1 **Manipulation of plasma *myo*-inositol in broiler chickens: effect on growth performance,**
2 **dietary energy, nutrient availability and hepatic function**
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17 Abbreviated title: **Plasma *myo*-inositol in broiler chickens**

18   **ABSTRACT**

19   This study investigated the effects of graded levels of *myo*-inositol (INS) in diets containing

20   two levels of available P, on growth performance, nutrient retention, liver N, fat and vitamin

21   E contents, INS and alkaline phosphatase (ALP) concentrations in blood plasma. One

22   hundred and twenty male Ross 308 broilers were allocated to 60 small floor pens each

23   holding 2 birds. Two basal mash diets were formulated to be nutritionally adequate for chicks

24   at that age, with one diet designed to have the recommended available P content (RP) (4.8

25   g/kg non-phytate P), and the other diet containing low available P (LP) (2.5 g/kg non-phytate

26   P). The two basal diets were split in three batches each and two of the batches were

27   supplemented with INS at 3.0 and 30 g/kg diet, with the remaining batch of each basal diet

28   not supplemented, giving a total of six experimental diets. Diets were fed *ad libitum* to 10

29   pens from 7 to 21 d age following randomization. Feeding RP diets improved (P < 0.001) the

30   birds' growth performance, mineral availability, and blood plasma ALP. Feeding RP diets

31   reduced (P < 0.001) apparent metabolizable energy (AME), dry matter and fat availability,

32   blood plasma INS and hepatic vitamin E. Dietary INS did not (P > 0.05) influence bird

33   growth, dietary AME or nutrient retention coefficients. Feeding INS linearly increased (P <

34   0.05) liver weight and hepatic N content, but linearly reduced (P < 0.05) hepatic fat

35   concentration. It also linearly increased (P < 0.001) the INS concentration in blood plasma,

36   but did not influence (P > 0.05) the endogenous losses (measured as sialic acid concentration)

37   in excreta. Dietary INS did not influence (P > 0.05) the hepatic vitamin E concentration but

38   increased (P < 0.001) the ALP in the blood of birds fed 30 g/kg INS. In conclusion, high-

39   level dietary INS supplementation did not affect bird growth performance, mineral

40   availability, and endogenous losses and there were no interactions between INS and P.

41   **Key words:** *myo*-inositol, phytate, phytase, antioxidants, alkaline phosphatase

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61   INTRODUCTION

62   The beneficial effects of dietary phytases (PHY) when fed to poultry are well documented

63   (Selle and Ravindran, 2007). Phytate is considered an anti-nutritional factor binding minerals

64   and proteins into indigestible complexes (Cowieson et al., 2004; Pirgozliev et al., 2007).

65   Phytase not only releases more available energy and nutrients to the birds, but also

66   hydrolyses dietary phytate. Due to the conditions in bird's gastrointestinal tract (GIT), as well

67   as the catalytic properties of supplementary microbial PHY, it is unlikely that phytate is

68   completely dephosphorylated into free *myo*-inositol (INS) and inorganic phosphate (Wyss et

69   al., 1999). Recent studies in pigs (Kühn et al., 2016) and poultry (Cowieson et al., 2015;

70   Beeson et al., 2017; Sommerfeld et al., 2017) have demonstrated that feeding supra doses of

71   third generation *E. coli* PHY increases INS concentrations in the digesta and excreta of

72   animals. These results indicate potential further phytate hydrolysis to free INS, consequently

73   released in the digestive tract of broiler chickens. The biological importance of INS is well

74   documented, with the involvement in cell survivability and growth, lipid metabolism, and

75   insulin sensitivity the most relevant for poultry (Lee and Bedford, 2016; Huber, 2016).

76   Although a number of studies on the effects of supplementary INS in broilers are published,

77   the results for nutrient availability and performance are inconsistent. Some authors found an

78   increase in growth performance in response to dietary INS (Żyła et al., 2013; Pirgozliev et al.,

79   2017; Sommerfeld et al., 2017), while others (Pearce, 1975; Farhadi et al., 2017) did not. In

80   addition, Cowieson et al. (2013) reported an interaction between INS and exogenous PHY as

81   addition of INS to either the normal or low P diet improved feed efficiency only in the

82   presence of PHY. Farhadi et al. (2017) and Sommerfeld et al. (2017) reported no impact of

83   supplementary INS on dietary P digestibility and bone mineralisation in poultry. However,

84   Pirgozliev et al. (2017) observed an interaction as the increase of dietary INS content had no

85   effect on P digestibility in the absence of PHY but it depressed P digestibility in the diets

86   containing PHY.

87   The amount of INS supplemented to diets in the aforementioned experiments was between

88   0.1 and 0.75%. This is similar or slightly higher than the theoretical range of INS released in

89   the gut of chickens after feeding a commercial level of PHY. However, different dietary

90   sources would have different phytate contents with varying bioavailability, and different PHY

91   may possess different abilities to hydrolyse dietary phytate, explaining the discrepancies in

92   the data published.

93   In view of the above, the objectives of the present study were to quantify the response of bird

94   growth performance, dietary metabolisable energy and nutrient digestibility as a result of

95   feeding a high level of supplementary INS in diets that contain low (LP) or recommended

96   (RP) levels of dietary P. Endogenous losses (measured as sialic acid; SA) in excreta, hepatic

97   vitamin E content, liver composition, alkaline phosphatase (ALP) and INS content in the

98   blood of broiler chickens were also determined.

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100   **MATERIALS AND METHODS**

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102   ***Birds and Housing***

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104   The experiment was conducted at the National Institute of Poultry Husbandry and approved

105   by the Harper Adams University Research Ethics Committee. A total of 130 male Ross 308

106   broilers were obtained from commercial hatchery (Cyril Bason Ltd, Craven Arms, UK),

107   allocated to a single floor pen and offered a standard wheat-based broiler starter feed

108   formulated to meet Ross 308 nutrient requirements (Aviagen Ltd., Edinburgh, UK). At 7d

109   age, 120 birds were allocated to 60 small floor pens each holding 2 birds. Each of the pens

110   had a solid floor and were equipped with an individual feeder and drinker. Feed and water

111   were offered *ad libitum* to birds throughout the experiment. Each diet was offered to birds in

112   10 pens in a randomized block design. The birds were fed the experimental diets from 7 to

113   21d age, when the experiment ended. Room temperature and lighting regime met commercial

114   recommendations (Aviagen Ltd, Edinburgh, UK). For the last 4 d of the study, the solid floor

115   of each pen was replaced with a wire mesh in order to enable excreta collection. The well-

116   being of the birds was checked regularly every day.

