P and Ca digestibility is increased in broiler diets supplemented with the high phytase HIGHPHY wheat (*Triticum aestivum* L.)

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Short title: HIGHPHY phytase wheat in broiler diets

Abstract

Around 70% of total seed phosphorus is represented by phytate which must be hydrolysed to be bioavailable in non-ruminant diets. The limited endogenous phytase activity in non-ruminant animals make it common practice to add an exogenous phytase source to most poultry and pig feeds. The mature grain phytase activity (MGPA) of cereal seeds provides a route for the seeds themselves to contribute to phytate digestion, but MGPA varies considerably between species and most varieties in current use make negligible contributions. Currently, all phytases used for feed supplementation and transgenic improvement of MGPA are derived from microbial enzymes belonging to the group of histidine acid phosphatases (HAP). Cereals contain HAP phytases, but the bulk of MGPA can be attributed to phytases belonging to a completely different group of phosphatases, the purple acid phosphatases (PAPhy). In recent years, increased MGPAs were achieved in cis-genic barley holding extra copies of barley *PAPhy* and in the wheat HIGHPHY mutant, where MGPA was increased to ~6200 FTU/kg. In the present study, the effect of replacing 33%, 66% and 100% of a standard wheat with HIGHPHY wheat was compared to a control diet with and without 500 FTU of supplemental phytase. Diets were compared by evaluating broiler performance, ileal Ca and P digestibility and tibia development, using 9 replicate pens of 4 birds per diet over 3 weeks from hatch. There were no differences between treatments in any tibia or bird performance parameters, indicating the control diet did not contain sufficiently low levels of phosphorus to distinguish effect of phytase addition. However, in a comparison of the two wheats, the ileal Ca and P digestibility coefficients for the 100% HIGHPHY wheat diets are 22.9 and 35.6 % higher respectively, than for the control diet, indicating the wheat PAPhy is functional in the broiler digestive tract. Furthermore, 33% HIGHPHY replacement of conventional wheat, significantly

improved Ca and P digestibility over the diet supplemented exogenous phytase, probably due to the higher phytase activity in the HIGHPHY diet (1804 versus 1150 FTU). Full replacement by HIGHPHY gave 14.6 and 22.8 % higher ileal digestibility coefficients for Ca and P respectively, than for feed supplemented with exogenous HAP Phytase at 500 FTU. This indicates that *in planta* wheat PAPhys has promising potential for improving P and mineral digestibility in animal feed.

Keywords: phytase, wheat, broiler diet, P digestibility, Ca digestibility.

Implications (max 100 words)

Phytase is routinely added to broiler diets, but all current available enzymes are HAP phytases. This study shows that purple acid phosphatase phytase within HIGHPHY wheat stays functional in broiler mash feed and that HIGHPHY wheat significantly improves coefficients of Ca and P digestibility compared to both conventional wheat and conventional wheat supplemented with standard commercial levels of exogenous phytase. This study adds fundamental, initial data into the use of acid phosphatase phytase in broilers, and the use of plant breeding to improve phytase activity of grains.

