JAAN

Wageningen Academic Publishers

Journal of Applied Animal Nutrition, 2022 online **ARTICLE IN PRESS** **OPEN ACCESS**

Effect of phytase supplementation on plasma and organ *myo*-inositol content and erythrocyte inositol phosphates as pertaining to breast meat quality issues in chickens

H. Whitfield[1], C. Laurendon[1], S.J. Rochell[2], S. Dridi[2], S.A. Lee[3], T. Dale[3], T. York[3], I. Kuehn[4], M.R. Bedford[3] and C.A Brearley[1*]

[1]School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, United Kingdom; [2]University of Arkansas, Center of Excellence for Poultry Science, University of Arkansas, 1260 W. Maple, POSC O-406, Fayetteville, AR 72701, USA; [3]AB Vista, Woodstock Ct, Marlborough, Wiltshire, SN8 4AN, United Kingdom; [4]AB Vista, Feldbergstrasse 78, 64293 Darmstadt, Germany; c.brearley@uea.ac.uk

Received: 5 October 2021 / Accepted: 25 January 2022

RESEARCH ARTICLE

POULTRY

Abstract

'Woody breast' (WB) and 'white striping' in broiler meat is a global problem. With unknown etiology, WB negatively impacts bird health, welfare and is a significant economic burden to the poultry industry. New evidence has shown that WB is associated with dysregulation in systemic and breast muscle-oxygen homeostasis, resulting in hypoxia and anaemia. However, it has been observed that phytase (Quantum Blue (QB) a modified, *E. coli*-derived 6-phytase) super dosing can reverse dysregulation of muscle-oxygen homeostasis and reduces WB severity by ~5%. The objective of this study was to assess whether levels of Ins(1,3,4,5,6)P_5, the main allosteric regulator of haemoglobin, are influenced by changes in plasma *myo*-inositol arising from super dosing with phytase. To enable this, methods suitable for measurement of *myo*-inositol in tissues and inositol phosphates in blood were developed. Data were collected from independent trials, including male Ross 308 broilers fed low and adequate calcium/available phosphate (Ca/AvP) diets supplemented with QB at 1,500 phytase units (FTU)/kg, which simultaneously decreased gizzard InsP_6 (P<0.001) and increased gizzard *myo*-inositol (P<0.001). Similarly, male Cobb 500 broiler chicks fed a negative control (NC) diet deficient in AvP, Ca and sodium or diet supplemented with the QB phytase at 500, 1000 or 2,000 FTU/kg increased plasma (P<0.001) and liver (P=0.007) *myo*-inositol of 18d-old birds at 2,000 FTU/kg. Finally, QB supplementation of Cobb 500 breeder flock diet at 1,250 FTU/kg increased blood *myo*-inositol (P<0.001) and erythrocyte Ins(1,3,4,5,6)P_5 (P=0.011) of their 1d-old hatchlings. These data confirmed the ability of phytase to modulate inositol phosphate pathways by provision of metabolic precursors of important signalling molecules. The ameliorations of WB afforded by super doses of phytase may include modulation of hypoxia pathways that also involve inositol signalling molecules. Elevations of erythrocyte Ins(1,3,4,5,6)P_5 by phytase supplementation may enhance systemic oxygen carrying capacity, an important factor in the amelioration of WB and WS myopathy.

Keywords: broiler, inositol, phytase, meat quality

1. Introduction

Woody breast (WB) meat is a myopathy that poses a threat to global food security, first described as hard, out-bulging tissue with pale areas and white striping on the ventral surface of the *pectoralis major* of broilers (Sihvo *et al.*, 2014). The exact etiology is not known, however, there is evidence that WB causes degeneration and necrosis of muscle tissue, connective tissue and fat cells (Sihvo *et al.*, 2014). Recent mechanistic data indicated that the WB

myopathy is related to systemic and local breast muscle hypoxia (Greene *et al.*, 2019). Given that hypoxic conditions can limit the regenerative capacity of muscle, preferentially replacing degenerated fibres with lipid and fibrotic tissue (Hoppeler and Vogt, 2001), this is plausible.

Studies have shown significant impacts on the health and welfare of affected modern broilers leading to on-farm culling and mortality, resulting in higher economic costs to the global poultry industry (Cauble *et al.*, 2020). It has been estimated that the cost to the US poultry industry is more than US$200 million per year (Kuttappan *et al.*, 2016). A myriad of factors contribute to these, including losses: condemnation, down-grading, lower meat yield and decreases in protein content combined with increases in fat and collagen content. Rejection at the consumer level due to poor appearance of the meat resulting from the negative meat characteristics is common (Chatterjee *et al.*, 2016; Kuttappan *et al.*, 2012; Mudalal *et al.*, 2015; Tijare *et al.*, 2016).

Phytic acid [*myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); InsP_6] is a well-established anti-nutrient in plant feedstuffs (Harland and Oberleas, 1987), present as phytate (any salt of InsP_6) which stores phosphorus (P). Availability of P to chickens is dependent on the breakdown of InsP_6 into less phosphorylated phosphates and *myo*-inositol. Phytase enzymes are routinely added to the diets of non-ruminants to increase the digestibility of amino acid- and phosphate-containing components of animal feed (Agbede *et al.*, 2009; Cowieson *et al.*, 2006; Ingelmann *et al.*, 2018). The efficacy of phytase is commonly assessed in animal feeding, and has led to the observation that increases in animal performance are often greater than that expected from release of phosphate alone (Walk *et al.*, 2013). It has been posited that these extra-phosphoric effects may arise from the liberation of *myo*-inositol from phytate into the gastrointestinal tract (Gonzalez-Uarquin *et al.*, 2020; Walk *et al.*, 2018). It has been shown that the Quantum Blue (QB) phytase enhances haematological parameters in channel catfish by improving the expression of oxygen-sensing genes (Peatman and Beck, 2016). In addition, QB supplementation can reduce the severity of WB by over 5%, reversing the dysregulated expression profile of oxygen-homeostasis related genes in myopathic birds which cause low oxygen and haemoglobin levels that increase WB severity (Greene *et al.*, 2019). Several studies have related increases in plasma *myo*-inositol with increasing phytase (Ajuwon *et al.*, 2020; Cowieson *et al.*, 2017; Schmeisser *et al.*, 2017; Sommerfeld *et al.*, 2018a,b) and a recent study (Gonzalez-Uarquin *et al.*, 2020) reported increases in kidney inositol by supplementing 1,500 phytase units (FTU)/kg phytase. However, the metabolic consequences for particular tissues and organs of increased *myo*-inositol provision by enzymes are not wholly defined – not least because of the considerable synthetic capacity of liver and kidney tissue for de novo *myo*-inositol biosynthesis (Hasegawa and Eisenberg Jr., 1981). The effect of dietary phytase on gene expression of *myo*-inositol transporters in the small intestine, liver, kidney and hepatic function has been demonstrated (Gonzalez-Uarquin *et al.*, 2020; Pirgozliev *et al.*, 2019; Walk *et al.*, 2018).

Given these findings, it is plausible that changes in tissue inositol phosphates accompany changes in *myo*-inositol status. The measurement of endogenous inositol phosphates in animal tissues and the establishment of their relationship with *myo*-inositol is problematic without recourse to metabolic labelling with radioactive precursors. A method using post-column complexation of inositol phosphate with ferric ion in perchloric acid and UV detection at 290 nm (Phillippy and Bland, 1988) is widely used for measurement of inositol phosphates in seeds, grains and beans (Raboy, 2003) and digestive tract chymus (Beeson *et al.*, 2017; Pontoppidan *et al.*, 2012; Sommerfeld *et al.*, 2018a,b; Zeller *et al.*, 2015, 2016). In the following trial, this method was used to measure inositol phosphates in chicken erythrocytes and a HPLC method is described for measurement of *myo*-inositol therein.

The objective of the following study was to determine whether super dosing of phytase liberated *myo*-inositol from phytate, which can then be taken up by the blood plasma to support Ins(1,3,4,5,6)P_5 production in chicken erythrocytes to enhance oxygen availability and help alleviate WB myopathy.

2. Materials and methods

The studies were approved by the Animal Care and Use Committee of the University of Arkansas, under Protocol 16084. This was an extension of a study published by Greene *et al.* (2019). Some of the experiments formed partial studies (Lee *et al.*, 2017, 2018). For these latter experiments, the trials were reviewed by the Drayton Animal Health Welfare and Ethical Review Body and conducted according to the Animals (Scientific Procedures) Act 1986.