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118   Diets and Treatments

119   Six corn-soy-based diets were offered to the birds during the experiment. Two basal diets

120   were formulated to be nutritionally adequate for chicks at that age (12.90 MJ/kg ME, 216

121   g/kg CP), with one diet designed to have the recommended available P content (4.8 g/kg non-

122   phytate P), and the other diet containing relatively low available P (2.5 g/kg non-phytate P)

123   (Table 1). The two basal diets were then split in three batches each, and two of the batches

124   were supplemented with *myo*-inositol (Sigma-Aldrich, Inc., St. Louis, MO 63103, USA)

125   (Table 2) at 3.0 and 30 g/kg diet, respectively, with the remaining batch of each basal diet not

126   supplemented, to give a total of six experimental diets. All diets were fed as a mash.

127   Sampling and Measurements

128   Birds were weighed on d1, in order to obtain information on the average birds weight at the

129   start of the study. Birds and feed were then weighed on d7, and d21 in order to determine

130   average daily feed intake (ADFI), average daily weight gain (ADG) and to calculate the

131   gain:feed ratio (G:F) on a pen basis. Excreta were quantitatively collected each day for the

132   last four days of the experiment (in order to avoid evaporation losses), immediately dried at

133   60∘C and then milled through 0.75 mm screen. At the end of the study, one bird per pen,

134   selected at random, was electrically stunned and blood was obtained in heparin tubes from

135 the jugular vein. The livers from the same birds were collected immediately after, weighed,

136 and freezed prior to analysis.

137 *Chemical Analysis*

138 Dry matter (DM) in feed and excreta samples was determined by drying of samples in a

139 forced draft oven at 105◦C to a constant weight (AOAC, 2000; method 934.01). Crude

140 protein ($6.25 \times N$) in samples was determined by the combustion method (AOAC, 2000;

141 method 990.03) using a LECO FP-528 N (Leco Corp., St. Joseph, MI). Oil (as ether extract)

142 was extracted with diethyl ether by the ether extraction method (AOAC, 2000; method

143 945.16) using a Soxtec system (Foss UK Ltd.). The gross energy (GE) value of feed and

144 excreta samples was determined in a bomb calorimeter (model 6200; Parr Instrument Co.,

145 Moline, IL) with benzoic acid used as the standard. Phosphorus and Ca in feed and excreta

146 samples were determined by inductively coupled plasma emission spectrometry, ICP (Optima

147 4300 DV Dual View ICP-OE spectrometer, Perkin Elmer, Beaconsfield, UK) (Tanner et al.,

148 2002).

149 Hepatic vitamin E was analysed at the IDEXX BioResearch Vet Med Labor GmbH

150 (Ludwigsburg, Germany). Vitamin E in liver was determined by means of high performance

151 liquid chromatography (Hess et al., 1991). The ALP in blood plasma was analysed at the

152 APHA Laboratory (Shrewsbury, UK) following standard procedure using a Randox Immolite

153 Analyser and the associated Randox kit (Recommendations of the German Society for

154 Clinical Chemistry).

155 The concentration of excreta SA was determined by the periodate-resorcinol method as

156 described by Jourdian et al. (1971). The procedure detects total, free and glycosidically bound

157 N-acety neuraminic (sialic) acid.

158 For analysis of INS, samples of milled feed (0.1g) were extracted in 5 mL of 20 mM EDTA,

159 100 mM NaF, pH 10, on a rotary shaker for 15 min followed by sonication in a bath sonicator

160 for 15 min. The samples were held at 4◦C for 2 h before centrifugation at 14,000 x g for 15

161 min. The supernatant was filtered through a 13 mm x 45 µm pore PTFE filter (Kinesis Ltd,

162 UK) and diluted 50-fold in 18.2 MOhm.cm water. Inositol was determined by HPLC pulsed

163 amperometry (HPLC-PAD) on a gold electrode at 30◦C after separation by 2-dimensional

164 HPLC (Dionex DX-600 HPLC System). Samples (20 µL) were injected onto a 4 mm x 50

165 mm CarboPac PA1 column (Dionex, UK) arranged in series with a 4 mm x 250 mm

166 CarboPac MA1 column with 4 mm x 50 mm guard column of the same material. The flow

167 rate of the 150 mM NaOH eluent was 0.4 mL min^{-1}. After 1.5 min, the flow through the

168 CarboPac PA1 column was switched to 750 mM NaOH, at 0.4 mL min^{-1} to elute more

169 strongly retained sugars to waste. Eluent from the CarboPac MA1 column (150 mM NaOH)

170 was directed to an ED50 electrochemical detector (Dionex) configured with a gold electrode

171 and operating a standard Dionex carbohydrate waveform. After 11.5 min, the CarboPac PA1

172 column was switched back into the 150 mM NaOH flow (in series with the CarboPac MA1

173 column) to condition the column for a further 8.5 min, before initiation of the next injection

174 sequence.

175 Inositol eluted at approximately 10.5 min. Injection of INS standards (0.01-0.2 nmole in 20

176 µL) and integration of the peak yielded a linear calibration curve typically with $r^2 > 0.995$

177 and slope of approximately 100 nC.min nmol^{-1}.