Introduction

Around 70% of total seed P and 2-4% of the cereal seed dry weight is represented by phytate (Lott *et al.*, 2001). In order for phytate bound P to be bioavailable in non-ruminant diets it needs to be hydrolyzed. Phytases (myo-inositol hexakisphosphate 3- and 6-phosphohydrolase; EC 3.1.3.8 and EC 3.1.3.26) are phosphatases that can initiate the stepwise hydrolysis of phytate (InsP6, myoinositol-(1,2,3,4,5,6)-hexakisphosphate). Unfortunately, non-ruminant animals have limited phytase activity in their digestive tract (Morgan *et al*., 2015) so it is common practice to add an exogenous phytase source to most poultry and pig feeds (Dersjant-Li *et al*., 2015). Moreover, the mature grain phytase activity (MGPA) of cereal seeds varies considerably between species (Brinch-Pedersen *et al*., 2014). Non-triticeae tribe cereals like maize and oats have low MGPAs ranging from 15 to 42FTU/kg in maize and oats respectively (Eeckhout and De Paepe, 1994). Seeds of these species provide basically no contribution to phytate digestion in non-ruminant feed. In contrast, due to an evolutionary gene duplication and neo-functionalization of the major phytase gene, triticea tribe cereals have much higher MGPAs, ranging from 582 FTU/kg in barley (*Hordeum vulgare* L.), 1193 FTU/kg in wheat (*Triticum aestivum* L.) and tangible 5130 FTU/kg in rye (*Secale cereale* L.) (Eeckhout and De Paepe, 1994; Madsen *et al*., 2013). However, efficacy of intrinsic cereal phytase faces three challenges to efficacy in the gastrointestinal tract: high temperatures used in broiler feed processing (60-105°C) (Silversides and Bedford, 1999), degradation of the enzyme by pepsin secretions and the highly acidic pH of the proventriculus. It is therefore common practice to supplement the feed with an extrinsic enzyme selected for high efficacy under these conditions. Broiler diets are supplemented at a standard inclusion level of 500 FTU kg^{-1}, with the activity being based on the standard measurement at pH 5.5 (AOAC, 2000), although recent

evidence suggests further benefits may be derived through increasing the dose to 1500 FTU/kg (Walk *et al*., 2013). As alternative to supplementing the diet with phytase, the MGPA of the feed crop can be increased. This was achieved through *in planta* expression of microbial phytase in transgenic crops (Henrik Brinch-Pedersen *et al*., 2002). Soybean expressing *Aspergillus niger* phytase and maize expressing *E. coli* phytases both improved P digestibility when evaluated in broiler and pig feeding studies respectively (Denbow *et al*., 1998; Nyannor *et al*., 2007).

Currently, all phytases used for feed supplementation are microbial enzymes belonging to the group of histidine acid phosphatases (HAPs) (Lei *et al*., 2007). Similarly, microbial HAP phytases have been favored for increasing MGPA through transgene overexpression. However scientific initiatives in recent years have led to a substantially increased knowledge base on the complement of phytases in cereals. Cereals contain HAP phytases, but the bulk of MGPA can be attributed to phytases belonging to a completely different group of phosphatases, the purple acid phosphatases (Dionisio *et al*., 2011; Dionisio *et al.*, 2007). So far, experiences with this type of phytase (PAPhys) in animal feed are very limited (Brejnholt *et al*., 2011). In addition to being able to hydrolyze phytate, a successful feed phytase must be able to function under feed relevant conditions. This includes sufficient proteolytical resistance in the digestive tract and relevant pH and temperature profiles. For wheat grain PAPhy, the pH optimum is 5.5 ± 0.14 and the temperature optimum curve is quite broad, with optimum at 55°C ± 1.8°C. With phytate as substrate, the Km for wheat grain PAPhy is 35 µM (Dionisio *et al*., 2011). These biochemical parameters are in comparable range to two out of seven commercial phytase products evaluated in broiler feed simulation studies, the *Aspergillus niger* based phytase, Natuphos and the *Perniphora lycii* phytase, Ronozyme NP (Menezes-Blackburn *et al*.,

2015). Both products are today commonly used in broiler diets, although new generation commercial phytase additives tend to be derived from *E. Coli* or *Buttiauxella* spp. (Plumstead *et al*., 2012). So far, the potentials of wheat PAPhy have been supported using purified and recombinant PAPhy in feed simulations and by using standard wheat in broiler feeding trials (Brejnholt *et al*., 2011; Morgan *et al*., 2015). Unfortunately, the level of MGPA in standard wheat until recently has been too limited for efficient phytate P utilization in broiler feed.