Birds, diets and treatments

The design of the feeding trials from which blood, plasma, kidney, liver and muscle tissue were obtained for this study have been previously described (Greene *et al.*, 2019; Lee *et al.*, 2017, 2018).

Briefly, samples obtained from Greene *et al.* (2019) were used to measure the effect of phytase and *myo*-inositol supplementation on plasma and liver *myo*-inositol levels in 576, Cobb 500 broiler chickens at d18, d36 and d56 of age, housed in 48 pens with 12 birds per pen. Treatments were fed for the duration of the study and included a nutritionally adequate control group (PC), the PC supplemented with

0.3% *myo*-inositol, a negative control (NC) deficient in available P and Ca by 0.15 and 0.16%, respectively, NC fed plus the commercial enzyme QB (AB Agri, Marlborough, UK) at doses of 500 FTU/kg, 1000 FTU/kg, or 2,000 FTU/kg in feed. Bloods were collected from eight randomly selected birds per treatment for further analysis. The composition of the experimental diets is shown in Table 1.

Gizzard, kidney, liver and muscle tissue were obtained from Lee *et al.* (2017), from 576 Ross 308 broilers, housed in 32 pens with 18 birds per pen from 1-42 d. Diets were fed in two phases (0-21 and 21-42 d) as four treatment groups; low and adequate levels Ca and AvP diets with and without QB at 1,500 FTU/kg. Eight samples of each tissue were taken from randomly selected birds in each of the treatment groups. The composition of starter and grower diets is shown in Table 2.

Erythrocytes and plasma used for identification of inositol phosphates in erythrocytes were obtained from Lee *et al.* (2018). The composition of the starter and grower diets is shown in Table 3.

Erythrocytes were obtained from a trial performed at the Centre of Excellence for Poultry Science, University of Arkansas (Fayetteville, AR, USA), where Cobb 500 and 700

Table 1. Ingredient and nutrient composition of experimental diets taken from Greene *et al.* (2019), (as-is basis).

	Starter phase		Grower phase		Finisher phase	
	Diet 1-2	Diet 3-6	Diet 1-2	Diet 3-6	Diet 1-2	Diet 3-6
Ingredient (%)[1]						
Corn	60.100	61.720	65.070	66.690	67.088	68.708
Soy bean meal (46%)	33.382	33.112	28.286	28.016	25.833	25.563
Poultry fat	2.473	1.899	2.821	2.248	3.616	3.042
Dicalcium phosphate	1.610	0.792	1.481	0.663	1.284	0.466
Limestone	1.015	1.130	0.981	1.096	0.919	1.034
Salt	0.355	0.282	0.359	0.285	0.361	0.288
Sodium bicarbonate	0.120	0.120	0.120	0.120	0.120	0.120
DL-methionine	0.330	0.328	0.285	0.283	0.249	0.247
L-lysine HCl	0.244	0.248	0.233	0.237	0.181	0.185
L-threonine	0.102	0.102	0.096	0.096	0.082	0.082
Choline chloride (60%)	0.031	0.028	0.029	0.026	0.028	0.026
Vitamin premix[2]	0.100	0.100	0.100	0.100	0.100	0.100
Trace mineral premix[3]	0.100	0.100	0.100	0.100	0.100	0.100
Selenium premix[4]	0.020	0.020	0.020	0.020	0.020	0.020
Santoquin	0.020	0.020	0.020	0.020	0.020	0.020
Calculated nutrients (%)						
Dry matter	88.12	87.94	87.99	87.81	87.98	87.80
AMEn (kcal/kg)	3,035	3,035	3,108	3,108	3,180	3,180
Crude protein	21.20	21.20	19.10	19.10	18.00	18.00
AID Lys	1.18	1.18	1.05	1.05	0.95	0.95
AID Met	0.61	0.61	0.54	0.54	0.50	0.50
AID TSAA	0.89	0.89	0.80	0.80	0.74	0.74
AID Thr	0.77	0.77	0.69	0.69	0.65	0.65
AID Trp	0.22	0.22	0.19	0.19	0.18	0.18
AID Arg	1.27	1.27	1.12	1.12	1.05	1.05
AID Ile	0.79	0.79	0.71	0.70	0.66	0.66
AID Val	0.86	0.86	0.78	0.78	0.74	0.74
Total calcium	0.90	0.74	0.84	0.68	0.76	0.60
Total phosphorus	0.71	0.56	0.66	0.51	0.61	0.46
Available phosphorus	0.45	0.30	0.42	0.27	0.38	0.23
Phytate phosphorus						
Sodium	0.20	0.17	0.20	0.17	0.20	0.17
Potassium	0.89	0.88	0.80	0.80	0.75	0.75
Chloride	0.30	0.25	0.30	0.25	0.29	0.24
Magnesium	0.17	0.17	0.16	0.16	0.15	0.15
Copper	16.85	16.86	16.21	16.22	15.90	15.90
Selenium	0.20	0.20	0.20	0.20	0.20	0.20
Choline	1,750	1,750	1,650	1,650	1,600	1,600
Linoleic acid	1.17	1.20	1.27	1.30	1.31	1.34
Analysed nutrients (%)						
Crude protein	21.75	21.00	18.90	18.65	18.75	18.70
Phytate phosphorus			0.22	0.22	0.22	0.22

[1] AID = apparent ileal digestibility; AMEn = actual metabolisable energy; TSAA = total sulfur amino acids.

[2] Supplied per kilogram of diet: manganese, 100 mg; magnesium, 27 mg; zinc, 100 mg; iron, 50 mg; copper, 10 mg; iodine, 1 mg.

[3] Supplied per kilogram of diet: vitamin A 30,863 IU; vitamin D_3 22,045 ICU; vitamin E 220 IU; vitamin B_{12} 0.05 mg; menadione 6.0 mg; riboflavin 26 mg; d-pantothenic acid 40 mg; thiamine 6.2 mg; niacin 154 mg; pyridoxine 11 mg; folic acid 3.5 mg; biotin 0.33 mg.

[4] Supplied 0.12 mg of selenium per kg of diet.

Table 2. Composition of starter and grower broiler diets taken from Lee *et al.* (2017).

	Starter (0-21 d)		Grower (21-42 d)	
	Adequate CaP	Low CaP	Adequate CaP	Low CaP
Ingredient (g/kg)[1]				
Maize	582	600	661	677
Soybean meal 48	366	363	291	288
Soy oil	11.8	5.9	17.9	12.8
NaCl	4.6	4.6	3.6	3.6
DL-methionine	2.9	2.9	2.4	2.4
L-lysine HCl	1.5	1.6	0.9	0.9
Limestone	11.8	9.0	9.3	7.4
Mono Ca phosphorus	14.5	8.3	8.7	3.0
Premix[2,3]	5.0	5.0	5.0	5.0
Calculated nutrient content				
Crude protein	222	222	192	191
ME (MJ/kg)	12.6	12.6	13.1	13.1
Calcium	9.0	7.0	7.0	5.4
Phosphorus	7.3	5.9	5.7	4.4
Available phosphorus	4.4	3.0	3.0	1.7
Na	2.0	2.0	1.6	1.6
Dig. methionine	5.7	5.7	4.9	4.9
Dig. methionine + cysteine	9.0	9.0	7.8	7.8
Dig. lysine	12.2	12.2	9.8	9.8
Dig. tryptophan	2.4	2.3	2.0	2.0
Dig. threonine	7.7	7.7	6.6	6.6
Dig. arginine	13.6	13.6	11.5	11.5
Dig. isoleucine	8.5	8.5	7.2	7.2
Dig. valine	9.3	9.3	8.1	8.0
Dig. glycine	8.3	8.3	7.2	7.2
Analysed content calcium	8.5	6.7	6.9	5.3
Phosphorus	7.1	5.8	4.8	3.9

[1] Dig. = Digestible; ME = metabolisable energy.
[2] Starter premix – supplied per kg of diet: manganese 100 mg; zinc 80 mg; iron (ferrous sulphate) 20 mg; copper 10 mg; iodine 1.0 mg; molybdenum 0.50 mg; selenium 0.25 mg; vitamin A 13.5 mg; vitamin D3 5 mg; vitamin E 100 mg; vitamin B1 3 mg; vitamin B2 10 mg; vitamin B6 3.0 mg; vitamin B12 30 mg; hetra 5.0 mg; nicotinic acid 60 mg; pantothenic acid 15 mg; folic acid 1.5 mg; biotin 251 mg; choline chloride 250 mg.
[3] Grower premix – same as starter premix, except vitamin A, 10.0 mg.

breeder hens (63-65 weeks-old) were fed diets supplemented with QB at 0, 1,250 or 3,000 FTU/kg from where eggs were obtained and chicks hatched after transfer at embryonic day 18 (unpublished data). There were 30 experimental units per treatment, except for treatments 1 (Cobb 500, 0 phytase) and 4 (Cobb 700, 0 phytase), where limited availability and poor hatch of fertile source eggs resulted in 16 and 8 experimental units, respectively. Each experimental unit consisted of a pooled sample of 0.4 ml blood from three randomly selected, humanely euthanised chicks on the day of hatch. The composition of the layer diet is shown in Table 4 and the dietary treatments are shown in Table 5. Only the blood samples from Cobb 500 hens (treatments 1-3) were analysed for erythrocyte inositol phosphates.

