	30.5	0.64	27.2	28.5	29.7	0.80	<0.001	0.085
Plasma									
Transferrin, mg/mL	45.0	38.0	2.22	43.0	42.4	39.1	2.82	<0.05	0.554
Ferritin, ng/mL	66.2	80.8	4.8	67.9	74.5	78.1	6.32	<0.05	0.395
Fe, µg/mL	1.10	1.37	0.09	1.14	1.21	1.36	0.11	<0.05	0.290

634 [1] Data are means of 8 replicate pigs.
635 [2] Analyzed total Fe of the 50 mg Fe/kg and 300 mg Fe/kg diets were 105 mg/kg and 320 mg/kg
636 respectively.
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Table 5. Main effects of supplemental phytase and iron on tissue mineral concentrations and on the normalised relative abundance of duodenal nutrient transporter mRNA[1]

	Fe (mg/kg)[2]			Phytase (FTU/kg)				P-value	
	50	300	SEM	0	500	2,500	SEM	Fe	Phytase
Bone									
Ash weight, %	37.21	37.04	0.42	36.42[a]	36.74[ab]	38.21[b]	0.52	0.781	<0.05
Ca, %	14.27	14.30	0.16	13.96[a]	14.21[ab]	14.69[b]	0.20	0.878	<0.05
P, %	6.67	6.69	0.07	6.49[a]	6.62[a]	6.93[b]	0.09	0.854	<0.01
Fe, mg/kg	77.5	85.2	2.31	78.4	80.1	85.6	2.86	<0.05	0.189
Liver									
Wet weight, g	434	459	9.2	452	451	436	11.4	0.056	0.950
Fe, mg/liver	18.66	34.24	1.92	24.88	28.90	25.58	2.40	<0.001	0.435
Fe, mg/kg DM	177.54	298.40	17.48	217.36	259.25	237.30	21.17	<0.001	0.372
Duodenal transporters									
DMT1[2]	1.88	0.83	0.19	1.37	1.33	1.36	0.23	<0.01	0.773
SMIT2[3]	1.23	1.35	0.17	1.22	1.46	1.09	0.20	0.852	0.445

[1] Data are means of 8 replicate pigs.
[2] Analyzed total Fe of the 50 mg Fe/kg and 300 mg Fe/kg diets were 105 mg/kg and 320 mg/kg respectively.
[a-b] Means within a row that do not share a common superscript are significantly different ($P < 0.05$)
[2] DMT1 = divalent metal transporter 1.
[3] SMIT2 = Na/myo-inositol transporter 2.

Table 6. Interactive effects of supplemental phytase and iron on ileal $InsP_6$ hydrolysis (%) and inositol phosphate ($InsP_{2-6}$) and myo-inositol concentration (nmol/mg Ti) in weaner pigs[1]

Fe (mg/kg)[2]	Phytase (FTU/kg)	$InsP_6$ hydrolysis (%)	$InsP_6$	$InsP_5$	$InsP_4$	$InsP_3$	$InsP_2$	MYO
50	0	9.9[a]	1584	312[a]	240	131	218	232
50	500	67.6[bc]	720	130[bcd]	319	234	332	426
50	2,500	78.1[c]	380	49[d]	100	93	313	853
300	0	44.2[b]	1060	212[b]	302	162	232	312
300	500	64.1[bc]	774	144[bc]	304	177	219	271
300	2,500	75.0[c]	495	64[cd]	162	101	252	495
	SEM	6.64	156.0	20.6	44.4	29.8	39.0	107.4
Main effects								
Fe	50	51.9	895	164	220	153	291	503
	300	61.1	777	140	256	147	234	355
	SEM	4.04	82.8	11.6	25.2	17.3	22.6	73.9
Phytase	0	27.0	1322[a]	262	271[a]	147[ab]	225	266[a]
	500	65.9	747[b]	137	311[a]	206[a]	275	348[a]
	2,500	76.5	437[b]	56	131[b]	97[b]	287	673[b]
	SEM	4.89	102.1	14.0	30.2	21.4	28.0	91.5
P-value								
Fe		0.108	0.302	0.146	0.328	0.804	0.082	0.518
Phytase		<0.001	<0.001	<0.001	<0.01	<0.01	0.244	<0.001
Fe x Phytase		<0.05	0.059	<0.01	0.618	0.419	0.274	0.135

[1] Data are means of 8 replicate pigs.