In the recent years, increased MGPAs were achieved in cis-genic barley holding extra copies of barley *PAPhy* and in the wheat HIGHPHY mutant (Brinch-Pedersen *et al*., 2012; Holme *et al*., 2012). In cis-genic barley, the MGPA was increased to ~3500 FTU/kg but in HIGHPHY wheat, MGPA was increased to ~6200 FTU/kg, leading to increased interest in the viability of this cultivar as a feed wheat. In vitro investigation into heat stability of phytase in HIGHPHY grains has been previously estimated by incubating fine flour at 80 °C in 100 % relative humidity for 10, 20 and 40 min. In this setup, residual activity after 10, 20 and 40 min were 70, 42 and 22 %, respectively (Brinch-Pedersen *et al*., 2012). While this finding indicates PAPhy in HIGHPHY wheat fulfills the requirement for heat stability of commercially viable phytase enzymes, it is also vital to establish whether the PAPhy fulfils the second criteria of resistance to proteolytic degradation in the upper intestine before undertaking large scale evaluation of PAPhy efficacy in HIGHPHY wheat. Therefore, the aim of this study was to investigate the impact of substituting standard wheat with HIGHPHY wheat in broiler diets on phosphorus release from phytate in diets containing marginally low levels of available phosphorus.

Materials and Methods

Wheat materials

Wheat grains used in the feeding trial were standard field grown wheat *Triticum aestivum* L. cv Skagen with a phytase activity on 1060 FTU/kg and HIGHPHY *Triticum aestivum* L. with a phytase activity on 6196 FTU/kg.

Birds and Husbandry

Institutional and UK national NC3R ARRIVE guidelines for the care, use and reporting of animals in research (Kilkenny *et al*., 2010) were followed and all experimental procedures involving animals were approved by the University's College of Arts and Science ethical review committee.

Male Ross 308 broilers (n = 180) from a 43-week-old breeder flock were obtained from a commercial hatchery at day of hatch. Chicks were placed in groups of 4 per pen, bedded on clean wood shavings and randomized by weight across treatment groups. Birds were allowed ad libitum access to the treatment diets and water for the duration of the trial. The room was thermostatically controlled to produce an initial temperature of 32°C on d1 and reduced to reach 21°C by day 14 based on bird behaviour. The lighting regime was set to 23 hours on day one and reduced by one hour per day until day 6, where 18 hours of light (in two blocks, including an uninterrupted 4-hour stretch of darkness) was maintained for the remainder of the study. All birds sampled were euthanized by cervical dislocation on d21 post-hatch.

154 *Dietary Treatments*

155 Birds were fed mash diets from d0 to d21. Diets were commercially formulated by a UK-
156 based specialist nutrition solution company, using a matrix based on the Avian Ross 308
157 guidelines. The five dietary treatments were based on a control diet containing a putative
158 marginally low P supply with standard wheat, no added phytase and no HIGHPHY wheat. A
159 phytase containing, positive control was added to allow comparison with commercial
160 standards, which provided an easily adequate P supply through use of standard wheat, with
161 500 FTU/kg Quantum Blue Phytase but again with no HIGHPHY wheat. Three further diets
162 were as per control but with replacement of standard wheat with HIGHPHY wheat at either
163 33%, 66% or 100%. There were 9 replicate pens per diet.

164 Diet formulations are presented in Table 1. Diets were mixed in house using a ribbon mixer.
165 Titanium dioxide was added to all diets at 5g/kg inclusion as an inert marker for digestibility
166 measures. All diets were analyzed for dry matter and protein content (calculated as nitrogen
167 multiplied by 6.25) by the AOAC standard methods (930.15 and 990.03, respectively) and
168 gross energy (via bomb calorimetry; Robbins and Firman, 2006). Amino acid content of diets
169 and protein sources was determined using a Biochrom 30 amino acid analyser based on ion
170 exchange chromatography. Briefly, samples were oxidised with performic acid prior to acid
171 hydrolysis with nor-leucine added as an internal standard, and then analysed against
172 prepared standards. P, Ca and titanium dioxide content of the diets were analyzed by
173 inductively coupled plasma-optical emission spectroscopy (ICP-OES) following an aqua
174 regia digestion step (AOAC 985.01, Leytem *et al*. 2006; Morgan *et al*., 2014). Analyzed
175 values for protein, amino acids, DM, energy and mineral content are shown in Table 2. Total
176 phytate content was analyzed by a K-Phyt assay kit (Megazyme^{TM}, Wicklow, Ireland, UK)
177 which quantitatively measured available phosphorus release from the samples. Briefly,