Blood collection for measurement of erythrocyte inositol phosphates

Blood was collected in lithium heparin tubes and plasma obtained by centrifugation at 1,500×*g* for 10 min. Cells were washed in phosphate-buffered saline by centrifugation and suspended to the original blood volume. Whole blood or washed cells were mixed with two volumes of ice-cold 1M $HClO_4$ held on ice for 15 min with repeated mixing and centrifuged at 14,000×*g* for 10 min at 4 °C. The supernatant (one volume) was diluted with four volumes of 10 mM NaF, 20 mM EDTA (disodium salt) pH 10 and packed in dry-ice and sent to the University of East Anglia for analysis of inositol and inositol phosphates.

Extraction and measurement of digesta inositol phosphates

Inositol phosphates were extracted from digesta with modifications to the method of Zeller *et al.* (2015). Briefly, milled, freeze-dried digesta (100 mg) were extracted with 5 ml 100 mm NaF, 20 mM disodium EDTA, pH 10, for 30 min with shaking, followed by 30 min in a bath sonicator at approximately 10 °C and a further 2 h standing at 4 °C. The extract was centrifuged at 9,000×*g* for 15 min at 4 °C and filtered through a 13 mm 0.45 µm pore size PTFE syringe filter (Cole-Parmer Instrument Company Ltd, St. Neots, UK). Inositol phosphates were analysed according to the method of Whitfield *et al.* (2020). Inositol phosphate standards were obtained from Cayman Chemical Company (Ann Arbor, MI, USA), Merck Millipore (Watford, UK), SiChem (Bremen, Germany) or were kindly provided by Barry Potter, University of Oxford, UK.

Table 3. Composition of starter and grower broiler diets taken from Lee *et al.* (2018).

	Starter (0-21 d)	Grower (21-42 d)
Ingredients (g/kg)		
Wheat	633.0	735.7
Soybean meal 48	308.5	205.2
Soy oil	27.1	35.9
Salt	3.9	3.9
DL methionine	1.8	0.8
Lysine HCl	2.1	2.1
Threonine	0.2	0.0
Limestone	12.8	9.7
Mono Ca phosphorus	6.0	2.1
Premix[1]	4.0	4.0
Monteban G100	0.6	0.6
Quantum Blue[2]	0.1	0.1
Nutrient composition (%)		
Crude protein	21.85	17.90
ME (MJ/kg)	12.45	12.97
Calcium	0.98	0.78
Phosphorus	0.71	0.59
Phytate phosphorus	0.23	0.21
Available phosphorus	0.46	0.37
Fat	4.12	5.04
Crude fibre	2.60	2.50
Methionine	0.50	0.34
Methionine + cysteine	0.88	0.67
Lysine	1.28	1.00
Tryptophan	0.27	0.22
Threonine	0.80	0.62
Sodium	0.19	0.19
Chloride	0.33	0.33

[1] Starter premix – supplied per kg of diet: manganese 100 mg; zinc 80 mg; iron (ferrous sulphate) 20 mg; copper 10 mg; iodine 1.0 mg; molybdenum 0.50 mg; selenium 0.25 mg; retinol (vitamin A) 13.5 mg; cholecalciferol (vitamin D3) 5 mg; tocopherol (vitamin E) 100 mg; thiamine (vitamin B1) 3 mg; riboflavin (vitamin B2) 10 mg; pyridoxine (vitamin B6) 3.0 mg; cobalamin (vitamin B12) 30 mg; hetra 5.0 mg; nicotinic acid 60 mg; pantothenic acid 15 mg; folic acid 1.5 mg; biotin 251 mg; choline chloride 250 mg. Grower premix – same as starter, except retinol (vitamin A) 10.0 mg.

[2] Quantum Blue was included at 100 g/t, with an expected activity of 500 FTU/kg, into all diets. Phytase matrix applied: 0.15% available phosphorus, 0.165% calcium and 0.035% sodium.

Table 4. Composition of layer diets taken from a trial performed at the University of Arkansas, 2019 (Unpublished data).

	Layer diets
Ingredients (g/kg)[1]	
Corn	655.6
DDGS	119.5
SBM 46.5	89.8
MBM, 50%	56.3
Limestone course	55
Fat	7.5
Limestone	6.0
Salt	1.8
MHA Alimet	1.6
Lysine	1.0
Breeder VTM	1.6
Other	0.4
Calculated nutrient content (%)	
Dry matter	87.7
AMEn (kcal/kg)	2,807
Crude protein	16.81
Dig. Lys	0.62
Dig. Met	0.42
Dig. Thr	0.47
Dig. Trp	0.13
Dig. Arg	0.81
Dig. Val	0.63
Total calcium	2.98
Total phosphorus	0.59
Available phosphorus	0.41

[1] AMEn = actual metabolisable energy; DDGS = distillers dried grains with solubles; Dig. = digestible; MBM = meat bonemeal; MHA = DL-methionine hydroxy analog free acid; SBM = soyabean meal; VTM = vitamin trace mineral mixture.

Table 5. Description of experimental treatments taken from a trial performed at the University of Arkansas, 2019 (Unpublished data).

n (actual/target)[1]	Line (age, wks.)	Phytase level in breeder feed (FTU/kg)
16/30	C500 (63)	0
30/30	C500 (63-65)	1,250
30/30	C500 (63-65)	3,000
8/30	C700 (63)	0
30/30	C700 (63-65)	1,250
30/30	C700 (63-65)	3,000

[1] Each experimental unit consisted of group weight or pooled samples from 3 to 5 chicks. Actual number of experimental units for Treatments 1 and 4 were less than intended to limited availability and poor hatch of fertile source eggs.

Measurements of inositol in liver tissue and plasma

Liver tissue (100 mg) was homogenised using a T10 ULTRA-TURRAX® homogeniser (IKA, Königswinter, Germany) fitted with a S10N-8G-ST probe, in 1 ml ice-cold 5% w/v perchloric acid. The extract was centrifuged at 20,000×*g* for 10 min at 4 °C and the supernatant was analysed.

Inositol in plasma and liver was determined after dilution of the perchloric acid extracts in 18.2 MOhm/cm water by two-dimensional HPLC with detection by pulsed amperometry on a gold working electrode on either a Dionex (Sunnyvale, CA, USA) DX500 HPLC with ED50 electrochemical detector and Ag/AgCl reference electrode or Antec (Antec Scientific, Zoeterwoude, the Netherlands) carbohydrate analyser fitted with a HyREF cell. Validation was afforded by spiking the extracted plasma with 1-10 µM *myo*-inositol. The significant regression (r^2>0.996) and gradient 80.6 nC/nmol was almost identical to that obtained with simple inositol standards in water (r^2>0.999) with a gradient of 79.7 nC/nmol. The detector response was stable for large sets of samples: in another example experiment correlation coefficients of r^2>0.998 and 0.992 with gradients of 90.7 and 90.1 nC/nmol, were obtained, respectively, for calibration curves run at the start and the end of a set of >100 samples. Chromatographic conditions were as described by Pirgozliev *et al.* (2019). This method measures *myo*-inositol at single pmol levels on the column and can resolve the isomers D-*chiro*-inositol, *myo*-inositol and *scyllo*-inositol.

Measurements of muscle p473-Akt and total muscle Akt

Liver tissue was obtained from a feeding trial described in Greene *et al.* (2019) which measured the level of pAkt and Akt genetic signalling, which is involved in the adaptive response to hypoxia. Tissue (100 mg) was homogenised using a T10 ULTRA-TURRAX (IKA), fitted with a S10N-8G-ST probe, in 1 ml ice-cold RIPA buffer with phosphatase and protease inhibitors. Duplicate gels were probed with anti-p-473-Akt or anti-Akt antibodies and, following imaging for these, gels were stained with Amido Black for comparison of gel loading. All blotting procedures were performed according to methods reported by Greene *et al.* (2019). Gel data was exported as .tif files and the pixel intensity of bands, corresponding to p473-Akt and total Akt, were measured using LI-COR Image Studio software. Liver samples were obtained from two chickens per treatment to allow comparison with inositol measurements.

Statistics

Data were analysed by one- or two-factorial ANOVA in GraphPad Prism v.6 (GraphPad Software, San Diego, CA, USA). Treatment effects were indicated at $P<0.05$, and *post-hoc* tests (Tukey) to separate means were applied. Linear regression between Ins(1,3,4,5,6)P_5 and inositol was performed in StatPlus v.7 (AnalystSoft Inc., Walnut, CA, USA) with normality of residuals, linearity and homoscedasticity confirmed. Measured values were reported as means and standard errors. Chromatography data was processed as described by Madsen *et al.* (2019) without sampling or smoothing.

3. Results

Detection of inositol phosphates in washed erythrocytes

This was considered to be the first study on avian erythrocyte inositol phosphates in an animal feeding trial setting. Figure 1A shows analysis of inositol phosphates extracted from washed erythrocytes of 21d-old birds obtained from the study of Lee *et al.* (2018). Inositol phosphates were not detected in freshly collected plasma. Spiking the erythrocyte extracts with a set of standards, prepared by acid hydrolysis of InsP_6, identified the principal inositol phosphate of erythrocytes as Ins(1,3,4,5,6)P_5 (InsP_5(2-OH)). A chromatogram of the standards is shown in Figure 1B. Smaller peaks with the chromatographic mobility of D- and/or L-inositol 3,4,5,6-tetrakisphosphate [D- and/or L-Ins(3,4,5,6)P_4] and InsP_6 were also detected (Figure 1A inset). InsP_3 peaks were detected that eluted in the position of D-Ins(1,4,5)P_3 and D-Ins(3,4,5)P_3 (Figure 1C and 1D). Ins(1,3,4,6)P_4 was also detected as a minor InsP_4 (Figure 1D).

Effect of phytase supplementation in layer diets on erythrocyte Ins(1,3,4,5,6)P_5 of hatchlings

Inositol phosphate metabolism of chick erythrocytes has been studied by radiolabelling (Stephens and Downes, 1990; Stephens *et al.*, 1988, 1989) but not in a feeding trial scenario. The effect of maternal diet on hatchling erythrocytes was tested. Table 6 shows phytase supplementation in parent birds increased Ins(1,3,4,5,6)P_5 in the blood of 1 d-old hatchlings [$F(2,72)=4.780$, $P=0.0112$]. Ins(1,3,4,5,6)P_5 was increased from 939 to 1,110 nmol/ml with 1,250 FTU/kg but was unaltered at 3,000 FTU/kg. D- and/or L-Ins(3,4,5,6)P_4 was present at levels 5.5-6.3% that of Ins(1,3,4,5,6)P_5.

Table 6 shows *myo*-inositol levels from the samples where Ins(1,3,4,5,6)P_5 measurements were made. Phytase supplementation in parent birds increased *myo*-inositol content in the blood of 1d-old hatchlings [$F(2,72)=8.191$, $P=0.0006$]. *Myo*-inositol was increased from 290 to 381 nmol/ml with 1,250 FTU/kg phytase but not altered by 3,000 FTU/kg phytase.

Linear regression of Ins(1,3,4,5,6)P_5 vs *myo*-inositol (Figure 2) was significant ($F(1,73)=22.39$, $P=0.00001$) and yielded a Pearson correlation coefficient $r=0.485$ and equation:

y (InsP_5) = 1.110 × x (inositol) + 674.

Effect of dietary phytase and gizzard InsP_6 on gizzard myo-inositol

The effect of phytase on tissue inositol is rarely tested (Gonzalez-Uarquin *et al.*, 2020). Analysis of samples obtained from Lee *et al.* (2017), where four diets containing low or adequate Ca /AvP with zero or 1,500 FTU/kg of phytase was described (Table 7). Phytase reduced the InsP_6 content of the gizzard from 4,311 to 211 nmol/g in the low Ca/AvP diet and from 3,698 to 403 nmol/g in the adequate Ca/AvP diet. There was no effect of calcium nor any interaction between calcium and phytase. Phytase increased inositol from 719 to 2,121 nmol/g in the low Ca/AvP diet and from 509 to 1,756 nmol/g in the adequate Ca/AvP diet. There was no effect of calcium nor interaction of calcium and phytase. Inositol was not altered in liver, kidney or muscle tissue; 18,613, 8,054 and 1,107 nmol/g, respectively.

Effect of dietary phytase on plasma and liver inositol

To test further the influence of phytase supplementation on inositol levels of distal organs, samples were analysed from a larger scale experiment with Cobb 500 broiler chicks fed graded and higher levels of phytase in NC diet or PC diet with or without added inositol (Table 8). The dietary treatments for birds from which these samples were obtained are shown in Table 1 (Greene *et al.*, 2019).

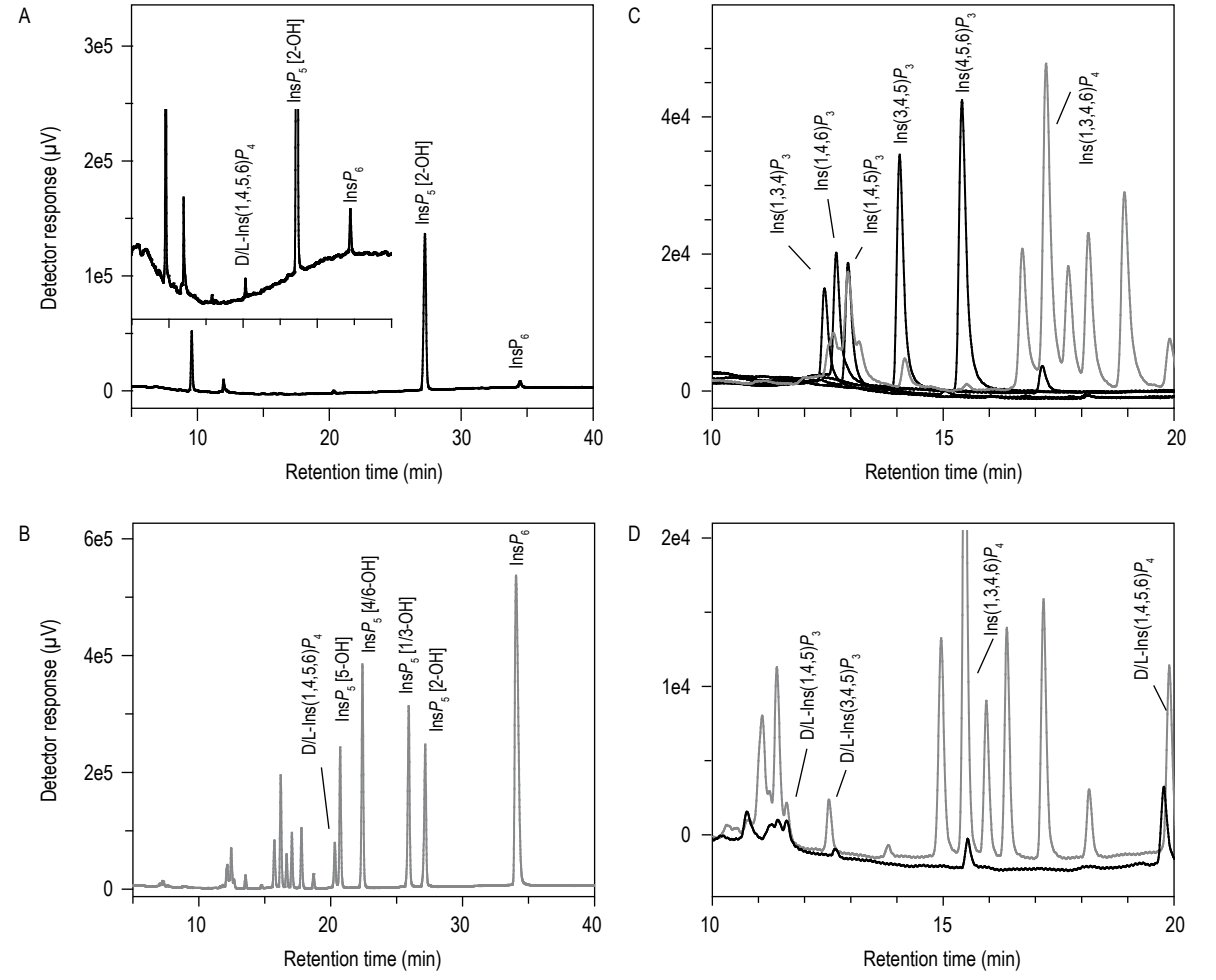

Figure 1. Inositol phosphates in chicken erythrocytes. (A) inositol phosphates extracted with perchloric acid were resolved by HPLC, the inset shows an exploded chromatogram; (B) an acid-hydrolysate of InsP_6; (C) the acid hydrolysate (grey) overlaid with individual inositol phosphate standards (black) run separately; (D) Co-elution of erythrocyte inositol phosphates (black) with peaks in an acid-hydrolysate (grey). The profile shown, (A) is typical of data obtained with >100 samples of erythrocytes from 22 d-old birds from an experiment by Lee *et al.* (2018) and is typical of whole blood of 1 d-old hatchings, this study.

Table 6. Effect of phytase in feed of breeder flock on blood *myo*-inositol and erythrocyte inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P_5) of d1 hatchlings.[1]

Treatment	*myo*-inositol (nmol/ml)	Ins(1,3,4,5,6)P_5 (nmol/ml)
Control[2]	290[b]	939[b]
Phy 1250[3]	381[a]	1,110[a]
Phy 3000[4]	332[ab]	1,061[ab]
Pooled SEM	9	22
P-value	<0.001	0.011

[1] Means in column not sharing a common superscript are significantly different (P<0.05).
[2,3,4] Treatment means; n=16, 30 and 29 experimental units (one experimental unit represents pooled samples of three birds).

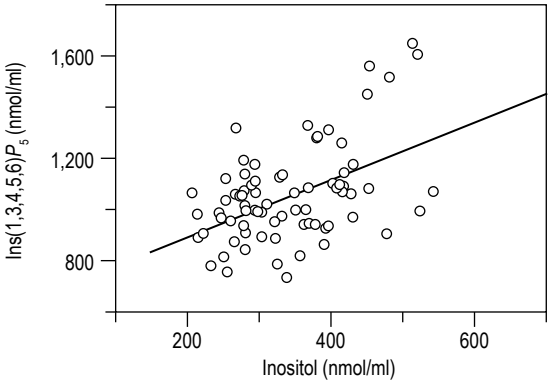

Figure 2. Correlation of *myo*-inositol and Ins(1,3,4,5,6)P_5 levels in chick blood. Inositol phosphates were measured in erythrocytes of 1d-old hatchings of breeder flock fed diet shown in Table 4 with treatments shown in Table 5.

Table 7. Effect of phytase and Ca/AvP on gizzard phytate and *myo*-inositol (nmol/g DM), and tissue *myo*-inositol (nmol/g FW)[2].

Dietary treatment		Gizzard phytate[1]	Gizzard inositol[1]	Liver inositol[2]	Kidney inositol[2]	Muscle inositol[2]
Ca/AvP	Phytase (FTU)					
Low	0	4,311[a]	719[b]	18,992	7,643	1,110
	1,500	211[b]	2,121[a]	17,831	9,763	1,260
Adequate	0	3,698[a]	509[b]	18,420	7,057	876
	1,500	403[b]	1,756[a]	19,310	7,753	1,006
Pooled SEM		374	191	507	489	74
Pooled mean		2,116	1,276	18,613	8,054	1,107
P-value						
Ca		0.553	0.361	0.681	0.246	0.128
Phytase		<0.001	<0.001	0.944	0.210	0.434
Ca × Phy		0.263	0.805	0.430	0.518	0.923

Treatment means; [1] n=8 birds per treatment [2] n=5 birds per treatment. [a-b] Means in column not sharing a common superscript are significantly different ($P<0.05$). Total calcium, total phosphorus and available phosphorus levels (g/kg) in starter d0-21 diets were: 7.0, 5.9, 3.0 for the low Ca/AvP treatment and 9.0, 7.3, 4.4 for the adequate Ca/AvP diet. The values for the grower d21-42 diets were: 5.4, 4.4, 1.7 for the low Ca/AvP diet and 7.0, 5.7, 3.0 for the adequate Ca/AvP diet.

Effects on muscle physiology of 56 d-old birds from this trial have been previously described (Greene *et al.*, 2019). In the present study, measurements were made on tissues from 18, 36 and 56 d.

For 18 d-old birds there was a significant difference in plasma *myo*-inositol ($F(5,47)=12.95$; $P<0.0001$) between treatments, but not for 36 or 56 d-old birds. Supplementation of the PC diet with *myo*-inositol (3 g/kg) increased plasma *myo*-inositol in 18 d-old birds from 140 to 353 nmol/ml. Addition of phytase to the NC diet increased plasma *myo*-inositol linearly between 500 and 2,000 FTU/kg, from 153 to 279 nmol/ml ($P=0.0002$). For 18 d-old birds, there was a significant difference in liver inositol ($F(5,39)=3.780$; $P<0.0069$) between treatments. Supplementation of the PC diet with *myo*-inositol increased liver *myo*-inositol from 11,667 to 17,930 nmol/g fresh weight. Supplementation of the NC diet with 2,000 FTU/kg phytase increased *myo*-inositol from 13,255 to 17,627 nmol/g. There was no effect at 36 or 56 d.

Effect of dietary phytase and dietary supplementation with inositol on Akt phosphorylation in liver

Signalling-related gene expression was investigated, specifically, for Akt (protein kinase B). The mRNA levels and phosphorylation status of this gene/protein and its upstream and downstream effectors are elevated in WB (Greene *et al.*, 2019). Western blotting of liver tissue showed a marked increase in p473-Akt with phytase supplementation of the NC diet, but only at the lowest dose (Figure 3). The figure shows the Akt phosphorylation status and inositol values for tissue from two birds for each of the treatments analysed in Table 8.

Table 8. The effect of phytase and *myo*-inositol supplementation on plasma and liver *myo*-inositol[1] of broiler chickens at 18, 36 and 56 days of age.[1,2,3,4]

Treatment[3]	Plasma d18 (nmol/ml)	Plasma d36 (nmol/ml)	Plasma d56 (nmol/ml)	Liver d18 (nmol/g FW)	Liver d36 (nmol/g FW)	Liver d56 (nmol/g FW)
1 PC	140[c,#]	208	284	11,667[b]	16,674	18,747[#]
2 PC + inositol	353[a]	308	260	17,930[a]	18,670	20,188[#]
3 NC	153[b,c]	257	320	13,255[b]	18,123	17,138[#]
4 NC + 500	206[b]	268	334	15,356[b]	18,350	19,965
5 NC + 1000	239[b]	285	260	16,085[b]	17,983	19,126
6 NC + 2,000	279[a,b]	278	267	17,627[a]	17,838	19,942
Pooled SEM	15	15	15	581	285	388
Pooled mean	228	267	287	15,389	17,939	19,185
P-value	<0.0001	0.5155	0.6027	0.0069	0.4554	0.5828

[1] FW = fresh weight; PC = positive control; NC = negative control.

[2] Means in column not sharing a common superscript are significantly different ($P<0.05$).

[3] Samples obtained from an experiment by Greene *et al.* (2019). Total calcium, total phosphorus and available phosphorus levels in starter d 1-18, grower d 19-36 and finisher d 37-56 diets 1 and 2 were: 0.90, 0.71, 0.45; 0.84, 0.66, 0.42 and 0.76, 0.61, 0.38, respectively. The values for diets 3-6 were, for starter, grower and finisher: 0.74, 0.56, 0.30; 0.68, 0.51, 0.27 and 0.60, 0.46, 0.23.

[4] Treatment means; n=8 individuals per treatment, except # n=7.

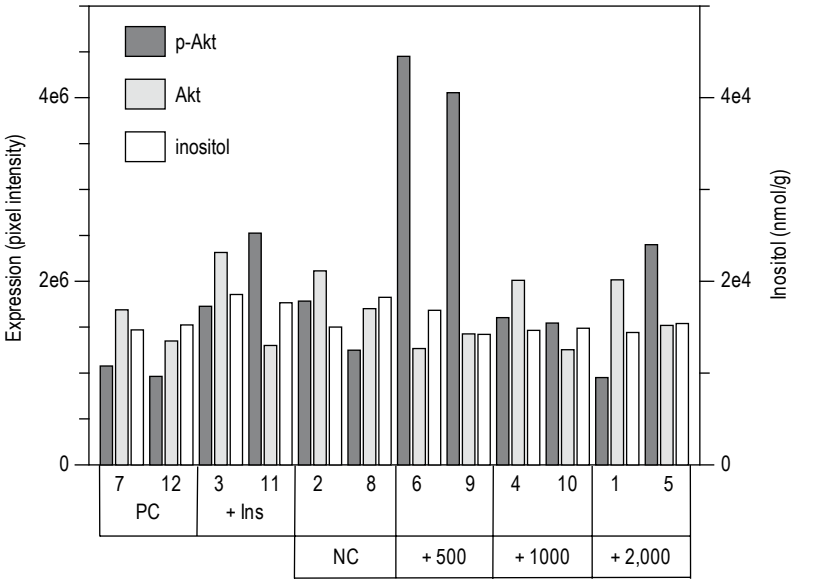

Figure 3. *Myo*-inositol and Akt status at 18d of liver of individual birds fed diets with or without supplemental *myo*-inositol (3 g/kg) or phytase (FTU/kg). Liver tissue from individual birds (labelled 1-12) fed diets shown in Table 1 from an experiment by Greene *et al.* (2019) were analysed for inositol content and for Akt and phosphorylated Akt (p-Akt) protein levels by Western blot. The pixel intensity of p473-Akt is scaled by a factor of 10 to aid comparison beside Akt. Treatments are those of Table 8.

4. Discussion

According to the current literature, this was the first study to show that phytase-mediated changes in plasma *myo*-inositol were correlated with increases in erythrocyte Ins(1,3,4,5,6)P_5 levels. Although phytases are now routinely added to poultry diets worldwide, a formal connection between plasma *myo*-inositol and tissue inositol phosphate or phosphatidylinositol phosphate level has not yet been demonstrated. Several studies have measured the effects of phytase or *myo*-inositol supplementation on *myo*-inositol content of the gastrointestinal tract (Beeson *et al.*, 2017; Pirgozliev *et al.*, 2019; Schmeisser *et al.*, 2017; Sommerfeld *et al.*, 2018a,b; Walk *et al.*, 2014) and animal performance traits (Cowieson *et al.*, 2013; Pirgozliev *et al.*, 2019; Zeller *et al.*, 2015, 2016). However, relatively few have measured the effects on plasma *myo*-inositol (Cowieson *et al.*, 2014; Sommerfeld *et al.*, 2018a) and only recently has any effect on *myo*-inositol in other tissues been reported (Gonzalez-Uarquin *et al.*, 2020; Greene *et al.*, 2019, 2020) The effect of dietary supplementation with *myo*-inositol or phytase-treatment extends to changes in blood metabolites (Cowieson *et al.*, 2013) and alterations in lipids (Żyła *et al.*, 2012). These studies showed how inositol released from dietary phytate may have specific physiological effects in different organs. Given the extensive literature on the intracellular signalling function of inositol phosphates and phosphatidylinositol phosphates in animals in the context of endocrine control of metabolism (Jones and Varela-Nieto, 1999; Manning, 2010), it is remarkable that studies have not examined whether inositol released in the digestive tract may be reused for the intracellular signalling purposes in distal organs.

Blood is an obvious tissue in which to test the consequence of phytase- or *myo*-inositol-supplementation on increases in *myo*-inositol. Not only do erythrocytes have a fundamental role of oxygen transport, they are a historic 'model-system' of inositol phosphate and inositol phospholipid research, one studied principally by radiolabelling methods established in the 1950's (Hokin and Hokin, 1953). The most detailed studies on chick erythrocyte inositol phosphate metabolism have been provided in the radiolabelling work of Stephens *et al.* (1988, 1989, 1990) and Stephens and Downes (1990). These studies employed *myo*-[2-^{3}H]inositol- and [^{32}P] orthophosphate-labelling of isolated, washed erythrocytes, methods that are not applicable to animal feeding trials. Consequently, alternative methods have been developed in the current study. Several observations arose from the application of the methods described above: (1) inositol phosphates were not detectable in chicken plasma, a result consistent with human plasma (Letcher *et al.*, 2008; Wilson *et al.*, 2015); (2) the identities of the inositol phosphates detected in this study were exactly those identified by Stephens *et al.* (1988, 1989); (3) the isomers of inositol phosphates detected in erythrocytes were distinct from the commonly observed products of gastro-intestinal phytate degradation (Sommerfeld *et al.*, 2018a,b), that is to say, they were not simply transferred from the gastro-intestinal tract to the plasma to the erythrocyte; (4) the levels of

inositol phosphates that were detected (939-1,110 nmol/ml, for Ins(1,3,4,5,6)P_5)) closely matched the values (975-1,136 nmol/ml) in Table 2 and 3 of Stephens and Downes (1990). It was noted that an alternative approach, metal-dye detection HPLC, has been applied to the measurement of inositol phosphates in avian and reptilian erythrocytes (Casals *et al.*, 2002; Mayr, 1988; Radenberg *et al.*, 1989). Again, while this method has not been applied in a feeding trial, it confirmed the original identification of Ins(1,3,4,5,6)P_5 (Johnson and Tate, 1969) as the predominant inositol phosphate in avian erythrocytes (Johnson and Tate, 1969).

Significantly, the foregoing studies, employing four fundamentally different methodologies, concurred that the most abundant isomer of inositol phosphate in avian erythrocytes was Ins(1,3,4,5,6)P_5, an allosteric regulator of haemoglobin (Coates, 1975). Collectively, these studies illustrated how plasma inositol has a metabolic fate as precursor of inositol phosphates in avian erythrocytes and a related physiological context in modulation of haemoglobin.

The correlation of erythrocyte Ins(1,3,4,5,6)P_5 level with blood *myo*-inositol observed in the present study suggested that phytase may reduce the severity of WB myopathy and have a contributory *myo*-inositol-mediated signalling effect on other organs. The increase in liver *myo*-inositol seen with phytase dose in 18 d-old birds suggested that younger birds may be particularly responsive to the dietary interventions demonstrated in the present study. Gonzalez-Uarquin *et al.* (2020) reported phytase effects at 1,500 FTU/kg, but not at 3,000 FTU/kg, on kidney inositol at 22 d of age, but no effect of phytase on liver inositol. The magnitude of the values reported by Gonzalez-Uarquin *et al.* (2020) were very similar to those reported currently, when converting dry weight to wet weight by a factor 4.167, as per the EFSA FEEDAP Panel (2016). The effect observed at 18 d of age may, in part, have reflected the observation that younger animals are metabolically more adaptive and, therefore, more responsive to dietary interventions (Norin and Metcalfe, 2019). At 18 d of age, levels of *myo*-inositol in the blood and liver are low, therefore, treatments that increase *myo*-inositol availability can fill the blood and tissues to capacity. At 18, 36 and 56 d of age, observations showed that, as the birds aged, they had higher levels of *myo*-inositol in both blood and liver. Consequently, with age and proper *myo*-inositol provision early in life, plasma and tissue levels may reach a maximal capacity, after which a potential feedback mechanism switches off *myo*-inositol accumulation in the animal, as previously discussed by Herwig *et al.* (2021).

The significance of erythrocyte and distal organ responsiveness to dietary *myo*-inositol, liver p-Akt at 18 d, may be related to the signalling function of inositol phosphates or inositol phospholipids in these tissues. There does not appear to be any studies on tissue inositol phosphate or inositol phospholipids in poultry feeding trials, but it should be noted that gene expression and Western blot are common proxy assessments of inositol phosphate and phospholipid signalling in poultry (Greene *et al.*, 2019, 2020; Schmeisser *et al.*, 2017). Greene *et al.* (2019) observed transcriptional increases in muscle PI3-kinase isoforms PI3KA, PI3KB and effectors Akt and mTOR in birds exhibiting muscle myopathies which, along with significant increases in expression of HIF-1α, a master regulator of the adaptive response to hypoxia, confirmed the local hypoxic status of the muscle tissue in birds with myopathies. The muscle phenotypes of these myopathies activate hypoxia signalling pathways in the affected tissue. Systemic responses include dysregulation of oxygen sensing genes in blood and muscle tissue. Inadequate vascularisation of rapidly growing *pectoralis major* muscle may contribute to oxidative stress responses of the affected tissues (Abasht *et al.*, 2016).

5. Conclusions

Dietary phytase supplementation increased *myo*-inositol content in the digestive tract, plasma and tissue of young birds and, moreover, increased *myo*-inositol and inositol phosphate levels in erythrocytes of hatchlings of a breeder flock, when fed phytase. The phytase-mediated increases in Ins(1,3,4,5,6)P_5 observed could impact haemoglobin function (Coates, 1975). Phytase supplementation has previously shown to reverse hypoxic responses of blood and tissues in animals showing WB phenotypes (Greene *et al.*, 2019) and modified fatty acid profiles (Cauble *et al.*, 2020). It is plausible that the amelioration of WB symptoms by phytase in poultry diets may, in part, reflect the oxygen carrying capacity of blood, which was influenced by changes in dietary-generated plasma *myo*-inositol and tissue (blood-) responsive Ins(1,3,4,5,6)P_5 levels. The findings of the present study should prompt further research into this mechanistic pathway, as elucidating the relationship between plasma *myo*-inositol, erythrocyte Ins(1,3,4,5,6)P_5 and phytase on broiler *myo*pathies is required.

Acknowledgements

AB Vista funded the experiments at the University of Arkansas and Drayton Animal Health Ltd. AB Vista had no role in conducting the research or the generation of data therefrom. Hayley Whitfield, Caroline Laurendon and Charles A. Brearley would like to thank Biotechnology and Biological Sciences Research Council UK (BBSRC) LINK Award BB/N002024/1 for their funding along with financial support from AB Vista. The authors would like to thank Barry Potter at the University of Oxford for provision of inositol phosphate standards.

Authors can confirm that all relevant data are included in the article and/or its supplementary information files.

Conflict of interest

Sophie Lee, Tom Dale, Tara York, Imke Kuehn and Mike Bedford are employees of AB Visa.

References

Abasht, B., Mutryn, M.F., Michalek, R.D. and Lee, W.R., 2016. Oxidative stress and metabolic perturbations in wooden breast disorder in chickens. PLoS ONE 11: e0153750.

Agbede, J.O., Kluth, H. and Rodehutscord, M., 2009. Studies on the effects of microbial phytase on amino acid digestibility and energy metabolisability in caecectomised laying hens and the interaction with the dietary phosphorus level. British Poultry Science 50: 583-591.

Ajuwon, K.M., Sommerfeld, V., Paul, V., Däuber, M., Schollenberger, M., Kühn, I., Adeola, O. and Rodehutscord, M., 2020. Phytase dosing affects phytate degradation and Muc2 transporter gene expression in broiler starters. Poultry Science 99: 981-991.

Beeson, L.A., Walk, C.L., Bedford, M.R. and Olukosi, O.A., 2017. Hydrolysis of phytate to its lower esters can influence the growth performance and nutrient utilization of broilers with regular or super doses of phytase. Poultry Science 96: 2243-2253.

Casals, I., Villar, J.L. and Riera-Codina, M., 2002. A straightforward method for analysis of highly phosphorylated inositols in blood cells by high-performance liquid chromatography. Analytical Biochemistry 300: 69-76.

Cauble, R.N., Greene, E.S., Orlowski, S., Walk, C., Bedford, M., Apple, J., Kidd, M.T. and Dridi, S., 2020. Research note: dietary phytase reduces broiler woody breast severity via potential modulation of breast muscle fatty acid profiles. Poultry Science 99: 4009-4015.

Chatterjee, D., Zhuang, H., Bowker, B., Sanchez-Brambila, G. and Rincon, A., 2016. Instrumental texture characteristics of broiler pectoralis major with the wooden breast condition. Poultry Science 95: 2449-2454.

Coates, M.L., 1975. Hemoglobin function in the vertebrates: an evolutionary model. Journal of Molecular Evolution 6: 285-307.

Cowieson, A., Aureli, R., Guggenbuhl, P. and Fru-Nji, F., 2014. Possible involvement of *myo*-inositol in the physiological response of broilers to high doses of microbial phytase. Animal Production Science 55: 710-719.

Cowieson, A.J., Acamovic, T. and Bedford, M.R., 2006. Phytic acid and phytase: implications for protein utilization by poultry. Poultry Science 85: 878-885.

Cowieson, A.J., Ptak, A., Mackowiak, P., Sassek, M., Pruszynska-Oszmalek, E., Zyla, K., Swiatkiewicz, S., Kaczmarek, S. and Jozefiak, D., 2013. The effect of microbial phytase and *myo*-inositol on performance and blood biochemistry of broiler chickens fed wheat/corn-based diets. Poultry Science 92: 2124-2134.

Cowieson, A.J., Roos, F.F., Ruckebusch, J.P., Wilson, J.W., Guggenbuhl, P., Lu, H., Ajuwon, K.M. and Adeola, O., 2017. Time-series responses of swine plasma metabolites to ingestion of diets containing *myo*-inositol or phytase. British Journal of Nutrition 118: 897-905.

European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2016. Scientific opinion on the safety and efficacy of inositol as nutritional additive for dogs and cats. EFSA Journal 14: e04511.

Gonzalez-Uarquin, F., Molano, E., Heinrich, F., Sommerfeld, V., Rodehutscord, M. and Huber, K., 2020. Research note: jejunum phosphatases and systemic *myo*-inositol in broiler chickens fed without or with supplemented phytase. Poultry Science 99: 5972-5976.

Greene, E., Flees, J., Dadgar, S., Mallmann, B., Orlowski, S., Dhamad, A., Rochell, S., Kidd, M., Laurendon, C., Whitfield, H., Brearley, C., Rajaram, N., Walk, C. and Dridi, S., 2019. Quantum blue reduces the severity of woody breast myopathy via modulation of oxygen homeostasis-related genes in broiler chickens. Frontiers in Physiology 10: 1251.

Greene, E., Mallmann, B., Wilson, J.W., Cowieson, A.J. and Dridi, S., 2020. Monitoring phytate hydrolysis using serial blood sampling and feather *myo*-inositol levels in broilers. Frontiers in Physiology 11: 736.

Harland, B.F. and Oberleas, D., 1987. Phytate in foods. World Review of Nutrition and Dietetics 52: 235-259.

Hasegawa, R. and Eisenberg Jr., F., 1981. Selective hormonal control of *myo*-inositol biosynthesis in reproductive organs and liver of the male rat. Proceedings of the National Academy of Science of the USA 78: 4863-4866.

Herwig, E., Walk, C., Bedford, M., Schwean-Lardner, K. and Classen, H., 2021. Contrasting the effects of phytase and pure *myo*-inositol on the performance, digestibility, blood and egg yolk inositol levels and digestion physiology of laying hens. British Poultry Science 62(4): 517-527.

Hokin, M.R. and Hokin, L.E., 1953. Enzyme secretion and the incorporation of P32 into phospholipides of pancreas slices. Journal of Biological Chemistry 203: 967-977.

Hoppeler, H. and Vogt, M., 2001. Muscle tissue adaptations to hypoxia. Journal of Experimental Biology 204: 3133-3139.

Ingelmann, C.J., Witzig, M., Mohring, J., Schollenberger, M., Kuhn, I. and Rodehutscord, M., 2018. Effect of supplemental phytase and xylanase in wheat-based diets on prececal phosphorus digestibility and phytate degradation in young turkeys. Poultry Science 97: 2011-2020.

Johnson, L.F. and Tate, M.E., 1969. The structure of *myo*-inositol pentaphosphates. Annals of the New York Academy of Sciences 165: 526-532.

Jones, D.R. and Varela-Nieto, I., 1999. Diabetes and the role of inositol-containing lipids in insulin signaling. Molecular Medicine 5: 505-514.

Kuttappan, V., Hargis, B. and Owens, C., 2016. White striping and woody breast myopathies in the modern poultry industry: a review. Poultry Science 95: 2724-2733.

Kuttappan, V.A., Lee, Y.S., Erf, G.F., Meullenet, J.F.C., McKee, S.R. and Owens, C.M., 2012. Consumer acceptance of visual appearance of broiler breast meat with varying degrees of white striping. Poultry Science 91: 1240-1247.

Lee, S.A., Dunne, J., Febery, E., Brearley, C.A., Mottram, T. and Bedford, M.R., 2018. Exogenous phytase and xylanase exhibit opposing effects on real-time gizzard pH in broiler chickens. British Poultry Science 59: 568-578.

Lee, S.A., Dunne, J., Mottram, T. and Bedford, M.R., 2017. Effect of diet phase change, dietary Ca and P level and phytase on bird performance and real-time gizzard pH measurements. British Poultry Science 58: 290-297.

Letcher, A.J., Schell, M.J. and Irvine, R.F., 2008. Do mammals make all their own inositol hexakisphosphate? Biochemical Journal 416: 263-270.

Madsen, C.K., Brearley, C.A. and Brinch-Pedersen, H., 2019. Lab-scale preparation and QC of phytase assay substrate from rice bran. Analytical Biochemistry 578: 7-12.

Manning, B.D., 2010. Insulin signaling: inositol phosphates get into the akt. Cell 143: 861-863.

Mayr, G.W., 1988. A novel metal-dye detection system permits picomolar-range h.p.l.c. analysis of inositol polyphosphates from non-radioactively labelled cell or tissue specimens. Biochemical Journal 254: 585-591.

Mudalal, S., Lorenzi, M., Soglia, F., Cavani, C. and Petracci, M., 2015. Implications of white striping and wooden breast abnormalities on quality traits of raw and marinated chicken meat. Animal 9: 728-734.

Norin, T. and Metcalfe, N.B., 2019. Ecological and evolutionary consequences of metabolic rate plasticity in response to environmental change. Philosophical Transactions of the Royal Society B: Biological Sciences 374: 20180180.

Peatman, E. and Beck, B., 2016. From floor sweepings to fish flesh-phytase super dosing in the US catfish industry. In: Walk, C.L., Kuhn, I., Stein, H.H., Kidd, M.T. and Rodhutscord, M. (eds.) Phytate destruction-consequences for precision animal nutrition. Wageningen Academic Publishers, Wageningen, the Netherlands, pp. 237-250.

Phillippy, B.Q. and Bland, J.M., 1988. Gradient ion chromatography of inositol phosphates. Analytical Biochemistry 175: 162-166.

Pirgozliev, V., Brearley, C.A., Rose, S.P. and Mansbridge, S.C., 2019. Manipulation of plasma *myo*-inositol in broiler chickens: effect on growth performance, dietary energy, nutrient availability, and hepatic function. Poultry Science 98: 260-268.

Pontoppidan, K., Glitsoe, V., Guggenbuhl, P., Quintana, A.P., Nunes, C.S., Pettersson, D. and Sandberg, A.S., 2012. *In vitro* and *in vivo* degradation of *myo*-inositol hexakisphosphate by a phytase from *Citrobacter braakii*. Archives of Animal Nutrition 66: 431-444.

Raboy, V., 2003. myo-Inositol-1,2,3,4,5,6-hexakisphosphate. Phytochemistry 64: 1033-1043.

Radenberg, T., Scholz, P., Bergmann, G. and Mayr, G.W., 1989. The quantitative spectrum of inositol phosphate metabolites in avian erythrocytes, analysed by proton n.m.r. and h.p.l.c. with direct isomer detection. Biochemical Journal 264: 323-333.

Schmeisser, J., Seon, A.A., Aureli, R., Friedel, A., Guggenbuhl, P., Duval, S., Cowieson, A.J. and Fru-Nji, F., 2017. Exploratory transcriptomic analysis in muscle tissue of broilers fed a phytase-supplemented diet. Journal of Animal Physiology and Animal Nutrition 101: 563-575.

Sihvo, H.-K., Immonen, K. and Puolanne, E., 2014. Myodegeneration with fibrosis and regeneration in the pectoralis major muscle of broilers. Veterinary Pathology 51: 619-623.

Sommerfeld, V., Kunzel, S., Schollenberger, M., Kuhn, I. and Rodehutscord, M., 2018a. Influence of phytase or *myo*-inositol supplements on performance and phytate degradation products in the crop, ileum, and blood of broiler chickens. Poultry Science 97: 920-929.

Sommerfeld, V., Schollenberger, M., Kuhn, I. and Rodehutscord, M., 2018b. Interactive effects of phosphorus, calcium, and phytase supplements on products of phytate degradation in the digestive tract of broiler chickens. Poultry Science 97: 1177-1188.

Stephens, L., Hawkins, P.T., Carter, N., Chahwala, S.B., Morris, A.J., Whetton, A.D. and Downes, P.C., 1988. L-*myo*-inositol 1,4,5,6-tetrakisphosphate is present in both mammalian and avian cells. Biochemical Journal 249: 271-282.

Stephens, L.R., Berrie, C.P. and Irvine, R.F., 1990. Agonist-stimulated inositol phosphate metabolism in avian erythrocytes. Biochemical Journal 269: 65-72.

Stephens, L.R. and Downes, C.P., 1990. Product-precursor relationships amongst inositol polyphosphates. Incorporation of [32P]Pi into *myo*-inositol 1,3,4,6-tetrakisphosphate, *myo*-inositol 1,3,4,5-tetrakisphosphate, *myo*-inositol 3,4,5,6-tetrakisphosphate and *myo*-inositol 1,3,4,5,6-pentakisphosph. Biochemical Journal 265: 435-452.

Stephens, L.R., Hawkins, P.T. and Downes, C.P., 1989. An analysis of *myo*-[3H]inositol trisphosphates found in *myo*-[3H]inositol prelabelled avian erythrocytes. Biochemical Journal 262: 727-737.

Tijare, V.V., Yang, F., Kuttappan, V., Alvarado, C., Coon, C. and Owens, C., 2016. Meat quality of broiler breast fillets with white striping and woody breast muscle myopathies. Poultry Science 95: 2167-2173.

Walk, C.L., Bedford, M.R. and Olukosi, O.A., 2018. Effect of phytase on growth performance, phytate degradation and gene expression of *myo*-inositol transporters in the small intestine, liver and kidney of 21 day old broilers. Poultry Science 97: 1155-1162.

Walk, C.L., Bedford, M.R., Santos, T.S., Paiva, D., Bradley, J.R., Wladecki, H., Honaker, C. and McElroy, A.P., 2013. Extra-phosphoric effects of superdoses of a novel microbial phytase. Poultry Science 92: 719-725.

Walk, C.L., Santos, T.T. and Bedford, M.R., 2014. Influence of superdoses of a novel microbial phytase on growth performance, tibia ash, and gizzard phytate and inositol in young broilers. Poultry Science 93: 1172-1177.

Whitfield, H., White, G., Sprigg, C., Riley, A.M., Potter, B.V.L., Hemmings, A.M. and Brearley, C.A., 2020. An ATP-responsive metabolic cassette comprised of inositol tris/tetrakisphosphate kinase 1 (ITPK1) and inositol pentakisphosphate 2-kinase (IPK1) buffers diphosphosphoinositol phosphate levels. Biochemical Journal 477: 2621-2638.

Wilson, M.S.C., Bulley, S.J., Pisani, F., Irvine, R.F. and Saiardi, A., 2015. A novel method for the purification of inositol phosphates from biological samples reveals that no phytate is present in human plasma or urine. Open Biology 5: 150014.

Zeller, E., Schollenberger, M., Kuhn, I. and Rodehutscord, M., 2016. Dietary effects on inositol phosphate breakdown in the crop of broilers. Archives of Animal Nutrition 70: 57-71.

Zeller, E., Schollenberger, M., Witzig, M., Shastak, Y., Kuhn, I., Hoelzle, L.E. and Rodehutscord, M., 2015. Interactions between supplemented mineral phosphorus and phytase on phytate hydrolysis and inositol phosphates in the small intestine of broilers. Poultry Science 94: 1018-1029.

Żyła, K., Mika, M., Duliński, R., Koreleski, J., Świątkiewicz, S. and Piironen, J., 2012. Effects of inositol, inositol-generating phytase B applied alone, and in combination with 6-phytase A to phosphorus-deficient diets on laying performance, eggshell quality, yolk cholesterol, and fatty acid deposition in laying hens. Poultry Science 91: 1915-1927.