[2] Analyzed total Fe of the 50 mg Fe/kg and 300 mg Fe/kg diets were 105 mg/kg and 320 mg/kg respectively.

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

Table 7. Main effects of supplemental phytase and iron on portal and peripheral plasma myo-inositol concentration (nmol/mL) in weaner pigs[1]

		Blood	
Fe (mg/kg)[2]	Phytase (FTU/kg)	Portal	Peripheral
Fe	50	32.5	56.7
	300	31.3	56.5
	SEM	2.42	3.74
Phytase	0	25.2[a]	45.6[a]
	500	32.1[ab]	55.1[ab]
	2,500	38.3[b]	69.1[b]
	SEM	3.00	4.65
P-value			
Sampling site[3]		<0.001	
Fe		0.727	0.975
Phytase		<0.05	<0.01

[1] Data are means of 8 replicate pigs.
[2] Analyzed total Fe of the 50 mg Fe/kg and 300 mg Fe/kg diets were 105 mg/kg and 320 mg/kg respectively.
[3] Portal vs peripheral paired analysis (portal = 31.41 nmol/mL, peripheral = 56.89 nmol/mL).
[a-c] Means within column that do not share a common superscript are significantly different ($P < 0.05$).

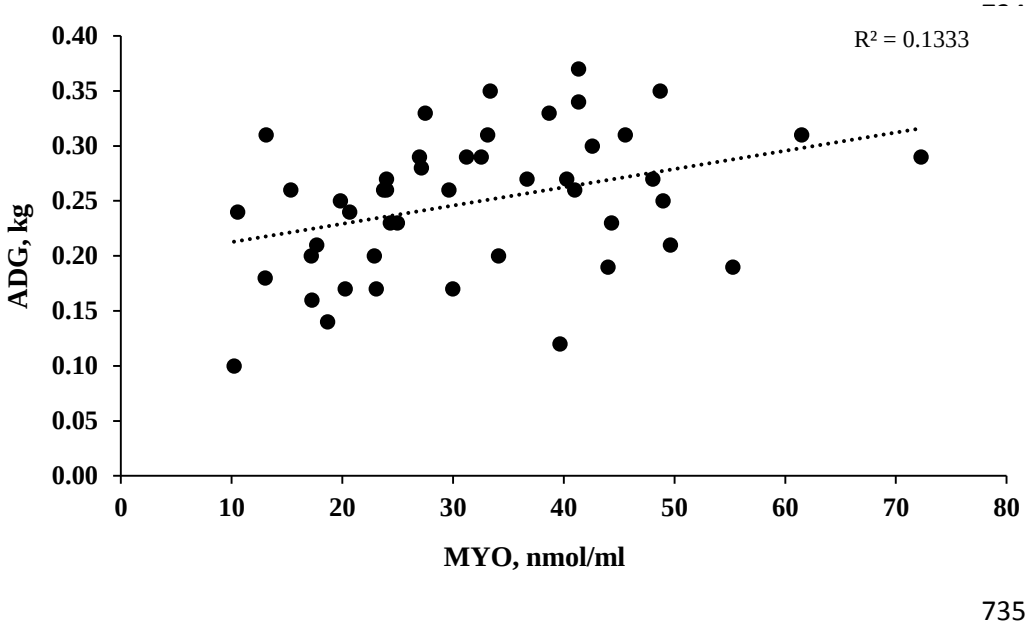

Figure 1. Relationship between portal plasma myo-inositol concentration and ADG in weaner pigs.

Values represent data from individual pigs (n = 48). Pearson product-moment correlation coefficient = 0.365; P-value < 0.05.