178 inositol phosphates were acid extracted followed by treatment with a phytase specific for
179 IP_6-IP_2 and alkaline phosphate added to ensure release of the final phosphate from myo-
180 inositol phosphate (IP_1). The total phosphate released was measured using a modified
181 colorimetric method and expressed as grams of phosphorus per 100 g of sample material.
182 Phytase activity was analyzed according to the method of Engelen *et al*. (2001). Dietary
183 phytase levels were approximately 600, 1050, 1800, 4000 and 6000 FTU/kg (Table 2). The
184 diets had total phytate levels ranging from approximately 10-12g/kg DM (Table 2), dietary
185 Ca levels of approximately 7.8g/kg DM (as dicalcium phosphate and limestone) (Table 1)
186 and non-phytate-P levels of approximately 2.48g/kg DM (Table 2).

187 *Response Variables*

188 Birds were weighed by pen on arrival and d7, 14 and 21 and fed from individual bags to
189 allow feed intake to be measured.

190 On d21 birds were euthanised and ileum digesta contents from all birds per pen were
191 collected by gentle digital pressure into one pot per section of tract per pen. Digesta samples
192 were freeze dried and ground to a fine powder before analysis. Calcium, phosphorus and
193 titanium dioxide content of the digesta was determined by ICP-OES following aqua regia
194 digestion as previously discussed for the diets. The following equation was then used to
195 calculate total Ca, P or Ti content:

196 (Ca, P or Ti in sample (mg/L))*(volume of sample (ml)/ weight of sample (g))/1000. The
197 apparent ileal digestibility coefficient was calculated by:

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

199 Gross energy content of the digesta was measured as described previously for diets and
200 apparent ileal metabolisable energy (AME) was calculated by the following equation:

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

Nitrogen content of the digesta was analysed by Dumas method and metabolisable nitrogen was calculated using the following equation:

Diet N - Digesta N*(Diet Ti/Digesta Ti). AME was also corrected to zero N balance (AMEn) using the figure of 34.4Kg/g N retained as detailed by Hill and Anderson (1958).

Tibia bones (separated at the tibiotarsal junction and the tibiofemoral junction) were collected from the left leg of three birds per pen. Flesh and adherent tissue was carefully removed by hand leaving the cartilage caps intact. Bone strength of all tibia bones was analysed using a TA.XT plus texture analyser (Stable Microsystems, Guildford, UK) set up with a 50kg load cell and 3 point-bend fixture. The texture analyzer was set to measure force in compression with the test speed set at 1mm/sec, and trigger force set at 7g (0.069N). Supports of the fixture were set at 26mm to accommodate for the bone length. The texture analyser was calibrated using a 5kg weight. The defleshed bone was placed on the fixtures, a test was run and the peak force in Newtons was recorded.

Following analysis for breaking strength, the tibias were defatted by the Soxhlet method for 6 hours (Soxtherm, C. Gerhardt UK Ltd). The defatted tibias were oven dried at 105°C for constant weight. The dried samples were then cooled and weighed into a pre-weighed ceramic crucible, ashed in a muffle furnace for approximately 14 hours at 650°C, cooled in a desiccator and then reweighed. Bone ash was calculated as ash weight as a percentage of dry bone weight.

Calculations

Statistical analysis of data

Statistical analysis was carried out using SPSS v.22. After KS testing to confirm normality, data were analysed using one-way ANOVA to test the equality of the means to investigate the effect of dietary treatment on performance, tibia strength and mineralisation, ileal Ca and P digestbility and phytate hydrolysis. Statistical significance was declared at $p<0.05$. Duncan post hoc testing was used to elucidate differences between diets.