178 For INS measurement in plasma, plasma samples were mixed with 2 volumes of ice-cold 5%

179 w/v perchloric acid and held on ice for 20 min to precipitate protein. The samples were

180 centrifuged at 16,000 x g for 15 min at 4∘C and the supernatant diluted 50-fold in 18.2

181 MOhm.cm water before analysis (20 µL injection) by HPLC-PAD.

182 Calculations and Statistical Analysis

183 Average daily feed intake, ADG and G:F ratio were calculated for the experimental period

184 from d7 to d21 on a pen basis. The AME of the diets was calculated following total collection

185 technique. The total tract digestibility coefficient of each of the studied nutrients was

186 calculated as the difference between the intake and the excretion of the nutrient, divided by

187 their respective intake based on data obtained for the last 4 days during collection period as

188 previously described (Whiting et al., 2018). The quantity of P and Ca retained in the body

189 (g/d) was obtained by multiplying of P and Ca intake and their total tract digestibility

190 coefficients.

191 Statistical analysis was performed using GenStat 19th edition statistical software (IACR

192 Rothamstead, Hertfordshire, England). A randomised block two-way analysis of variance

193 was performed using a 2×3 factorial structure to investigate the main treatment factors (P $\times$

194 INS inclusion levels) and their interaction. When there were statistically significant

195 differences in INS, the treatment sum of squares were partitioned to test the linear effects.

196 Differences were reported as significant at $P < 0.05$ and trends were noted at $P \leq 0.1$.

RESULTS

198 ***Broiler Growth Performance, Dietary Metabolizable Energy and Nutrient Digestibility***

199 Birds fed adequate available P diets, had greater (P < 0.001) BW (by 22.4 %), ADFI (by 16.2

200 %) and a better G:F (by 11.1 %) (Table 3). Variation in growth performance, nutrient and

201 mineral availability, and AME were in the expected range for a study involving broiler

202 chickens at this age and fed a similar diet formulation (Abdullah et al., 2016; Whiting et al.,

203 2017). Coefficients of variation (CV %) of ADFI, ADG, and G:F were 6.9 %, 9.2 %, and 7.3

204 %, respectively. The variation in dietary AME was relatively low (CV = 2.5 %), ranging from

205 14.32 to 14.68 MJ/kg DM and was not affected by dietary INS content (P > 0.05) (Table 4).

206 Feeding diets low in available P improved (P < 0.001) dietary AME by 0.36 MJ/kg DM (by

207 2.5 %). Daily intake of dietary AME was improved (P < 0.001) by feeding RP diets, but was

208 not influenced (P > 0.05) by INS supplementation.

209 Nutrient retention and digestibility coefficients were not affected (P > 0.05) by dietary INS

210 content (Table 4). The CV in DMR, NR and FD coefficients were 3.0 %, 7.8 % and 4.0 %,

211 respectively. Feeding LP diets improved (P < 0.001) DMR and FD, but did not influence (P >

212 0.05) the NR coefficient (Table 4).

213 Digestibility coefficients and daily retention of P and Ca in broilers were not affected (P >

214 0.05) by INS supplementation (Table 5). The CV % of digestibility and retention data varied

215 from 10.0 to 15.0 %. Feeding LP diets improved the Ca digestibility coefficient (P < 0.001)

216 but did not affect P digestibility (P > 0.05). Feeding RP diets improved (P < 0.001) daily

217 retention of P and Ca.

218 ***Hepatic vitamin E, fat and N contents, SA secretion, inositol and alkaline phosphatase in***
219 ***blood plasma***

220 The CV in the variables presented in Table 6 were between 2.2 % for hepatic N concentration

221 and 22.9 % for INS in blood plasma. Feeding RP diets reduced (P < 0.001) the relative

222 weight of the liver of the birds, and relative hepatic fat concentration and content (P < 0.05).

223 However, it increased (P < 0.001) the relative hepatic N concentration but did not influence

224 (P > 0.05) hepatic N retention. The concentration of secreted SA was not affected (P > 0.05)

225 by any of the dietary treatments (Table 7). Feeding LP diets increased (P < 0.001) the hepatic

226 vitamin E and INS concentration in blood plasma. However, birds fed RP diets had an

227 increased (P < 0.001) ALP concentration in blood.

228 Feeding INS-supplemented diets linearly increased (P < 0.05) liver weight and hepatic N

229 content, but linearly reduced (P < 0.05) hepatic fat concentration (Table 6). It also linearly

230 increased (P < 0.001) the INS concentration in blood plasma, but did not influence (P > 0.05)

231 the SA concentration in excreta (Table 7). Dietary INS did not influence (P > 0.05) the

232 hepatic vitamin E concentration but increased (P < 0.001) the ALP in the blood of birds fed

233 30 g/kg INS (Table 7).

234 **DISCUSSION**

235 The aim of this study was to investigate the effects of three different levels of INS in LP and

236 RP diets, on growth performance, nutrient retention, liver N, fat and vitamin E contents, INS

237 and ALP concentrations in blood plasma. The low INS supplemented diet contained 3 g/kg

238 INS, which is the expected dose released in response to a commercial dosage of PHY in the

239 GIT of broilers. The high INS supplemented diet contained 30 g/kg INS and was designed to

240 emphasise the impact of INS on the studied variables. Previous studies on INS

241 supplementation of broiler diets have used doses from 0.1 to 0.75 % (1 to 7 g/kg) (Żyła et al.,

242 2004; Cowieson et al., 2013; Pirgozliev et al., 2007), but high dietary levels of INS has not

243 yet been studied.

244 There are relatively few reported measurements of INS in chicken plasma. Schmeisser et al.

245 (2013) recorded values of 0.199, 0.246 and 0.345 mM for birds fed positive control, negative

246 control and phytase supplemented at 1000 FTU/kg diets, while Sommerfield et al. (2017)

247 obtained values of 0.23 and 0.54 mM for birds fed control and INS-supplemented diets (3.8

248 g/kg, starter; 3.5 g/kg grower). The values obtained here for LP, and LP + 3 g/kg and 30

249 g/kg-supplemented diets were 0.583, 0.664 and 0.982 mM (Table 7). While these, perhaps,

250 seem high the factors that control plasma INS are poorly defined. The renal clearance of INS

251 in chickens is unknown, but in humans it has been shown that following intra-venous supply

252 of INS the renal clearance of INS is exceeded at 3-4 mM plasma INS, considerably higher

253 than the values measured here (Doughaday and Larner, 1953). The elevations of INS reported

254 in our study coincided with a reduced concentration of hepatic fat content in birds fed INS.

255 Early reports support this observation and found that dietary INS supplementation is effective

256 at depressing liver fat synthesis (Bull, 1968; Cough, 1968). A beneficial effect of

257 supplementing diets with INS to treat fatty liver syndrome in layers was also reported by

258 Parker and Deacon (1968). Holub (1986) describes the metabolism and the function of INS

259 and INS phospholipids in animals. A close negative correlation has been found between the

260   activity of the enzymes fatty acid synthetase and acetyi-CoA carboxylase and levels of

261   dietary INS, thus further supporting the observed results in this study. The total hepatic N

262   content increased primarily due to increased liver weight.

263   Cowieson et al. (2015) and Sommerfeld et al. (2017) reported greater INS in blood plasma of

264   PHY fed chickens compared to birds fed control diets. In agreement with Shastak et al.

265   (2014) and Zeller et al. (2015), in the present study, broilers fed LP diets had higher blood

266   plasma INS concentration, compared to birds fed RP diets. In agreement with our findings,

267   higher INS concentrations in blood plasma (Cowieson et al., 2015) and excreta (Beeson et al.,

268   2017) has been reported in chicks fed LP diets compared to birds fed RP diets. It has been

269   demonstrated that a reduced intake of dietary minerals, especially of P and Zn, could increase

270   the activity of intestinal alkaline phosphatase and PHY (Davies et al., 1970; Bitar &

271   Reinolds, 1972). This enhanced the digestibility of P in a subsequently fed diet (Moore &

272   Veum, 1982, 1983). In addition, Maenz & Classen (1998) demonstrated *in vitro*, that high

273   mineral concentration markedly decreased the activity of brush border phytase in chicks.

274   McComb et al. (1979) reported that many microbes adapt to low P concentrations by

275   increasing the synthesis of alkaline phosphatase. This finding suggests that the intestinal

276   microflora might be involved in the enhanced dietary P availability due to feeding LP diets.

277   Although not determined in this study, it seems likely that the activity of the intestinal

278   alkaline phosphatase and PHY in the gut of broilers fed LP diets is elevated and thus these

279   birds exhibited an improved phytate degradation compared to the birds fed RP diets. The

280   improved AME, DMR and FD of LP diets observed in the present study further support this

281   hypothesis.

282   The apparent total tract Ca and P digestibility coefficients were in the expected range

283   (Olukosi and Fru-Nji, 2014; Sommerfeld et al., 2017). The lack of difference between P

284   digestibility in the LP and RP diets was expected and can be explained by the same

285   bioavailability of the added dietary dicalcium phosphate. Similar to Olukosi and Fru-Nji

286   (2014), the LP diet exhibited lower Ca digestibility compared to RP diets. When LP diets are

287   fed the Ca:P ratio in the gut is relatively wide compared to the same ratio in RP diets.

288   However, the widening of Ca:P ratio is known to reduce Ca utilisation in broilers (Olukosi

289   and Fru-Nji, 2014; Farhadi et al., 2017). It seems that the decreased Ca utilization in diets

290   with wide Ca:P can thus be explained by the presence of a greater amount of Ca in the

291   intestine than can be used by the birds, leading to excessive Ca excretion or reduced

292   efficiency of Ca absorption.

293   The hepatic vitamin E concentration was in line with previous observations (Karadas et al.,

294   2010, 2014; Pirgozliev et al., 2015). Although the reduction in vitamin E concentration in RP

295   fed birds was not expected, the values were in the expected range for chickens at this age.

296   The increased vitamin E concentration in the liver of LP fed birds coincided with reduced

297   growth performance variables, thus suggesting that the relatively low feed intake of these

298   birds reduced the oxidative stress, thereby preventing vitamin E reserves from depletion.

299   The ALP concentration in blood was in agreement with the expected reference limits

300   (Meluzzi et al., 1992). The levels of ALP for birds fed RP diets were higher than those fed LP

301   diets, possibly due to increased growth rates and osteoblastic activity of chickens (Bell and

302   Freeman, 1971). ALP belongs to a group of enzymes with a low substrate specificity and

303   catalyses the hydrolysis of phosphate esters in a basic environment. This suggests that dietary

304   INS may reform INS phosphate isomers, provoking ALP synthesis, thus explaining the

305   results for the high inclusion INS diet. There was a correlation between ALP activity and the

306   rates of bone formation in mice (Dimai et al., 1998), suggesting that decreased bone

307   reabsorption and an improved bone density might be a reason for elevated ALP in the blood

308   of birds fed the high INS diets. A study by Cowieson et al. (2015) showed positive

309   correlations between bone ash and plasma INS after adding phytase to LP diets. Conversely,

310   there was no link between plasma INS levels and bone ash contents in birds fed diets

311   supplemented with either INS or PHY (Sommerfeld et al., 2017).

312   Here, no effects of INS supplementation on growth performance, energy, nutrient and

313   mineral utilisation were observed. The literature investigating the effects of supplemental INS

314   on broiler performance is ambiguous. Żyła et al. (2013) demonstrated that supplementation

315   with as little as 1 g/kg INS in wheat- and corn-based diets containing 1.5 g/kg of available P

316   improved growth of broilers of a similar age. Pirgozliev et al. (2017) reported an optimised

317   dietary AME and broiler growth at approximately 2.5 g/kg INS when feeding corn-based

318   diets containing 2.5 g/kg available P. Sommerfeld et al. (2017) found that supplementing 3.5

319   g/kg INS to P sufficient wheat-based diets improved feed efficiency in broilers during the

320   starter phase. However, Cowieson et al. (2013) found that the addition of INS to a diet low in

321   Ca and digestible P resulted in a negative effect on feed efficiency during the starter phase,

322   although during the finisher phase the effect became positive. Moreover, feeding INS reduced

323   feed intake (Cowieson et al., 2013) which is in contrast to the current work. Furthermore,

324   Cowieson et al. (2013) reported an interaction between INS and exogenous PHY, whereby

325   the addition of INS to either a RP or LP diet improved feed efficiency in older birds only in

326 the presence of PHY. Finally, Pearce (1975), Żyła et al. (2004), and Farhadi et al. (2017), did

327 not find any advantage in broiler growth rates when fed INS supplemented RP or LP diets.

328 The principal finding that large dietary INS significantly elevates plasma INS with neither

329 beneficial nor deleterious effect focuses attention on aspects of avian physiology concerned

330 with renal clearance of INS. The half-life of INS in plasma is rarely reported, but values of

331 approximately 2 h are evident for human adults in the data of Doughaday and Larner (1953)

332 and 5 h in neonatal infants (Phelps et al. 2013). With regards to INS measurement, the

333 methods elaborated here have been tested by the authors and found to be appropriate for

334 chicken muscle, liver and kidney. They should enable metabolomic and transcriptomic study

335 of tissue response to phytate-derived INS, providing means can be found to distinguish

336 between organ-specific de-novo synthesis of INS (from glucose 6-phosphate) and INS

337 derived from dietary phytate. In this regard, the known refractory nature of proximal, but not

338 distal, PI-3kinase signalling to insulin in chicken muscle (but not liver) remains a puzzle that

339 would benefit from measurement of plasma, liver and muscle INS and its metabolites.

340 Regardless of dietary P content, in the present experiment INS did not influence studied

341 mineral availability in agreement with previous reports (Farhadi et al., 2017; Sommerfeld et

342 al., 2017). However, comparing diets with and without PHY supplementation, Pirgozliev et

343 al. (2017) observed an interaction as the increase in dietary INS content had no effect on P

344 digestibility in the absence of PHY but it depressed P digestibility in the diets containing

345 PHY. In addition, increasing dietary INS content did not change SA concentration, which is

346 contradictory to previous findings (Pirgozliev et al., 2017). Since INS is involved in the

347 control of cell volume and osmolarity (Kane et al., 1992), it was expected that high dietary

348 levels might modulate mucin secretion. However, the dose range in this experiment was

349 greater compared to Pirgozliev et al. (2017), thus a physiological maximum response may be

350 met between 7.5 and 30 g/kg of dietary INS supplementation.

351 Dietary PHY supplementation in RP diets has been shown to improve INS concentration in

352 blood but not overall growth performance (Cowieson et al., 2015). Conversely, in the same

353 study the dietary PHY supplementation in LP diets did not affect plasma INS concentration

354 but did improve overall growth performance variables. Similarly, Beeson et al. (2017)

355 reported overall improvement in growth performances of PHY fed birds, although there was

356 PHY by available P interaction for INS in excreta. Thus, there was no apparent correlation

357 between feed efficiency and INS and INS phosphate esters concentration in excreta. In

358 addition, feeding LP diet resulted in a lower feed efficiency but higher INS concentration in

359 ileal digesta. Sommerfeld et al. (2017) reported highest INS concentration in blood plasma of

360 birds fed INS supplemented diets, approximately 35 % higher than the INS in blood plasma

361 of PHY only fed birds, but there was no difference between the overall feed efficiency

362 between these diets.

363 In conclusion, this experiment has confirmed the expected biological effects of diets that

364 differ in available P contents. However, dietary INS did not affect bird growth performance,

365 mineral availability, and endogenous losses. There was no observed interaction between

366 available P and INS in any of the variables studied. Further investigation of plasma, liver and

367 muscle INS and its metabolites is warranted to distinguish dietary from tissue specific de-

368 novo synthesised INS.

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374

375   Table 1. Ingredient composition (g/kg 'as fed') of the basal experimental diets[1]

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Ingredients	g/kg	g/kg
Corn	604	600
Soybean meal 48 % CP	300	300
Corn gluten meal	40	40
Vegetable oil	20	20
Salt	3.6	3.6
DL Methionine	4.2	4.2
Lysine HCl	3.0	3.0
Limestone	15.2	6.7
Dicalcium Phosphate	6.0	18.5
Vitamin Mineral premix[2]	4.0	4.0
	1000	1000
Calculated values (as fed)		
Crude protein (N x 6.25), g/kg	216	216
Crude fat, g/kg	47	47
ME, MJ/kg	12.87	12.82
Calcium, g/kg	9.4	9.2
Av Phosphorus, g/kg	2.5	4.8
Lysine	13.6	13.6
M+C	11.4	11.4
Determined values		
DM, g/kg	0.903	0.903
GE, MJ/kg	16.87	16.80
Crude protein (N x 6.25), g/kg	213	200
Crude fat, g/kg	41	42
Ca, g/kg	9.5	10.2
P, g/kg	4.5	7.4
Na, g/kg	1.2	1.4

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378
379   [1]The INS was added on the top of this formulation.
380   [2]The Vitamin and mineral premix contained vitamins and trace elements to meet the requirements specified by
381   NRC (1994). All the experimental diets were designed to be low in P. The premix provided (units/kg diet):
382   retinol 3600 µg, cholecalciferol 125 µg, α-tocopherol 34 mg, menadione 3 mg, thiamine 2 mg, riboflavin 7 mg,
383   pyridoxine 5 mg, cobalamin 15 µg, nicotinic acid 50 mg, pantotenic acid 15 mg, folic acid 1 mg, biotin 200 µg,
384   iron 80 mg, copper 10 mg, manganese 100 mg, cobalt 0.5 mg, zinc 80 mg, iodine 1 mg, selenium 0.2 mg and
385   molybdenium 0.5 mg.

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391 Table 2. Determined *myo*-inositol (INS) and total P content in the experimental diets
392

Diets	INS g/kg	P g/kg
LP	0.248	4.5
LP + 3 g/kg INS	2.482	4.5
LP + 30 g/kg INS	27.055	4.5
RP	0.280	7.4
RP + 3 g/kg INS	2.426	7.4
RP + 30 g/kg INS	28.163	7.4

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394 Analysis were performed in duplicates.

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398   Table 3. Body weight (BW), average daily feed intake (ADFI), average daily gain (ADG) and
399   gain to feed ratio (G:F) of broiler chickens fed the experimental diets.
400

Treatment factor	BW kg/bird (21d age)	ADFI kg/bird (7-21d)	ADG kg/bird (7-21d)	G:F kg/kg (7-21d)
av P (g/kg)				
2.5	0.594	0.766	0.461	0.601
4.8	0.727	0.890	0.594	0.668
SEM	0.0092	0.0105	0.0089	0.0085
INS (g/kg)				
0	0.656	0.817	0.522	0.636
3	0.662	0.838	0.530	0.628
30	0.664	0.829	0.530	0.639
SEM	0.0112	0.0128	0.0109	0.0104
p-Value				
av P	<0.001	<0.001	<0.001	<0.001
INS	NS	NS	NS	NS
av P x INS	NS	NS	NS	NS
CV %	7.6	6.9	9.2	7.3

401
402   av P, calculated dietary available P; INS, added dietary *myo*-inositol; CV %, coefficient of
403   variation.
404   There were 10 observations per treatment, based on two birds per observation.
405   Means within a column with no common superscript differ significantly.
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412 Table 4. Apparent metabolisable energy (AME), dry matter retention (DMR), nitrogen
413 retention (NR), and fat digestibility (FD) of the experimental diets.
414

Treatment factor	AME (MJ/kg DM)	AME:GE	AME intake (MJ)	DMR	NR	FD
av P (g/kg)						
2.5	14.68	0.870	10.17	0.761	0.647	0.774
4.8	14.32	0.852	11.51	0.743	0.627	0.751
SEM	0.067	0.0040	0.161	0.0042	0.0090	0.0056
INS (g/kg)						
0	14.52	0.863	10.71	0.753	0.634	0.760
3	14.57	0.865	11.02	0.756	0.641	0.766
30	14.41	0.856	10.78	0.746	0.636	0.763
SEM	0.082	0.0049	0.198	0.0051	0.0111	0.0069
p-Value						
av P	<0.001	<0.05	<0.001	<0.05	NS	0.006
INS	NS	NS	NS	NS	NS	NS
av P x INS	NS	NS	NS	NS	NS	NS
CV %	2.5	2.5	8.2	3.0	7.8	4.0

415
416
417 av P, calculated dietary available P; INS, added dietary *myo*-inositol; CV %, coefficient of
418 variation.
419 There were 10 observations per treatment, based on two birds per observation.
420 Means within a column with no common superscript differ significantly.
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425 Table 5. Digestibility coefficients and retention of dietary Ca and P.

426

Treatment factor	Digestibility		Retention (g/d)	
	Ca	P	Ca	P
av P (g/kg)				
2.5	0.396	0.503	2.89	1.72
4.8	0.549	0.524	4.99	3.47
SEM	0.0111	0.0094	0.109	0.059
INS (g/kg)				
0	0.473	0.507	3.89	2.53
3	0.470	0.510	3.98	2.61
30	0.475	0.524	3.94	2.64
SEM	0.0135	0.0116	0.133	0.072
p-Value				
av P	<0.001	NS	<0.001	<0.001
INS	NS	NS	NS	NS
av P x INS	NS	NS	NS	NS
CV %	12.8	10.1	15.1	12.4

427

428 av P, calculated dietary available P; INS, added dietary *myo*-inositol; CV %, coefficient of
429 variation.
430 There were 10 observations per treatment, based on two birds per observation.
431 Means within a column with no common superscript differ significantly.
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435 Table 6. Liver weight, concentration and retention of hepatic fat and N of the experimental
436 birds.

437

Treatment factor	Weight		Concentration (g/kg)		Retention (g)	
	Liver (g)	Liver (% body weight)	Fat	N	Fat	N
av P (g/kg)						
2.5	19.3	3.1	11.8	12.2	0.22	0.24
4.8	19.4	2.6	10.1	12.4	0.20	0.24
SEM	0.40	0.08	0.34	0.05	0.007	0.005
INS (g/kg)						
0	18.4[a]	2.7	11.6	12.2	0.21	0.22[a]
3	19.7[ab]	2.8	10.9	12.3	0.21	0.24[b]
30	20.1[b]	2.9	10.2	12.4	0.20	0.25[b]
SEM	0.49	0.10	0.42	0.06	0.009	0.006
p-Value						
av P	NS	<0.001	<0.001	<0.001	0.008	NS
INS	0.048	NS	0.090	0.084	NS	0.028
av P x INS	NS	NS	NS	NS	NS	NS
CV %	11.4	15.6	17.3	2.2	18.2	12.2

438
439 av P, calculated dietary available P; INS, added dietary *myo*-inositol; CV %, coefficient of
440 variation.
441 There were 10 observations per treatment, based on two birds per observation.
442 Means within a column with no common superscript differ significantly.
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445 Table 7. Sialic acid (SA) concentration in excreta, *myo*-inositol (INS) and alkaline
446 phosphatase (ALP) in blood, and hepatic vitamin E of chickens fed the experimental diets.
447

Treatment factor	SA (mg/g)	INS (nmol / mL)	ALP (U/ml)	Vitamin E (µg/g)
av P (g/kg)				
2.5	0.756	834	5817	29
4.8	0.768	653	13446	22
SEM	0.0201	31.1	661.6	1.04
INS (g/kg)				
0	0.765	584[a]	8519[a]	25
3	0.743	663[a]	8620[a]	25
30	0.781	982[b]	11756[b]	26
SEM	0.0246	38.1	810.3	1.3
p-Value				
av P	NS	<0.001	<0.001	<0.001
INS	NS	<0.001	0.010	NS
av P x INS	NS	NS	NS	NS
CV %	14.4	22.9	37.6	22.6

448
449 av P, calculated dietary available P; INS, added dietary *myo*-inositol; CV %, coefficient of
450 variation.
451 There were 10 observations per treatment, based on two birds per observation.
452 Means within a column with no common superscript differ significantly.
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REFERENCES

Abdulla, J., S.P. Rose, A.M. Mackenzie, M.W. Mirza, and V. Pirgozliev. 2016. Exogenous tannase improves feeding value of diet containing field beans (Vicia faba) when fed to broilers, Brit. Poult. Sci. 57: 246–250, http://dx.doi.org/10.1080/00071668.2016.1143551

AOAC (2000) Official methods of analysis of the association of agricultural chemists, 17th edition. Washington (US): Association of Official Analytical Chemists Inc.

Beeson, L. A., C. L. Walk, M. R. Bedford, and O. A. Olukosi. 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. Poult. Sci. online available. doi: 10.3382/ps/pex012

Bell, D.J., and B.M. Freeman, 1971. Physiology and biochemistry of the domestic fowl. Academic Press, London, England

Bull, H.S. 1968. Fatty liver syndrome in laying hens. Proc. 23rd Ann. Texas Nutrition Conf, p. 219 - 225

Bitar, K., and J.G. Reinhold. 1972. Phytase and alkaline phosphatase activities in intestinal mucose of rat, chicken, calf and man. Biochimica et Biophysica Acta, 268: 442-452.

Cowieson, A. J., T. Acamovic, and M. R. Bedford. 2004. The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. Br. Poult. Sci. 45:101–108.

Cowieson, A.J., A. Ptak, P. Mackowiak, M. Sassek, E. Pruszynska-Oszmalek, K. Żyła, S. Swiatkiewicz, S. Kaczmarek, and D. Józefiak. 2013. The effect of microbial phytase and inositol on performance and blood biochemistry of broiler chickens fed wheat/corn-based diets. Poult. Sci. 92:2124–2134.

Cowieson, A. J., R. Aureli, P. Guggenbuhl, and F. Fru-Nji. 2015. Possible involvement of inositol in the physiological response of broilers to high doses of microbial phytase. Anim. Prod. Sci. 55:710-719.

Couch, J. R. 1968. Fatty liver syndrome, summary of papers presented before the 1968 Texas Nutritional Conference. Feedstuffs, 40 (49): 48 - 51

Davies, M., G.M. Ritcey, and I. Motzok. 1970. Intestinal phytase and alkaline phosphatase of chicks: Influence of dietary calcium, inorganic and phytate phosphorus and vitamin D3. Poult. Sci. 49:1280-1286.

Dimai H.P., T.A. Linkhart, S.G. Linkhart, L.R. Donahue, W.G. Beamer, C.J. Rosen, J.R. Farley, and D.J. Baylink. 1998. Alkaline phosphatase levels and osteoprogenitor cell numbers suggest bone formation may contribute to peak bone density differences between two inbred strains of mice, Bone, 22 (3): 211-216

Doughaday, W.H. and J. Larner. 1954. The renal excretion of inositol in normal and diabetic human beings. J Clin Invest. 133(3): 326–332.

529   Farhadi, D., A. Karimi, Gh. Sadeghi, J. Rostamzadeh, and M. R. Bedford. 2017. Effects of a
530   high dose of microbial phytase and myo-inositol supplementation on growth performance,
531   tibia mineralization, nutrient digestibility, litter moisture content, and foot problems in broiler
532   chickens fed phosphorus-deficient diets, Poultry Science, http://dx.doi.org/10.3382/ps/pex186
533
534

535   Hess, D., H.E. Keller, B, Oberlin, R. Bonfanti, and W. Schuep. 1991. Simultaneous
536   determination of retinol, tocopherols, carotene and lycopene in plasma by means of high
537   performance liquid chromatography on reversed phase. Int. J. Vitam. Nutr. Res. 61:232-238
538

539   Holub, B. J. 1986. Metabolism and function of myo-inositol and inositol phospholipids, Ann.
540   Rev. Nutr., 6: 563 – 597.
541

542   Huber, K. 2016. Cellular inositol metabolism. Pages 53–60 in Phytate destruction. C. L.
543   Walk, I. Kühn, H. H. Stein, M. T. Kidd and M. Rodehutscord, eds., Wageningen Academic
544   Publishers, Wageningen.
545

546   Kane, M.T., M. Norris, and R.A.P. Harrison. 1992. Uptake and incorporation of INS by
547   preimplantation mouse embryos. J. Reprod Fertil. 95: 617-625.
548

549   Karadas, F., V. Pirgozliev, A.C. Pappas, T. Acamovic, and M. R. Bedford. 2010. Effects of
550   different dietary phytase activities on the concentration of antioxidants in the liver of growing
551   broilers. J. Anim. Physiol. Anim. Nutr. 94: 519–526.
552

553   Karadas, F., V. Pirgozliev, S. P. Rose, D. Dimitrov, O. Oduguwa, and D. Bravo. 2014.
554   Dietary Essential Oils Improve the Hepatic Anti- Oxidative Status of Broiler Chickens. Brit.
555   Poult. Sci. 55: 329–334. doi:10.1080/00071668.2014.891098.
556

557   Kühn, I., M. Schollenberger, and K. Männer. 2016. Effect of dietary phytase level on
558   intestinal phytate degradation and bone mineralization in growing pigs. J. Anim. Sci. 94:264–
559   267.
560

561   Lee, S. A., and M. R. Bedford. 2016. Inositol - An effective growth promotor? Worlds Poult.
562   Sci. J. 72:743–760.
563

564   Jourdian, G. W., L. Dean, and S. Roseman. 1971. A periodateresorcinol method for the
565   quantitative estimation of free sialic acids and their glycosides. J. Biol. Chem. 246:430–435.
566

567   Maenz, D.D. and H.L.Classen. 1998. Phytase activity in the small intestinal brush border
568   membrane 0f the chicken. Poultry Science, 77: 557-563.
569

570   McComb, R. B., G. N. Bowers and S. Posen. 1979. Alkaline Phosphatase, p. 866. New York:
571   Plenum Press.
572

573   Mellizzi, A., G. Primiceri, R. Giordani and G. Fabris, 1992. Determination of blood
574   constituents reference values in broilers. Poult. Sci., 71: 337 – 345
575

576   Moore R.J. and T.L. Veum. 1982. Effect of dietary phosphorus and yeast culture on the
577   utilization of phytate phosphorus by the rat. Nutr. Rep. Intern., 25 (2): 221-233
578

579 Moore R.J. and T.L. Veum. 1983. Adaptive increase in phytate digestibility by phosphorus-
580 deprived rats and the relationship of intetinal phytase (EC 3.1.3.8) and alkaline phosphatase
581 (EC 3.1.3.1) to phytate utilization. Brit. J. Nutr., 49: 145-152.
582
583 Moss, D. W., D. N. Baron, P. G. Walker and J. H. Wilkinson. 1971. Standardization of
584 clinical enzyme assays. J. Clin. Pathol. 24, 740-743.
585
586 Olukosi, O. A., and F. Fru-Nji. 2014. The interplay of dietary nutrient level and varying
587 calcium to phosphorus ratios on efficacy of a bacterial phytase: 2. Ileal and total tract nutrient
588 utilization. Poult. Sci. 93:3044–3052.
589
590 Parker, R.O. and L.E. Deacon (1968) Field cases of the fatty liver syndrome. Proc. Texas
591 Nutr. Conf. 23: 226 – 236
592
593 Pearce J. 1975. The effects of choline and inositol on hepatic lipid metabolism and the
594 incidence of the fatty liver and kidney syndrome in broilers. Brit. Poult. Sci. 16: 565-570.
595
596 Phelps D.L., R. M. Ward, R.L. Williams, K.L. Watterberg, A.R. Laptook, L.A. Wrage,
597 T.L. Nolen, T.R. Fennell, R.A. Ehrenkranz, B.B. Poindexter, C. M. Cotten, M.K. Hallman,
598 I.D. Frantz III, R.G. Faix, K.M. Zaterka-Baxter, A. Das, M.B. Ball, T. M. O'Shea, C. B.
599 Lacy, M.C. Walsh, S. Shankaran, P.J. Sánchez, E.F. Bell, R.D. Higgins. 2013.
600 Pharmacokinetics and Safety of a Single Intravenous Dose of myo-inositol in Preterm Infants
601 of 23 to 29 weeks. Pediatr. Res. 74: 721–729.
602
603 Pirgozliev, V.R., O. Oduguwa, T. Acamovic, and H. Bento. 2007. Effect of diets containing
604 Esherichia coli-derived phytase to young chickens and turkeys: effects on performance,
605 metabolisable energy, endogenous secretions and intestinal morphology. Poult. Sci. 86: 705–
606 713.
607
608 Pirgozliev V., F. Karadas, S.P. Rose, A. Beccaccia, M.W. Mirza and A.M. Amerah. 2015.
609 Dietary xylanase increases hepatic vitamin E concentration of chickens fed wheat based diet,
610 J. Anim. Feed Sciences 24: 80 – 84
611
612 Pirgozliev V., M.R. Bedford, S.P. Rose, I.M. Whiting, O.O. Oluwatosin, A.O. Oso, F.O. Oke,
613 S.G. Ivanova, G.P. Staykova. 2017. Phosphorus utilisation and growth performance of broiler
614 chicken fed diets containing graded levels of supplementary inositol with and without
615 exogenous phytase, J. World Poult. Res. 7 (1): 1 – 7
616
617 Recommendations of the German Society for Clinical Chemistry: Standardization of Methods
618 for the estimation of Enzyme Activity in Biological Fluids, J. Clinical Chemistry, Clinical
619 Biochemistry 8. 182 -192 (1972).
620
621 Shastak, Y., E. Zeller, M. Witzig, M. Schollenberger, and M. Rodehutscord. 2014. Effects of
622 the composition of the basal diet on the evaluation of mineral phosphorus sources and
623 interactions with phytate hydrolysis in broilers. Poult. Sci. 93:2548–2559
624
625 Sommerfeld V., S. Künzel, M. Schollenberger, I. Kühn, and M. Rodehutscord, 2017.
626 Influence of phytase or myo-inositol supplements on performance and phytate degradation
627 products in the crop, ileum, and blood of broiler chickens, Poult. Sci. – in press
628

629 Tanner, S.D., V.I. Baranov, and D.R. Bandura. 2002. Reaction cells and collision cells for
630 ICP-MS: a tutorial review. Spectrochimica Acta Part B: Atomic Spectroscopy, 57: 1361–
631 1452.
632
633 Whiting L.M., V. Pirgozliev, S.P. Rose, J. Wilson, A. M. Amerah, S.G. Ivanova, G.P.
634 Staykova, O.O. Oluwatosin, and A.O. Oso. 2017. Nutrient availability of different batches of
635 wheat distillers dried grains with solubles with and without exogenous enzymes for broiler
636 chickens, Poult. Sci. 96: 574–580, http://dx.doi.org/10.3382/ps/pew262
637
638 Whiting I., V. Pirgozliev, S. P. Rose, F. Karadas, M.W. Mirza and A. Sharpe, 2018. The
639 effect of temperature of storage of a batch of wheat distillers dried grains with solubles
640 samples on their nutritive value for broilers, British Poultry Science, DOI:
641 10.1080/00071668.2017.1380297, in press
642
643 Wyss, M., R. Brugger, A. Kronenberger, R. Rémy, R. Fimbel, G. Oesterhelt, M. Lehmann,
644 and A. P. van Loon. 1999. Biochemical characterization of fungal phytases (Ins-INS
645 hexakisphosphate phosphohydrolases): catalytic properties. Appl. Environ. Microbiol.
646 65:367–373.
647
648 Zeller, E., M. Schollenberger, M. Witzig, Y. Shastak, I. Kühn, L.E. Hoelzle, and M.
649 Rodehutscord. 2015. Interactions between supplemented mineral phosphorus and phytase on
650 phytate hydrolysis and inositol phosphates in the small intestine of broilers. Poultry science,
651 94(5), pp.1018-1029.
652
653 Żyła, K., R. Duliński, M. Pierzchalska, M. Grabacka, D. Józefiak, and S. Swiatkiewicz. 2013.
654 Phytases and myo-inositol modulate performance, bone mineralization and alter lipid
655 fractions in the serum of broilers. J. Anim. Feed Sci. 22:56-62.
656
657