Results and discussion

In the current study, the high phytase wheat, HIGHPHY was evaluated in a broiler feeding experiment. The MGPA phytase activity of HIGHPHY and conventional control wheat derives from the purple acid phosphatase phytase *PAPhy _ a* gene expressed during wheat grain development. Here for the first time, increasing levels of plant derived PAPhy phytase are evaluated and compared to a standard wheat supplemented with commercial HAP phytase, supplied via the enhanced *E. coli* HAP phytase product Quantum Blue

Mortality across the trial was 1.1% (2 birds), with no losses from any diet containing the HIGHPHY wheat, indicating there is no overall negative effect of the novel wheat cultivar on bird health. The feed intake, body weight gain and feed conversion rate (FCR) were evaluated weekly and cumulatively after 21 days of feeding and provide further evidence (Table 3) that the HIGHPHY wheat has no detrimental effect on health. The performance values recorded are poorer than would be expected for the age and strain of bird, due to the diets being fed as mash, increasing the feeding time for the same quantity of diet (Amerah *et al*., 2007). This reduction in performance may also effect bone size and strength, although bone ash was corrected for dry tibia weight, and mineral content

calulated as a proportion of tibia ash to reduce the effect of any discrepancies. Relatively large differences in FCR did not elicit a significance when analysed, as the small number of birds per pen, reduces the power of the facility when determining performance measures. However, although not significant, bird weight gain was highest for broilers fed diets where 100% of the standard wheat was replaced by HIGHPHY wheat, and FCR was incrementally improved with increasing inclusion of HIGHPHY.

Determination of AME and AMEn (of digesta, see table 3) did not reveal any significant differences between diets. Dietary analysis indicated nutritionally relevant differences in protein and phosphorus levels between the two wheats which may be shown to be impactful over longer feeding periods. As IP6 reacts with dietary proteins to form aggregates which are less accessible to proteases (Cheryan, 1980), protein digestion can be adversely affected by the presence of phytate (Vaintraub and Bulmaga, 1991). Therefore, the high protein content of the HIGHPHY wheat is worthy of further investigation via amino acid digestibility assessment. However, the analysed amino acid composition of the diets were very variable in this study, particularly for asp, which was lower than would have been expected for the 67% HIGHPHY diet. The analysed lysine was also notably low for the 33% HIGHPHY diet which may have compromised bird performance on this treatment. These values require further investigation in any future studies.

Interestingly, diet had no effect on tibia bone length, width, weight, strength or tibia mineral content (Table 4). This indicates that the level for marginally adequate phosphorus provision used can be set lower in experimental settings and that the study does not reveal the full potential of the experimental diets. Differences in individual bird bodyweight may have compromised these strength and size measures, as the larger birds would be

expected to have larger and stronger bones, increasing the number of birds analysed would potentially reduce this variability and therefore elucidate differences in bone strength. However, table 4 shows that coefficients of digestibility for both Ca and P were significantly improved by all phytase-containing diets over the non-phytase control diet. The data presented in table 4 shows that the HIGHPHY MGPA has a significant, positive impact on the amount of both Ca and P digested in the ileum at d21. The wheat PAPhy is functional in the broiler digestive tract and significantly more P and Ca were digested in birds fed diets containing 100% HIGHPHY wheat compared to those fed any other diet (Table 4). In a comparison of the two wheats, the ileal Ca and P digestibility coefficients for the 100% HIGHPHY wheat diets are 22.9 and 35.6 % higher, respectively, than for the control diet. Furthermore, 33% HIGHPHY replacement of conventional wheat, significantly improved Ca and P digestibility over the exogenous phytase supplemented diet. This finding may be explained by the phytase activity levels within each diet: table 2 shows 33% replacement of conventional wheat with HIGHPHY results in a substantially higher phytase activity levels than the diet containing exogenous HAP phytase (1804 versus 1150 FTU). However, it is important to note that the diets is this study were not formulated to be ideal for the exogenous phytase, as phytate can form insoluble salts in the ileum when Ca is higher, as it is in this study. Further improvements in phosphorus digestibility when phytase level is increased beyond the commercial level of 500 FTU ('super-dosing') are well established in poultry (Walk *et al*., 2013). Full replacement by HIGHPHY gives 14,6 and 22,8 % higher ileal digestibility coefficients for Ca and P respectively, than for feed supplemented with exogenous HAP Phytase. Strangely, although the intermediate replacement level (66% HIGHPHY) improves Ca and P digestibility over the control diet, it does not improve Ca and P digestibility compared to either the 33% HIGHPHY diet (lowest

293 replacement level), or the diet supplemented with exogenous HAP Phytase. These results
294 require further investigation.

295 The pH optimum for wheat grain PAPhy, is 5.5 which is higher than the optimum pH for
296 exogenous phytase used in this study (pH optima 4.5). It has been suggested that 60% of
297 phytate remains after the gizzard and may be hydrolyzed further along the gastrointestinal
298 tract (Morgan *et al*. 2015), and a higher pH optima may facilitate this phytate breakdown in
299 the small intestine where the pH tends to be higher. It may be that this PAP phytase will
300 have a synergistic effect on phytate degradation when fed in conjunction with a traditional
301 HAP phytase.

302 The in vitro investigations previously investigating temperature optimum curve of PAP
303 phytase (Brinch-Pedersen *et al.,* 2012). are not directly comparable to heat treatments
304 during feed production but indicate that HIGHPHY phytase activity can resist certain
305 temperature and moist treatments. When considered alongside findings from the current
306 study, there is evidence to justify further experiments establishing heat stability during feed
307 production for HIGHPHY to enable its full incorporation into pelleted pig and poultry diets.
308 Improvement of P and mineral digestibility in feed and food are challenging tasks.
309 However, given the severity of phosphate resource problem, environmental problems with
310 leaching of undigested phytate P and micronutrient deficiencies, the task can easily be
311 justified. Scientific initiatives in recent years have led to a substantially increased
312 knowledge base on the complement of phytases in cereals that can form the basis for
313 integrating nutrition, breeding, molecular biology and genetics. In the current article, we
314 have evaluated wheat with high MGPA in a broiler diet and found that that just 33%
315 replacement of standard wheat with HPW is required to significantly improve Ca and P
316 digestibility coefficients compared to conventional supplementation with exogenous

317 phytase. Replacement of standard wheat by 100% HIGHPHY further improved both Ca
318 and P digestibility. This indicates that *in planta* plant PAPhys has a promising potential for
319 improving P and mineral digestibility in animal feed, particularly where there are barriers to
320 the use of genetically modified plants or supplements.
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Table 1 Dietary formulations (%)

Diet	Control	Control + 500 FTU phytase	Control with 33% HPW[1]	Control with 67% HPW[1]	Control with 100% HPW[1]
Standard Wheat	56.71	56.70	37.61	18.61	0
Extruded Soya, 48% pr	35.00	35.00	35.00	35.00	35.00
Soya oil	3.78	3.78	3.78	3.78	3.78
Limestone	1.28	1.28	1.28	1.28	1.28
Salt	0.17	0.17	0.17	0.17	0.17
Sodium bicarbonate	0.26	0.26	0.26	0.26	0.26
Monocal phosphate, HCL	1.23	1.23	1.23	1.23	1.23
Lysine HCl	0.21	0.21	0.21	0.21	0.21
Methionine	0.32	0.32	0.32	0.32	0.32
Threonine	0.13	0.13	0.13	0.13	0.13
Econase XT	0.01	0.01	0.01	0.01	0.01
Quantum Blue Phytase	0	0.01	0	0	0
Vitamin Mineral Premix*	0.40	0.40	0.40	0.40	0.40
High Phytase Wheat	0	0	19.10	38.10	56.71
Titanium dioxide	0.5	0.5	0.5	0.5	0.5

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

[1] Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet)

398 **Table 2** Analysed content of diets and grain

Diet	Control	Control + 500 FTU phytase	Control with 33% HPW[1]	Control with 67% HPW[1]	Control with 100% HPW[1]	HIGHPHY Wheat	Control Wheat
DM (g/kg)	879.70	880.43	888.38	878.85	908.67	890.22	889.04
Ash (g/kg)	61.86	59.83	58.11	58.39	62.47	17.20	16.94
Protein (g/kg DM)	267.28	269.23	272.84	274.46	276.93	127.73	163.20
GE (MJ/kg DM)	19.61	19.62	20.27	20.53	20.45	18.73	18.94
Ca (g/kg DM)	7.83	7.96	7.73	7.82	7.82	0.93	0.80
P (g/kg DM)	5.84	5.70	5.24	5.55	5.58	3.86	2.37
Phytate (g/kg DM)	10.14	10.15	10.22	12.07	11.92	3.18	3.40
Phytate-P (g/kg DM)[2]	2.86	2.86	2.88	3.40	3.36	2.59	0.96
Non-phytate-P (g/kg DM)[3]	2.98	2.84	2.36	2.15	2.07	1.27	1.41
Total Phytase Activity (FTU/kg)[4]	605	1150	1804	3954	5925	1060	6196
Determined amino acid content (g/kg)							
CYS	6.031	5.398	5.437	6.448	6.963	5.544	4.444
ASP	17.610	17.147	15.642	12.492	21.286	6.497	6.454
THR	7.561	7.484	6.684	8.061	9.034	3.577	3.056
SER	8.431	8.334	7.765	9.909	10.169	5.678	4.494
GLU	42.077	38.556	38.324	44.916	50.346	39.656	30.748
GLY	7.945	7.897	7.624	8.307	9.563	5.169	4.302
ALA	7.830	7.815	7.485	8.185	9.435	4.445	3.797
VAL	9.229	9.037	8.533	9.151	11.016	5.836	4.533
MET	8.352	9.158	7.722	9.033	14.687	3.961	3.516
ILE	8.125	8.471	7.211	8.195	9.473	4.810	3.715
LEU	13.243	13.618	12.296	14.205	15.794	8.815	6.706

TYR	3.538	4.799	4.986	4.872	5.094	2.184	1.466
PHE	8.908	9.409	8.567	9.705	10.797	5.997	4.446
LYS	10.534	10.822	9.757	10.870	12.508	3.531	3.255
HIS	5.159	4.612	4.328	4.436	6.157	2.402	2.872
ARG	12.087	11.590	10.841	11.704	13.916	4.679	5.641

399 [1] Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet)

400 [2] Phytate-P was calculated as 28.2% of phytate (Tran and Sauvant, 2004).

401 [3] Non-phytate P was calculated as the difference between total P and phytate-P.

402 [4]Total phytase activity was analysed by a colorimetric enzymatic method and calculated as (net
403 optical density at 415nm*dilution volume)/(slope of standard curve*mass*incubation time) (Engelen
404 *et al*. 2001).

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411 **Table 3** Feed intake (FI), bodyweight gain (BWG) and feed conversion ratio (FCR), AME
412 and AMEn from birds fed varying replacement levels of HIGHPHY wheat from d0-21

Treatment	FI/bird (g)	BWG/bird (g)	FCR	AME (MJ/kg)	AMEn
Control	1238.0	764.5	1.63	12.8	11.6
Control plus 500FTU phytase	1162.3	722.2	1.57	12.9	11.8
Control with 33% HIGHPHY[1]	1139.0	737.8	1.54	13.0	11.9
Control with 67% HIGHPHY[1]	1124.2	726.9	1.49	13.2	12.0
Control with 100% HIGHPHY[1]	1073.9	790.4	1.46	13.1	11.9
SEM	24.03	11.47	0.030	0.17	0.17
p value	0.070	0.268	0.317	0.492	0.543

413 [1] Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet)

Table 4 Tibia bone measures and mineral content and coefficient of apparent ileal digestibility of Ca and P in birds fed varying replacement levels of HIGHPHY wheat at d21

Treatment	Bone parameters		Tibia Mineral content (g/dry tibia)		Apparent Ileal Digestibility Coefficient	
	% Tibia Ash	Tibia Strength (N)	Ca	P	Ca	P
Control	39.83	135.07	307.3	118.4	0.567[d]	0.561[d]
Control plus 500FTU phytase	39.92	119.9	298.2	116.9	0.608[c]	0.615[c]
Control with 33% HIGHPHY[1]	39.71	128.67	307.4	121.5	0.645[b]	0.703[b]
Control with 67% HIGHPHY[1]	39.93	127.18	287.4	113.3	0.618[bc]	0.644[c]
Control with 100% HIGHPHY[1]	40.13	131.8	314.3	122.9	0.697[a]	0.755[a]
SEM	0.462	5.144	12.68	4.81	0.0106	0.0168
p value	0.987	0.416	0.612	0.643	<0.001	<0.001

[1] Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet