Effects of Calcium Feeding Strategy, Dietary Adaptation Period Length, and Diet Composition on Phosphorus Availability for the Determination of True Phosphorus Utilization in Growing Broilers

by

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ABSTRACT

Standard methods have been proposed for the determination of P availability using the true P utilization (**TPU**) protocol. However, reported TPU data have been variable between common feedstuffs. Therefore, a series of 4 experiments were conducted using broilers from 19 to 26 d of age to elucidate the effects different methodological factors have on measures of P availability for the determination of corn TPU. The first and second experiments assessed the effects of differences in diet type related to the inclusion of purified ingredients on measures of P availability and gastrointestinal tract (**GIT**) histology after short (< 5 d) dietary adaption period lengths (**DAPL**). Intestinal histology was not influenced by diet type, but DAPL affected the P availability of the different diet types, including a diet formulated as a control for DAPL. This indicates that DAPL effects may be associated with more than just differences in diet type. The third experiment evaluated the effects of different Ca feeding strategies (low, high, or fixed Ca:total P [**tP**] ratio) on diet apparent P digestibility and the resulting estimates of TPU. Different Ca feeding strategies affected estimates of TPU with low Ca diets having higher estimates of TPU. The high Ca diets had the lowest TPU. Negative endogenous P losses (**EPL**) were predicted when titration diets were formulated with a fixed Ca:tP ratio The fourth experiment was conducted to assess the effects of Ca feeding strategy and DAPL on the incidence of predicted negative EPL. Digesta samples from gizzard, jejunum, and ileum were collected to determine how phytate and an inert marker

(TiO_2) flowed through the GIT. Negative EPL were predicted for diets with a fixed Ca:tP ratio, but not predicted when diet Ca concentrations were fixed at 0.35%. Data also indicated that the flow of phytate and TiO_2 varied in the anterior GIT resulting in inaccurate measurements of apparent phytate hydrolysis. These results indicated that DAPL, Ca feeding strategy, and the use of TiO_2 may contribute to the variability of TPU estimates. Future research should continue to address these factors to refine the methods used for generating TPU of common feedstuffs.

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LIST OF ABBREVIATIONS

∑IP..... Summation of all Inositol Phosphate Esters

25CTD..... 25% Corn Titration Diet

75CTD..... 75% Corn Titration Diet

A∑IPD..... Apparent Digestibility of the Summation of all Inositol Phosphate Esters

AIIP6H..... Apparent Ileal Phytate Hydrolysis

AIP6D..... Apparent Inositol Phosphate Digestibility

AIPD..... Apparent Ileal P Digestibility

APR..... Apparent P Retention

BWG..... Body Weight Gain

DAPL..... Dietary Adaptation Period Length

DMI..... Dry Matter Intake

EPL..... Endogenous P Losses

FI..... Feed Intake

GIT..... Gastrointestinal Tract

IP..... Inositol Phosphate

IP2..... Inositol Di-Phosphate

IP3..... Inositol Tri-Phosphate

IP4.....Inositol Tetra-Phosphate

IP5..... Inositol Penta-Phosphate

IP6.....Inositol Hexa-Phosphate

NRC..... National Research Council

NFD .....Nitrogen Free Diet

NPP.....Non-Phytate P

RBA .....Relative Bioavailability

SBM..... Soybean Meal

TIPD ..... True Ileal P Digestibility

tP..... Total P

TPI.....True P Indigestibility

TPR.....True P Retention

TPU..... True P Utilization

WPSA ..... World's Poultry Science Association

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I. INTRODUCTION

Phosphorus is the second most abundant mineral in diets fed to animals used for food production and must be supplied in adequate concentrations to ensure normal development and metabolic function (Suttle et al., 2010). The primary source of P in commonly used plant-based feedstuffs is phytate P (Eeckhout and de Paepe, 1994). However, phytate P concentrations and availability have been reported to vary between feedstuffs (Eeckhout and de Paepe, 1994; Leytem et al., 2008). To ensure that the P requirements of broilers are met, diets are formulated to meet non-phytate P (**NPP**) requirements and typically contain excess P as a safety margin. Formulating diets with excess P results in increased P excretion and additional dietary costs.

Due to growing concerns about excess P excretion resulting from animal agriculture and a desire to lower dietary costs, there has been a renewed interest in developing methods to more efficiently meet the P requirements of poultry (Rodehutscord, 2009). This requires transitioning from formulating diets on a NPP to a true P digestibility basis. True P utilization (**TPU**), including true ileal P digestibility (**TIPD**) and true P retention (**TPR**), of feedstuffs has been proposed by the World's Poultry Science Association (**WPSA**) as the best method to determine the true P availability of feedstuffs (WPSA, 2013). This method utilizes regression analysis to predict endogenous P losses (**EPL**), which allows for the correction of apparent P digestibility data into an estimate for the TPU of the test feedstuff. However, estimates for TPU and predictions of EPL have been highly variable between and within

laboratories for similar feedstuffs (Dilger and Adeola, 2006; Liu et al., 2013; Mutucumarana et al., 2014a,b, 2015a). It is possible that methodological aspects of these TPU protocols may be responsible for the generation of variable TPU data. Different methodologies used by researchers to estimate TPU include variations in Ca feeding strategy, dietary adaptation period length (**DAPL**), and differences in diet types relating to the inclusion of purified ingredients. Therefore, a series of experiments was conducted using growing broilers from 19 to 26 d of age to determine the effects different methodological factors have on measures of titration diet P availability and predictions of EPL and resulting estimates of TPU.

Titration diets for the determination of TPU contain varying concentrations of purified ingredients to limit P concentrations below the requirement of the animal. Observational evidence from our laboratory indicated that transitioning broilers from common starter diets to semipurified experimental diets may affect intestinal morphology thereby influencing P availability. Additionally, long DAPL when feeding nutrient deficient treatment diets incurs the risk of reduced feed intake and body weight gain, which may also influence P metabolism (Shastak et al., 2014). Furthermore, broilers have demonstrated an ability to adapt to P deficient diets as a means of maintaining P homeostasis (Yan et al., 2005). Therefore, research is warranted to assess the effects of different DAPL on the apparent P digestibility of titration diets used to determine TPU.

Dietary Ca concentration is the most significant factor affecting P availability (Selle et al., 2009). Therefore, careful consideration must be taken when selecting a Ca feeding strategy for the determination of TPU. The WPSA recommends feeding titration diets with a fixed Ca:total P (**tP**) ratio. However, researchers have used several different

Ca feeding strategies when assessing TPU including the direct method (Dilger and Adeola, 2006), fixed Ca:NPP ratio (Mutucumarana et al., 2014a,b), and fixed Ca:tP ratio (Liu et al., 2013). Estimates of TPU generated by these laboratories have been variable among similar feedstuffs (Dilger and Adeola, 2006; Liu et al., 2013; Mutucumarana et al., 2014b, 2015a). Many of these Ca feeding strategies also require diets with very low Ca concentrations, which may result in the overestimation of apparent P digestibility and TPU. Researchers utilizing a fixed Ca:tP ratio generated regression equations that predicted negative EPL (Iyayi et al., 2013; Liu et al., 2013). Therefore, research should be conducted to elucidate the effects of different Ca feeding strategies on the apparent P digestibility of titration diets, estimates of TPU, and prediction of EPL.

The research reported herein focused on a combination of these methodological factors with particular emphasis on the impact of DAPL and Ca feeding strategies on the apparent P digestibility of titration diets used to determine TPU. Corn was selected as the test P source due to its importance as a primary feedstuff in poultry diets. Data generated by these experiments should aid in explaining the variability commonly associated with predicted EPL and estimates of TPU as well as facilitate the implementation of methodologies to produce robust TPU data of feedstuffs for practical application in feed formulation.

II. LITERATURE REVIEW

PHOSPHORUS IN BROILER NUTRITION

Phosphorus is a macro-mineral necessary to optimize bone development and broiler growth performance. Currently, information on true P digestibility of common feedstuffs for broilers is limited and inconsistent. This is because true P digestibility is dependent on many factors related to the physiological state of the animal, test feedstuff, and the methodologies employed to evaluate P availability (Rodehutscord, 2009). Because of limited knowledge regarding feedstuff true P digestibility and the availability of supplemental P sources high in NPP, dietary P is provided to diets to meet the broiler's NPP requirement. The contribution of dietary phytate P to meet the P requirements of the animal is largely ignored as a consequence of this method, and therefore, broiler diets may contain excesses of 20 to 100% of the dietary P needed to ensure proper growth and bone development (Applegate and Angel, 2014). Over-supplementation of P in broiler diets has both economic and environmental consequences. Inorganic phosphate is an expensive nutrient due to a limited global supply coupled with high demand for inorganic P products for use as both fertilizers and feed supplements (Cordell et al., 2009; Donohue and Cunningham, 2009). Additionally, P-laden agricultural runoff resulting from the land application of poultry litter has resulted in the eutrophication of waterways (Honeyman, 1993). For these reasons, the limited understanding of the true P availability of feedstuffs has been deemed a major challenge to the future of animal agricultural production (Gross, 2010; Neset and Cordell, 2012).

The P requirement of modern broilers is described as a requirement for NPP (Ross 708 Broiler Nutrition Specification, 2014). Concentrations of NPP are calculated by subtracting phytate P from the total P concentration. Non-phytate P in feedstuffs originates from phospholipids, nucleic acids, adenosine triphosphate, and phosphate molecules (Veum, 2010). Formulating diets to meet NPP requirements is based upon the assumption that NPP concentrations are constant between feedstuffs and are 100% available for digestion and absorption (Rodehutscord, 2009). Additionally, the National Research Council (**NRC**) changed from listing available P values to NPP values of feedstuffs in consecutive editions of the Nutrient Requirements of Poultry without substantial differences in the published data itself (NRC, 1984, 1994). These terms are now considered to have different meanings, further complicating the understanding of true P availability of feedstuffs (Angel et al., 2002).

Furthermore, when formulating to meet the NPP requirement of broilers, phytate P is assumed to be completely unavailable. However, research has demonstrated that phytate P has some availability dependent on the feedstuff and dietary formulations utilized (Oloffs et al., 2000; Angel et al., 2002; Leske and Coon, 2002; Manangi and Coon, 2008). Because NPP and phytate P concentrations vary substantially between feed ingredients, the ability of broilers to utilize P is source dependent (Van der Klis and Versteegh, 1996; De Groote and Huyghebaert, 1997; Wendt and Rodehutscord, 2004). The formulation of diets solely on the basis of NPP oversimplifies the complex array of P moieties presented to the bird, neglecting the potential for more precisely meeting the bird's P needs. In order to more efficiently meet the P requirements of broilers, it is first necessary to better understand the digestion and absorption of both phytate P and NPP of

primary feedstuffs. A measure of P utilization which accounts for all forms of P will allow nutritionists to more precisely meet the P requirement of broilers limiting P excretion and reducing dietary costs.

PHOSPHORUS COMPOSITION OF FEEDSTUFFS

Total P, phytate P, and NPP concentrations, and the NPP to total P ratio vary between feedstuffs (Table 2.1). Variations in total P content are further complicated by differences in P storage forms associated with either plant- or animal-based ingredients. While P in rendered animal meals is almost exclusively NPP, phytate P generally comprises over 60% of the total P in plant feedstuffs (Eeckhout and De Paepe, 1994; Table 2.1). As the primary feedstuffs in global poultry diets are plant-based, diets typically contain over 1% phytate (Selle et al., 2009). Additionally, differences in feedstuff processing post-harvest also influence the P binding form in the ingredient. Because of the fermentation process (Cromwell, 1992), co-products, such as corn and wheat distillers dried grains with solubles have higher concentrations of NPP than phytate P (Mutucumarana et al., 2014b; Adebiyi and Olukosi, 2015). Understanding variations in NPP and phytate P concentrations among feedstuffs is critical because nutritionists formulate diets to meet the NPP requirement of broilers. In this system, the proportion of NPP related to total P concentrations of a feedstuff is the de facto representative of how much dietary P is considered available.

Phytate P distribution in the seed also varies between feedstuffs. Over 85% of corn phytate P is located in the germ (O'Dell et al., 1972). Conversely, phytate P is primarily (>80%) co-located with intrinsic phytases in the aleurone layer or outer brans of wheat and barley (McCance and Widdowson, 1944; Mollgaard, 1946; O'Dell et al.,

1972). The presence of intrinsic feedstuff phytases may affect P availability (McCance and Widdowson, 1942). Because these enzymes are heat and pH labile, they are believed to be inactivated during feed processing and digestion (Jongbloed and Kemme, 1990). In most oilseeds and grain legumes, phytate P is associated with protein globoids and distributed throughout the kernel (Erdman et al., 1979; Sebastian et al., 1998). In canola seeds, globoid crystals contain the majority of phytate P, which remains associated with the denatured protein after the seeds have been processed into canola meal (Yiu et al., 1983). Soybeans are unique because phytate P is equally localized throughout the seed (Sebastian et al., 1998). Phytate P concentrations also vary depending on crop maturity, cultivar, and agronomic conditions (Reddy et al., 1982). These factors, coupled with P binding form and distribution in the feedstuff, can greatly influence how P is digested and absorbed.

DIGESTION AND ABSORPTION OF PHOSPHORUS

Poultry diets composed of plant and animal based feedstuffs are theoretically sufficient to meet the P requirements of broilers. However, differences related to feedstuff P composition determine the efficacy of P utilization, and a majority of the P present in the diet is not readily digested or absorbed (Hill et al., 2008). Variability in concentrations of NPP and phytate P as well as differences in how these specific P binding forms are digested are a major concern with P supplied by common feedstuffs. In plant-based ingredients, P is primarily stored in the form of phytate P (Eeckhout and de Paepe, 1994). Therefore, in corn-soybean meal (**SBM**) diets phytate P represents one of the largest dietary sources of P. Phytate P has low availability in broilers because the orthophosphate groups must be enzymatically cleaved from phytate prior to absorption

(Ravindran et al., 1995). Due to insufficient endogenous production of phytase and the formation of indigestible chelates of phytate with dietary cations such as Ca, the availability of phosphate from phytate is typically less than 30% (Nelson, 1967; Mullaney et al., 2000). Because a significant proportion of the phytate P in a corn-SBM diet has limited availability, exogenous phytases are commonly supplemented to the diet to improve phytate P digestibility. Furthermore, poultry diets may also be formulated with ingredients with higher concentrations of NPP, like animal protein meals, distillers dried grains with solubles or inorganic P sources.

Phosphate absorption is dependent upon several factors, including the binding state of the phosphate molecule, the quantity of negatively and positively charged ions in the diet, and the dietary concentration of vitamin D. Free phosphate (HPO_4^{2-}) is absorbed by 1 of 2 methods including active transcellular or passive paracellular pathways (Danisi and Murer, 1992). Active absorption occurs over the apical membrane of intestinal tract enterocytes via a type IIb Na/phosphate-cotransporter (Hilfiker et al., 1998). Sodium alters the conformation of the cotransporter, which increases the cotransporter's affinity for phosphate. Once phosphate binds to the cotransporter, a second conformation change occurs, resulting in a transport competent cotransporter conformation similar to the Na/glucose cotransporter (Danisi and Murer, 1992). Phosphate then enters the cell against the concentration gradient due to the electrical gradient established by Na/K ATPase pump. Once in the enterocyte, phosphate is transported across the basolateral membrane into the blood by a Na independent transporter (Peerce, 1997).

Phosphorus transporters are affected by both the physiological state of the animal and the P, Ca, and vitamin D concentrations of the diet (Murer et al., 2004; Marks et al.,

2010; Proszkowiec-Weglarz and Angel, 2013). When plasma Ca concentrations are low, the parathyroid gland releases parathyroid hormone to increase Ca absorption from the GIT and renal tubules, as well as reabsorption of Ca from bone tissue. This action is mediated through the up-regulation of active vitamin D in the kidney (conversion of 25-hydroxycholecalciferol to 1, 25-dihydroxycholecalciferol) (Murer et al., 2004; Proszkowiec-Weglarz and Angel, 2013). This process acts as a hormonal regulator on intestinal enterocytes to increase Ca and phosphate absorption through the upregulation of their respective transporters (Marks et al., 2010). These findings are supported by evidence in the literature where broilers adapted to being fed P deficient diets by increasing their ability to digest and absorb dietary P (Yan et al., 2005).

However, dietary Ca concentrations are likely the most significant factor affecting the digestion, absorption, and excretion of dietary P. Phosphate absorption increases in concert with increased Ca absorption, but unlike Ca, P homeostasis is controlled to a greater extent by renal excretion (Klinefelter et al., 1984; Breves and Schröder, 1991). When parathyroid hormone increases active vitamin D synthesis, subsequently increasing phosphate and Ca absorption in the small intestine, phosphate reabsorption from glomular filtrate in the kidneys is reduced (Wideman et al., 1980; Wideman and Braun, 1981). This leads to an increase in P excretion in the urine. Dilger and Adeola (2006) observed lower true P retention compared with true ileal P utilization when broilers were fed low Ca (< 0.20%) diets. The negative effect of increased dietary Ca concentration on P availability has been well established (Selle et al., 2009). High Ca concentrations inhibit both phytate-P digestibility and P absorption. Phosphate is a negative anion, whereas *myo*-inositol *hexakis*-dihydrogen phosphate (phytate) is poly-anionic. When the pH of the GIT

is greater than 4, phosphate anions can chelate positively charged cations, forming insoluble complexes. These chelates are unavailable for enzymatic hydrolysis or absorption (Tamim and Angel, 2003). Dietary Ca concentrations have also been reported to influence the efficacy of endogenous enzymes, which further negatively affects phytate P digestibility (Applegate et al., 2003). Many factors such as P source, dietary P and Ca concentrations, GIT pH, and the presence or activity of exogenous and endogenous enzymes are involved with the digestion, absorption, and maintenance of P homeostasis in the animal. In order to quantify the effects of these factors on P availability, many different methodologies have been developed to assess the ability of broilers to digest and metabolize P.

PROTOCOLS FOR THE DETERMINATION OF AVAILABLE PHOSPHORUS

Several types of both qualitative and quantitative protocols have been utilized to generate P availability values for common feed ingredients (Shastak and Rodehutscord, 2013). These include relative bioavailability (**RBA**), apparent ileal phosphorus digestibility (**AIPD**), standardized ileal P digestibility, apparent P retention (**APR**), and standardized P retention (Shastak and Rodehutscord, 2013). Modern techniques have used regression to determine TPU. These data can be generated by either true ileal P digestibility (**TIPD**) or true P retention assays (**TPR**) (Rodehutscord 2009). Although, each method has purported benefits as well as limitations, data determined by these methods tend to be widely variable (Rutherfurd et al., 2002; Dilger et al., 2004; Rutherfurd et al., 2004; Dilger and Adeola, 2006; Selle et al., 2009).

Relative bioavailability protocols compare physiological changes of specific response criteria (growth rate, bone strength, and tibia or toe ash) generated by dietary increases in

the experimental P source with the same response criteria generated from feeding a reference P source (Hurwitz, 1964; Potter et al., 1995). However, the RBA method is limited in its use for feed formulations as results are only relative to a specific ingredient (Coon et al., 2002). Meta-analyses of response measurements and retention assays have been unable to sufficiently generate reliable P digestibility data that can be used for feed formulations (Rodehutscord, 2009).

Apparent P retention protocols were first proposed as an adaptation of nutrient balance protocols for the determination of metabolizable energy (Sibbald, 1982). Values generated by an APR assay allowed for the simultaneous determination of phytate P and NPP utilization for many different types of feedstuffs (Leske and Coon, 2002). Concerns with APR assays entail possible modification of P sources by hind-gut microflora as well as metabolic P excretion in the urine. Urinary P will increase when dietary P concentrations are above the requirement of the animal or when dietary Ca concentrations are low leading to the underutilization of absorbed P (Onyango et al., 2003; Driver et al., 2005; Liu et al., 2013).

To avoid the influence of post-ileal microbial fermentation and P excretion in the urine, researchers developed the AIPD protocol by adapting the well-established ileal amino acid digestibility assay (Ravindran et al., 1999; Adedokun et al., 2008, 2009). By removing the influence of P excretion from the kidneys, the response in AIPD to increasing concentrations of dietary P is linear over a wider range when compared with data generated by APR protocols (Rodehutscord et al., 2012). However, most proposed protocols require feeding diets with NPP concentrations below the requirement of the animal to avoid possible differences in AIPD and ARP. When researchers fed NPP

deficient diets, results were similar for P availability between mineral sources for AIPD and ARP assays (Shastak et al., 2012).

Standardizing AIPD and ARP values using EPL reduces the risk of reporting underestimated P availability values (Fan et al., 2001; Dilger and Adeola, 2006; Liu et al., 2012). Feedstuffs with low concentrations of P are more susceptible to the negative influence EPL have on P availability (Liu et al., 2012). The primary methods for the determination of EPL involve ad libitum or precision feeding of P-free diets or the use of the regression technique to extrapolate P-excretion responses to the point where no P is provided by the diet (Rutherfurd et al., 2002, 2004; Dilger and Adeola, 2006; Liu et al., 2012). While these methods have generated an estimate for EPL, reported values have been widely variable (-1,000 to 600 mg of P/kg DM intake [**DMI**]). Researchers have attributed this variability to a lack of standard methodology and nutrient differences between test ingredients (Liu et al., 2012; Mutucumarana et al., 2014a,b, 2015a,b).

VARIABILITY ASSOCIATED WITH PHOSPHORUS AVAILABILITY MEASUREMENTS

Nutritionists assume that the NPP fraction of the diet is 100% available, while phytate P is not available due to insufficient endogenous phytase production in broilers (Nelson, 1976). However, evidence from the literature demonstrates that broilers have an ability to utilize a portion of dietary phytate P depending on dietary Ca concentrations and experimental feedstuffs (Tamim and Angel, 2003; Leytem et al., 2008; Plumstead et al., 2008). Therefore, it is probable for nutritionists to more efficiently supply P to poultry diets to reduce P excretion and lower dietary costs. However, this would require a transition from formulating diets to meet the NPP requirements of poultry, and instead,

formulating diets using feedstuff TPU data. Determining the TPU of feedstuffs has been suggested by researchers as a replacement for using NPP as a representative of P availability because both NPP and phytate P contribute to the estimate of feedstuff TPU (Fan et al., 2001).

Like other methods to determine P availability, data generated on feedstuff TPU have been highly variable (Dilger and Adeola, 2006; Liu et al., 2013; Mutucumarana et al., 2014a,b, 2015a; Table 2.2). Additionally, inconsistent data have been reported for the AIPD of titration diets used to determine TPU (Dilger and Adeola, 2006; Liu et al., 2013; Mutucumarana et al., 2014a,b, 2015a). Differences in experimental conditions may have contributed to this variability, specifically Ca feeding strategy (Ca concentration and Ca:P ratio), DAPL, basal diet protein source, and the evaluated feedstuff (Dilger and Adeola, 2006; Liu et al., 2013; Mutucumarana et al., 2014a,b, 2015a).

Furthermore, variability associated with determining AIPD of titration diets may have been responsible for the prediction of negative EPL, which further increased the variability commonly associated with TPU estimates. Predictions of negative EPL resulted in inherently lower estimates for TIPD and TPR compared with AIPD and APR values for the same feedstuff (Iyayi et al., 2013; Liu et al., 2013; Mutucumarana et al., 2014a, 2015a). Lower corrected estimates for TIPD are contrary to the accepted understanding that true digestibility values should be higher than apparent digestibility values (Fan et al., 2001). Limited data, in addition to these inconsistencies, have precluded the use of TPU values in feed formulation. Therefore, a standard methodology should be used for the determination of the TPU of feedstuffs in order to reduce variability and to obviate the prediction of negative EPL.

WORLD'S POULTRY SCIENCE ASSOCIATION PROTOCOL

In order to develop a standard methodology, the Working Group 2 of the European Federation of Branches of the WPSA proposed a protocol for the determination of P availability (WPSA, 2013). The proposed protocol is based on the regression method and allows for the simultaneous prediction of EPL and estimation for TIPD of an ingredient (Fan et al., 2001; Dilger and Adeola, 2006). The WPSA Working Group proposed the following methodology: Diets are to be semipurified to limit the source of P in the diet and should be formulated to meet or exceed all nutrients with the exception of P and Ca. Treatment diets include at least 2 graded P concentrations for linear regression. A dietary adaptation period of at least 5 d should follow the transferring of broilers from a common starter diet to the P-deficient treatment diets. Digesta samples should be gently flushed from the terminal ileum of male broilers from fast-growing genetic strains during peak growth (21 to 28 d of age).

Generated data should provide a TIPD value of the test feedstuff which would be suitable for use in feed formulation. However, previous research on P availability and anecdotal evidence has raised concerns regarding several aspects of the proposed methodology. The use of regression analysis, length of dietary adaptation period, and a multitude of dietary effects (purified ingredients, Ca feeding strategy, and amino acid source and concentration) need to be further evaluated. Research aimed at investigating these methodological differences will determine if these factors are impeding the ability of the WPSA method to provide accurate and repeatable TPU data.

METHODOLOGICAL EFFECTS RELATED TO REGRESSION ANALYSIS AND DIETARY ADAPTATION PERIOD LENGTH

Regression

Using the regression method to determine P availability of feedstuffs may be advantageous as this method does not require the correction of apparent availability values using endogenous nutrient losses (Batterham et al., 1979; Fan et al., 2001; Dilger and Adeola, 2006). It has been suggested that TPU data are inherently less susceptible to variation than results obtained using AIPD or ARP methods alone (Fan et al., 2001). True P utilization values are generated using several titration diets, each with increasing P concentrations supplied by the test P source. Fan et al. (2001) associated the variability in previously published AIPD and ARP values to differences in dietary P concentrations. Additionally, regression methods require the use of purified ingredients in order to formulate titration diets with P concentrations below the requirement of the animal, which may affect broiler performance (Rodehutscord et al., 2012; Liu et al., 2013; Mutucumarana et al., 2014a,b). These factors, coupled with wide discrepancies in predicted estimates of EPL, are potential limitations of the regression method. The collection of additional data is warranted to better elucidate potential sources of variability.

While data are limited, TPU values have lacked homogeneity between similar feedstuffs (Table 2.2). Dilger and Adeola (2006) reported TIPD and TPR values for conventional SBM of 93.90 and 59.82%, respectively. Data published by Liu et al. (2013) indicated that SBM TIPD were dependent on dietary Ca:P ratios and ranged from 50.6 to 70.8%. Conversely, TPR data were not affected by the Ca:P ratio and were similar to

TPR values determined by Dilger and Adeola (2006). Predicted estimates for EPL also varied between work published by Dilger and Adeola (2006) and Liu et al. (2013) with the former reporting EPL averaging 235 mg/kg DMI and the latter reporting negative EPL. Negative EPL are a concern associated with the use of regression for the determination of P utilization.

Negative EPL have been reported in the literature with no clear consensus on the factors affecting the prediction of negative values by regression equations (Dänner et al., 2006; Rodehutscord et al., 2012; Shastak et al., 2012; Iyayi et al., 2013; Liu et al., 2013; Mutucumarana et al., 2014a,b; Table 2.2). Extrapolation of the regression line, coupled with variability in the influential first or last data points are likely responsible for the prediction of negative EPL (Mutucumarana et al., 2014a,b). Potential variability among the first or last observations in the regression series could be related to differences in diet type. Typically, the first observation is generated by feeding purified diets that tend to be highly deficient in both P and Ca. Conversely, the last observation represents diets with more of the test ingredient, P and Ca concentrations closer to the requirements of the animal, and a limited amount of purified ingredients. Additional research is warranted to determine the effects of diet type on the prediction of EPL and TPU estimates generated using regression analysis.

Adaptation Period

Researchers typically utilize DAPL between 3 to 10 d to mitigate any potential negative effects of transferring animals from diets containing common feedstuffs to semipurified treatment diets (Dilger and Adeola, 2006; Iyayi et al., 2013; Liu et al., 2013; Mutucumarana et al., 2014a,b). The standard methodology suggested by the WPSA

recommends a DAPL greater than 5 d (WPSA, 2013). Recently, published protocols assessing TIPD have employed dietary adaptation periods between 6 and 10 d (Dilger and Adeola, 2006; Shastak et al., 2012; Liu et al., 2013; Mutucumarana et al., 2014a,b). Although published data on the effects of different adaptation periods are limited, it is possible that the length of dietary adaptation periods could alter the physiological state of the animal. This alteration may influence P availability, particularly when feeding semipurified diets with P and Ca concentrations below the requirement of the animal. Liu et al. (2012) measured higher EPL from broilers fed P-free semipurified diets for 4 h compared with birds fed these diets for 72 h. Furthermore, dietary adaptation periods longer than 5 d have resulted in reduced or negative BW gain in broilers fed P-deficient semipurified diets (Dilger and Adeola, 2006; Mutucumarana et al., 2014a,b, 2015a). Diets resulting in BW loss were not only P and Ca deficient, but many were also deficient in amino acids. Additional research is warranted to clarify the effects of adaptation period length on the physiological state of the animal and P availability when feeding P-deficient, semipurified diets.

DIET EFFECTS RELATED TO PURIFIED INGREDIENTS AND NUTRIENT CONCENTRATIONS

Purified Ingredients

Regression analysis to estimate feedstuff TPU requires feeding titration diets where the test P source is increased at the expense of purified ingredients to maintain dietary P concentrations below the requirement of the animal (Fan et al., 2001). Previous research has indicated a possible negative effect of feeding diets comprised of purified ingredients on nutrient digestibility as well as endogenous nutrient losses (Becker et al.,

1955; Rochell et al., 2012; Kong and Adeola, 2013; Masey O'Neill et al., 2014, Shastak et al., 2014). Therefore, variability reported for the AIPD of titration diets to determine TPU may be related to differences in diet formulations, specifically the purified fraction of the diet (Dilger and Adeola, 2006; Liu et al., 2013; Mutucumarana 2014a,b, 2015a).

Possible explanations for this negative effect include irritation of the GIT, increased rate of passage, or adaptations of the gut microbiota to dietary corn starch and dextrose as these ingredients could act as fermentation substrates (Metzler et al., 2009; Masey O'Neill et al., 2014). Additionally, researchers demonstrated that when broilers were fasted for 3 d, feeding a semipurified starch-based diet impeded the recovery of GIT epithelium compared with broilers fed a control diet (Maneewan and Yamauchi, 2003; Maneewan and Yamauchi, 2004). Ren et al. (2012) force-fed either a 25 or 40 g bolus of corn starch to roosters and reported a lower true metabolizable energy value for the corn starch fed as the 40 g bolus (3.79 vs. 4.09 kcal/g). It has been suggested that chickens may have an inability to fully metabolize purified ingredients when comprising a large portion of the experimental diet (Ren et al., 2012; Masey O'Neill et al., 2014). Becker et al. (1955) observed increased morbidity and mortality in growing swine, which were attributed to the rapid fermentation of undigested purified ingredients in the experimental diets.

Additionally, Rochell et al. (2012) demonstrated increased rates of feed passage in broilers fed a diet containing purified ingredients compared with birds fed a corn SBM diet. An increased digesta rate of passage limits exposure of nutrients to digestive enzymes and enterocyte transporters (Mateos and Sell, 1981). Rochell et al. (2012) also observed a reduction in the digestibility of cysteine, a principle component of mucin

(Selle et al., 2000), when feeding the semipurified diet compared with the corn-SBM diet. Increased mucin production and excretion has been associated with higher endogenous nutrient losses (Nyachoti et al., 1997). Higher endogenous nutrient losses coupled with faster rates of passage may contribute to a reduction in P digestibility when feeding titration diets with higher concentrations of purified ingredients. However, more research is warranted to determine the extent to which diets with varying concentrations of purified ingredients negatively affects P digestibility.

Conversely, Shastak et al. (2014) demonstrated no negative effects on the P availability of mineral P sources when feeding broilers either a corn-SBM basal diet or a basal diet containing purified ingredients. Researchers have also observed increases in P utilization when feeding P-deficient semipurified diets (Liu et al., 2013). These authors suggested that the P digestibility increased when feeding low-P diets as a result of the ability of GIT adaptations (Yan et al., 2005) to maintain P homeostasis. Dietary adaptation period length should be standardized to mitigate both negative physiological effects as well as possible beneficial GIT adaptations associated with feeding nutrient deficient semipurified diets.

Calcium Concentration and Calcium to Phosphorus Ratio of Diet

When formulating titration diets, the concentration of nutrients other than the test nutrient may affect how the test nutrient is digested and absorbed. The effect of Ca feeding strategy on P availability is well documented (Günther and al-Masri, 1988; al-Masri, 1995; Tamim et al., 2004; Driver et al., 2005; Selle et al., 2009). Increasing the Ca concentration or the Ca:P ratio of the diet has been demonstrated to hinder P digestion, absorption, and utilization in monogastric animals (Hurwitz and Bar, 1971; Qian and

Kornegay, 1996; Qian et al., 1997; Liu et al., 1998; Brady et al., 2002; Dilger and Adeola, 2006; Liu et al., 2013). The negative effect of high dietary Ca concentrations on P utilization is related to the capacity of Ca cations to chelate phytate P, which results in the formation of insoluble Ca-phytate complexes. While not the most potent chelator of phytate (Cu > Zn > Ni > Co > Mn > Fe > Ca), Ca has by far the highest practical implications because Ca comprises up to 1% of poultry diets (Vohra et al., 1965). The chelation between Ca and phytate renders the phosphate groups on phytate less available to hydrolysis by endogenous and exogenous phytases. Therefore, less liberated phosphate is available for absorption (Tamim and Angel, 2003; Tamim et al., 2004; Plumstead et al., 2008). Furthermore, wide ratios of Ca:NPP (> 2.5) have been reported to decrease the availability of NPP due to the formation of Ca-phosphate chelates (Hurwitz and Bar, 1971, Shafey et al., 1993). Consequently, it is critical to understand the effects of both the Ca:tP ratio as well as the Ca:NPP ratios of the diet when determining the P utilization of a test ingredient (Van der Klis and Versteegh, 1996; Plumstead et al., 2008).

For the proposed standard P availability protocol, the working group recommended maintaining a Ca:tP ratio between 1.3:1 and 1.4:1 with finely ground limestone as the primary Ca source (WPSA, 2013). Maintaining this Ca:tP ratio was suggested by the working group on the basis of previous research in addition to the fact that a similar ratio is typically utilized in commercial poultry production (Cobb 500 Broiler Performance and Nutrition Supplement, 2012; Ross 708 Broiler Nutrition Specification, 2014). Unfortunately, it is impossible to simultaneously maintain the Ca:tP and Ca:NPP ratios when formulating titration diets using most feedstuffs. Because most P in cereal grains is stored as phytate P (Eeckhout and de Paepe, 1994), an increase in

dietary concentrations of the test ingredient inherently increases total P to a greater extent than NPP. Furthermore, the addition of Ca to maintain a constant Ca:tP ratio causes the Ca:NPP ratio to become progressively wider with each additional inclusion of the test feedstuff. Previous research utilizing Ca:NPP ratios greater than 4.0 resulted in decreased P availability (Smith and Kabaija, 1985; al-Masri, 1995; Qian et al., 1997). Additionally, negative EPL may be attributed to widening Ca:NPP ratios of titration diets that maintained a fixed Ca:tP ratio. Regression equations generated by Liu et al. (2013) predicted negative EPL when feeding 3 sets of titration diets with each set having a different but fixed Ca:tP ratio. Conversely, researchers reported positive EPL when Ca:NPP ratios of titration diets were fixed and also maintained below 2.2:1 (Dilger and Adeola, 2006; Mutucumarana et al., 2014a,b). Due to the pronounced influence Ca has on P and phytate P availability, it is critical that TPU research assess the effects of differences in Ca feeding strategies.

PHYTATE HYDROLYSIS

An improved understanding of P availability of feedstuffs requires a concomitant evaluation of phytate P hydrolysis. A better understanding of phytate hydrolysis could potentially elucidate the reasons for TPU variability because over half of total P in a typical broiler diet is supplied by phytate (Eeckhout and De Paepe, 1994). Even though phytate P is generally considered unavailable to broilers, several studies have demonstrated that P from phytate can be enzymatically hydrolyzed under certain dietary conditions (Tamim and Angel, 2003; Tamim et al., 2004). Dietary Ca concentrations have been suggested as the most influential factor affecting phytate hydrolysis (Selle and Ravindran, 2007; Selle et al., 2009). In addition to directly chelating phytate anions,

dietary Ca may also reduce the efficacy of mucosal phytase (McCuaig et al., 1972; Applegate et al., 2003).

Although data are limited, diet composition and DAPL may also influence phytate hydrolysis, which would affect feedstuff P utilization. Purified ingredients in low-P diets may provide a fermentation source for GIT microflora (Metzler et al., 2009). Bacteria could be a source of endogenous phytase production in the GIT resulting in additional phytate hydrolysis (Kerr et al., 2000). Additionally, allowing for long DAPL when feeding P-deficient diets may increase P availability. Broilers have demonstrated an ability to adapt to P and Ca deficient diets by increasing phytate hydrolysis and P absorption (Yan et al., 2005). If broilers or the GIT microflora are adapting to these low-P semipurified diets, increases in phytate degradation may be observed. Increased phytate P hydrolysis and subsequent absorption of liberated orthophosphate groups may be another source of variation in the AIPD of titration diets. Because TPU is estimated based on titration diet AIPD, any variation in titration diet AIPD likely results in variable TPU data. However, limited research has been conducted evaluating the interactive effect of purified ingredients, Ca feeding strategy, and DAPL on phytate hydrolysis in titration diets utilized to estimate feedstuff TPU.

PROPOSED RESEARCH/GAPS IN LITERATURE

Additional research is warranted to better understand how methodologies affect the dynamics of P availability for the development of TPU coefficients for common feedstuffs. Transitioning from formulating diets on a NPP to TPU basis will allow nutritionists to more efficiently provide P to broilers resulting in reductions in both P excretion and dietary costs. Researchers have proposed the use of regression analysis of

AIPD data for titration diets to determine TPU (WPSA, 2013). However, published evidence has identified limitations regarding P availability values determined using TIPD or TPR protocols. Specifically, variability between TPU values for similar feedstuffs, inconsistencies between TPU and previously reported measures of P availability, and wide discrepancies in the prediction of EPL have all been reported.

Variable TPU values and the prediction of negative EPL may be attributed to a multitude of factors specific to the experimental methodologies. These include DAPL or diet composition related to purified ingredients or Ca concentrations (Ca:P ratio). Determining the effects of differences in DAPL is especially critical. Currently, DAPL are suggested to be greater than 5 d, but this length may have negative physiological consequences on broilers due to the necessary feeding of nutrient deficient semipurified diets (Shastak et al., 2014). Additional evidence indicated that broilers might be able to adapt to these nutrient deficient diets (Yan et al., 2005), which may lead to aberrations in the AIPD of titration diets and resultant TPU estimations.

Formulating titration diets with different Ca feeding strategies may also be responsible for variability in the AIPD of titration diets. Dietary Ca content has been demonstrated to influence P availability, but several different Ca feeding strategies have been utilized when estimating TPU. The WPSA recommends that titration diets for the determination of TPU maintain a fixed Ca:P ratio of 1.4:1 Ca. However, further research is required to validate this recommendation because regression equations have predicted negative EPL when titration diets were formulated with a fixed Ca:P ratio.

Variability in TPU data may also be associated with the current limited knowledge of factors affecting phytate hydrolysis. Highly purified diets may alter the

histology of the GIT, modify microflora fermentation, or increase digesta transit time, all of which could influence phytate hydrolysis and subsequent measures of TPU. Moreover, broilers have also been reported to adapt to P-deficient semipurified diets by increasing phytate P utilization. Therefore, the use of inconsistent DAPL when feeding semipurified titration diets may be a potential source of commonly reported variability for feedstuff TPU. Additionally, differences in Ca feeding strategy, specifically dietary Ca concentrations likely influences phytate hydrolysis. Consequently, more research is warranted to determine how diet type, Ca feeding strategy, and DAPL affect phytate P hydrolysis.

In order to address these knowledge gaps, a series of experiments were conducted to elucidate the effects of differences in titration diet type, DAPL, and dietary Ca feeding strategy on titration diet P availability, phytate hydrolysis, and corn TPU. These data will aid in the development and standardization of protocols for the determination of accurate and robust feedstuff TPU estimates.

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Table 2.1 Concentrations of total phosphorus, phytate phosphorus, and non-phytate P and the non-phytate P to to
feedstuffs

Researchers	Feedstuff	Total P	Phytate P	Non-Phytate P	N
				%	
Mutucumarana et al., 2014a	Corn	0.25	0.18	0.07	
Mutucumarana et al., 2015a	Corn	0.26	0.19	0.07	
Eeckhout and De Paepe, 1994	Corn	0.28	0.19	0.09	
Mutucumarana et al., 2014a	Canola Meal	0.97	0.69	0.28	
Eeckhout and De Paepe, 1994	Canola Meal	1.12	0.40	0.72	
Mutucumarana et al., 2014b	Wheat	0.32	0.21	0.11	
Eeckhout and De Paepe, 1994	Wheat	0.33	0.22	0.11	
Mutucumarana et al., 2014b	Sorghum	0.24	0.18	0.06	
Mutucumarana et al., 2014b	Corn DDGS	0.82	0.38	0.44	
Mutucumarana et al., 2014b	Soybean Meal	0.65	0.43	0.22	
Mutucumarana et al., 2015a	Soybean Meal	0.67	0.47	0.20	
Dilger and Adeola, 2006	Soybean Meal	0.65	0.37	0.28	
Eeckhout and De Paepe, 1994	Soybean Meal	0.61	0.32	0.29	
Iyayi et al., 2013	Black-Eyed Pea Meal	0.35	0.23	0.12	
Iyayi et al., 2013	Peanut Flour	0.45	0.28	0.17	
Eeckhout and De Paepe, 1994	Peanut Meal	0.68	0.32	0.36	
Adebiyi and Olukosi, 2015	Wheat DDGS	0.65	0.15	0.50	
Mutucumarana et al., 2015b	Meat and Bone Meal	3.75 to 6.02	0.00	3.75 to 6.02	

Table 2.2 Variability associated with apparent ileal P digestibility (AIPD), apparent P retention (APR), true ileal P retention (TPR), and endogenous P losses (EPL) when fed to growing broilers

Researcher	Feedstuff	AIPD	APR	TIPD	T
		%			
Mutucumarana et al., 2014a	Corn	61 to 70	45 to 68	68	
Mutucumarana et al., 2015a	Corn	40 to 82	-	43 to 73	
Mutucumarana et al., 2014a	Canola Meal	51 to 68	54 to 70	47	
Mutucumarana et al., 2014b	Wheat	38 to 47	-	46	
Mutucumarana et al., 2014b	Sorghum	23 to 52	-	33	
Mutucumarana et al., 2014b	Corn DDGS	40 to 65	-	73	
Mutucumarana et al., 2014b	Soybean Meal	14 to 63	-	80	
Mutucumarana et al., 2015a	Soybean Meal	61 to 81	-	52 to 74	
Liu et al., 2013	Soybean Meal	64 to 89	74 to 91	46 to 71	53
Dilger and Adeola, 2006	Soybean Meal	71 to 88	33 to 54	94	
Iyayi et al., 2013	Black-eyed pea meal	58 to 78	22 to 31	29	
Iyayi et al., 2013	Peanut Flour	76 to 81	23 to 43	67	
Adebiyi and Olukosi, 2015	Wheat DDGS	57 to 63	68 to 79	94	
Mutucumarana et al., 2015b	Meat and Bone Meal	49 to 69	-	42 to 69	

[1]DDGS = Distillers Dried Grains with Solubles

III. INTERACTIVE EFFECTS OF DIETARY ADAPTATION PERIOD LENGTH AND TITRATION DIET TYPE ON APPARENT ILEAL PHOSPHORUS DIGESTIBILITY AND PHOSPHORUS RETENTION IN GROWING BROILERS

ABSTRACT

Two experiments were conducted to examine the effects of different corn titration diets and DAPL on intestinal histology, AIPD, and APR in Ross × Ross 708 male broilers from 20 to 24 d of age. It was hypothesized that purified ingredients in nutrient deficient titration diets may affect P availability with varying DAPL. In experiment 1, 1,152 broilers were utilized in a 3 × 3 factorial treatment structure with 3 diets (control, 25% corn titration diet [**25CTD**], or 75% corn titration diet [**75CTD**]) and 3 DAPL (0, 24, or 72 h). Experiment 2 was conducted with 576 broilers as a 4 × 3 factorial arrangement with 4 diets (control, 25CTD, 75CTD, or nitrogen free diet [**NFD**]) and 3 DAPL (24, 48, or 72 h). All diets contained purified ingredients except for the control diet, which had the same formulation as the common starter and served as a control for DAPL. The NFD diet was fed as a highly purified protein-free diet. Broilers were fed a common diet until 19 d of age and then transferred to experimental diets at 20 d of age. In experiment 1, diet type did not affect intestinal histology. However, diet type and DAPL each influenced ($P \leq 0.001$) diet AIPD. Higher ($P \leq 0.001$) AIPD were measured for the control diet compared with the 75CDT, and the 25CTD had the lowest AIPD. Following

a 24 h DAPL, AIPD was higher ($P.\leq.0.001$) than after a DAPL of 0 or 72 h. In experiment 2, diet type × DAPL interactions ($P.\leq.0.001$) were observed for APR of the control diet, 75CTD, and NFD, but not the 25CTD. Because APR of the control diet was affected by varying DAPL, factors other than differences in diet type may have been responsible for inconstancies in the measure of P availability. Therefore, a standard DAPL should be established to reduce this possible variability, but more research is warranted to further elucidate an optimum DAPL for the determination of P availability..

INTRODUCTION

Poultry diets are currently formulated to meet a NPP requirement in order to prevent dietary deficiencies related to ingredient variability (Applegate and Angel, 2014). This method creates diets that typically contain excess P as a safety margin, which results in higher feed costs and increased P excretion. In order for nutritionists to more efficiently meet the P requirements of broilers, the WPSA has proposed standard methodology for the determination of feedstuff TPU. To estimate TPU, researchers utilize regression analysis of AIPD and APR data of titration diets (Dilger and Adeola, 2006; Liu et al., 2013; Mutucumarana et al., 2014a, 2015). However, resulting estimates for TPU have been inconsistent and negative EPL were predicted. These inconsistencies may have been associated with variability in the measurements of titration diet AIPD and APR (Liu et al., 2013; Mutucumarana et al., 2014a, 2015).

In order to estimate TPU, titration diets are formulated to contain varying concentrations of purified ingredients to establish graded dietary P concentrations below the requirement of the animal (Fan et al., 2001). Summit titration diets typically contain more of the test P source while basal titration diets contain less of the test P source and more purified ingredients (Fan et al., 2001; Dilger and Adeola, 2006). A standard DAPL has yet to be established, but DAPL greater than 5 d are currently proposed to allow broilers sufficient time to acclimate to titration diets with semipurified ingredients (WPSA, 2013). However, long DAPL (> 5 d) when feeding P deficient diets may negatively affect broiler performance and P metabolism (Shastak et al., 2014). Conversely, feeding P deficient diets may also stimulate adaptive mechanisms in the GIT to increase P availability to maintain P homeostasis (Yan et al., 2005). Therefore, long

and inconsistent DAPL may be a potential cause of variability commonly associated with measuring P availability for the estimation of TPU.

It may be possible to preclude possible variable effects of long and inconsistent DAPL by utilizing shorter and standardized period lengths . However, short (< 5 d) DAPL have been avoided due to the potentially negative effects of purified ingredients on GIT physiology (Maneewan and Yamauchi, 2003, 2004). Broilers consuming diets with purified ingredient may also result in increases of endogenous nutrient losses, which may influence measures of nutrient availability (Liu et al., 2012; Ren et al., 2012; Kong and Adeola, 2013; Masey O'Neill et al., 2014). Currently, limited data exists on the interactive effects of feeding titration diets with various concentrations of purified ingredients on AIPD and APR after different DAPL.

It was hypothesize that differences in DAPL would affect AIPD and APR of diets representative of either summit or basal corn titration diets. Therefore, 2 experiments were conducted to determine the effects of differences in DAPL when feeding either a 25CTD or 75CTD. The effects of short DAPL on AIPD and APR of 2 other diets were assessed including a corn-SBM based diet serving as a control for DAPL and a NFD, which contained almost exclusively purified ingredients and no protein.

MATERIALS AND METHODS

All experimental protocols involving live birds (PRN 2011-1969; PRN 2013-2237) were approved by the Institutional Animal Care and Use Committee at Auburn University.

Bird Husbandry

In each experiment, Ross × Ross 708 male broilers (experiment 1 = 1,152; experiment 2 = 576) were obtained from a commercial hatchery and vaccinated for Marek's disease, Newcastle disease, and infectious bronchitis. In each experiment, broilers (12 per cage; 0.04 m^2/bird) were placed into grower battery cages (Petersime, Gettysburg, OH). Each cage (68 cm × 68 cm × 38 cm) was equipped with 1 trough feeder and 1 trough waterer. Battery cages were housed in a solid-sided facility equipped with forced-air furnaces and cooling pads to regulate the temperature. Room temperatures were set to 33^{o}C at placement and were decreased gradually to 27^{o}C by the conclusion of the experiment. A 23L:1D lighting schedule was used from 1 to 19 d of age, after which a 16L:8D lighting schedule was utilized to ensure adequate feed intake (**FI**) for digesta sample collections. Broilers were fed a common corn-SBM starter diet (AME_n, 3,090 kcal/kg; digestible Lys, 1.10%; digestible TSAA, 0.83%; digestible Thr, 0.80%; Ca, 0.95%; and NPP, 0.45%) until receiving experimental diets at 20 d of age. Birds and feed were weighed on a per cage basis at the beginning and termination of the experimental period for the determination of BW gain (**BWG**) and FI. Feed intakes were calculated using total bird d and presented on a g/bird/d basis. Due to the random selection of 3 birds per cage for ileal digesta collection after DAPL of 0, 24, and 48 h, BWG was not assessed in experiment 1.

Dietary Treatments

Broilers were randomly assigned to 1 of 3 dietary treatments (control, 25CTD, or 75CTD) in experiment 1 or 4 dietary treatments (control, 25CTD, 75CTD, or NFD) in experiment 2. The control diet was a corn-SBM diet and had identical formulation

specifications as the common starter diet except for the addition of inert markers (TiO_2 and Cr_2O_3). Therefore, broilers receiving the control diet served as a control for DAPL. Experiments that use regression analysis to estimate TPU and predict EPL require feeding experimental diets with increasing concentrations of the test ingredient (Fan et al., 2001). The 25CTD and 75CTD were formulated to represent possible minimum or maximum corn titration diets that could be used in the determination of corn TPU. These diets had similar nutrient concentrations as the control diet except for P. The NFD was formulated with over 85% purified ingredients (corn starch, dextrose, and purified cellulose) and fed as an example of a highly purified protein-free diet.

Additionally, P must be supplied by titration diets in concentrations below the requirement of the animal (WPSA, 2013). In the 25CTD, P concentrations were limited to 0.23% by substituting corn starch (46.3%) for corn. The 75CTD contained 0.74% corn starch and 0.32% P. In experiment 1, all diets were isonitrogenous (20% CP) with CP supplied by SBM in the control diet or soy protein concentrate in the 25CTD and 75CTD. In experiment 2, the NFD represented a diet comprised almost exclusively with purified ingredients but was still formulated to contain similar concentrations of P and Ca as the 75% corn diet. A blend of corn starch (45.3%), dextrose (38.5%), and purified cellulose (5%) were substituted for corn in the NFD, and P was supplied by deflourinated phosphate.

All diets were formulated to contain 0.95% Ca. This Ca concentration was used because the diet serving as a control for DAPL was formulated with the same nutrient specifications as the common starter diet. Fixed Ca:P ratios were not used as this would have required diets with very low Ca concentrations that differed between the 25CTD

and 75CTD. Differences in dietary Ca concentration have been reported to affect P availability (Applegate et al., 2003; Driver et al., 2005; Plumstead et al., 2008; Selle et al., 2009). Diets also contained 0.38% chromic oxide as a visual marker and 0.50% titanium dioxide as an indigestible marker. The common starter diet was provided in crumble form while all experimental diets were provided in mash form.

Sample Collection, Chemical Analyses, and Calculations

Experiment 1. The experiment was arranged as a 3 × 3 factorial with 3 diets (control, 25CTD, and 75CTD) and 3 DAPL (0, 24, and 72 h). Ileal digesta were collected after each of the 3 DAPL with the 0 h collection occurring at the first evidence of Cr_2O_3 in the excreta of the first cage sampled. Subsequent digesta collections occurred after DAPL of either 24 or 72 h after the 0 h collection. After each DAPL, 3 birds per cage were euthanized via CO_2 asphyxiation and digesta were collected by gently flushing out the contents of the ileum using deionized-distilled water. The sampled subsection of ileum was between 4 and 30 cm proximal to the ileo-cecal junction, which corresponded to the terminal two-thirds of the ileum for broilers of this BW. Digesta were pooled by cage and retained on ice before being frozen at -20°C until later analysis. Frozen samples were lyophilized (Virtis Genesis Pilot Lyophilizer, SP Industries, Warminster, PA) and finely ground to fit through a 1 mm screen with an electric coffee grinder to avoid significant loss due to the small sample size of the collected digesta.

Diet and digesta P concentrations were determined by a commercial laboratory (University of Missouri Agricultural Experiment Station Chemical Laboratory, Columbia, MO) in quadruplicates for diets and in duplicates for digesta via inductively coupled plasma optical emission spectroscopy (method 990.08; AOAC International,

2006). The experimental corn source was analyzed by a commercial laboratory (Enzyme Service and Consultancy, Tredomen Park, United Kingdom) to measure phytate P concentrations according to the Megazyme method (method K-PHYT, Megazyme International Ireland Ltd.). Samples were extracted in 0.66 M HCl overnight, followed by enzymatic dephosphorylation with phytase to yield *myo*-inositol monophosphate and 5 molecules of orthophosphate. The inositol ring was completely dephosphorylated by the action of alkaline phosphatase. Orthophosphate groups released from phytate were reacted with ammonium molybdate (5% wt/vol) to yield 12-molybdophosphoric acid. Finally, 12-molybdophosphoric acid and 1 M H_2SO_4 with ascorbic acid (10% wt/vol) reacted to form molybdenum blue. The concentration of molybdenum blue formed in the reaction was proportional to the concentration of orthophosphate in the original sample. The orthophosphate concentration was determined colorimetrically by measuring the increase in absorbance at 655 nm (Fiske and Subbarrow, 1925), and phytate content was calculated based on phytate having 28.2% P. Non-phytate P content of the corn was calculated as the difference between total P and phytate P. Dietary Ca concentrations were determined in quadruplicate using flame emission spectrometry (Fritz and Schenk, 1979) on a 1.0 g sample using an AAnalyst 700 spectrometer (Perkin Elmer Instruments, Waltham, MA). Lanthium oxide was added to samples prior to analysis to prevent Ca-phytate complexes from forming as chelated Ca ions are not able to be detected by the spectrometer.

Titanium dioxide concentrations were determined in quadruplicates and duplicates for diets and digesta, respectively, by a method based on that of Leone (1973). Briefly, 0.25 g of digesta or feed were added to threaded glass test tubes and ashed at

580°C for 10 h; 0.8 g of $NaSO_4$ was added to the ashed samples, which were diluted with 5 mL of H_2SO_4 and then heated at 130°C for 72 h; tube contents were diluted to 50 mL with distilled deionized water and held for 12 h at 25°C; 3 mL of feed samples or 1 mL of digesta samples plus 2 mL of 1.8 M H_2SO_4 were added to glass test tubes with 150 µL of H_2O_2; and after allowing 30 min for color development, absorbance was measured on a spectrophotometer (DU 730, Beckman Coulter, Brea, CA) at 410 nm. The efficacy of this assay was validated by verifying the recoveries of TiO_2 from the experimental diets were within a specific range (0.48 to 0.52%) of formulated values (0.50%).

Data generated from chemical analyses were used to calculate AIPD for each diet after each DAPL using the following equation in experiment 1:

$$AIPD = \left[1 - \left(\frac{TiO_{2\ Diet,}}{TiO_{2\ Digesta}}\right) \times \left(\frac{P_{Digesta}}{P_{Diet}}\right)\right] * 100$$

where $P_{Digesta}$ and P_{Diet} equal analyzed percent concentrations of P in the digesta and diets, respectively, and $TiO_{2\ Digesta}$ and $TiO_{2\ Diet}$ represent the analyzed percent concentrations of TiO_2 in the digesta and diets, respectively.

To determine the effects of diet type on small intestine histology, villus to crypt height ratios of the duodenum and jejunum were measured using the following methodology at the Auburn University Pathobiology Lab. During the 72 h digesta collection, 2 broilers per cage were randomly selected for analysis. A 2 cm section of the straight portion of the distal duodenum (3 cm distal the duodenal loop) and a 2 cm section of the jejunum (7 cm proximal to Meckel's diverticulum) were extracted from each bird. Using a 20 gauge needle, 10% formalin was gently infused into the lumen of each sample. Infused samples were affixed to filter paper and submerged into samples cups containing 30 mL of 10% formalin until further analysis. Fixed intestine samples were

embedded in paraffin and 4 micron sections were mounted, stained with hematoxylin and eosin, and coverslipped. Slides were scanned with Aperio ScanScope (20x objective, Leica Microsystems, Buffalo Grove, IL), and evaluated using Visiopharm Digital Pathology software (Visiopharm, Broomfield, CO). Calculations of villus to crypt height ratios were based upon measured circumference as a surrogate for height (assuming each transverse section to be approximately circular, $2 \times \pi$ will cancel out). The technician conducting these analyses was blinded to the experimental grouping.

Experiment 2. The experiment was arranged as a 4 × 3 factorial with 4 diets (control, 25CTD, 75CTD, and NFD) and 3 DAPL (24, 48, and 72 h). Three 24 h P retention assays were conducted from 20 to 21, 21 to 22, and 22 to 23 d of age, representing DAPL of 24, 48, and 72 h, respectively. Feed disappearance, net excreta weight, and excreta samples were collected after each 24 h period to calculate P intake and excretion. From the pan below each cage, 4 subsamples were collected (free from feed and feather contamination) from the total amount of excreta on the pan. Samples were homogenized and a representative 500 g sample was placed in a plastic bag for analysis. Samples of feed and excreta were frozen at -20°C until later analysis. Feed and excreta samples were lyophilized and ground through a cyclone mill (Cyclotec model number 1093, Foss North America, Inc., Eden Prairie, MN) equipped with a 1 mm screen to ensure a homogeneous mixture. Phosphorus contents of feed and excreta were determined utilizing the same procedure described in experiment 1. Percent P retention was determined using the following equations adapted from Leske and Coon (2002):

$$APR = \frac{(P\ intake - P\ excreted)}{P\ intake}$$

where APR represented apparent P retention (%). Phosphorus intake and excretion (g) data were determined from FI values and excreta output on a per bird basis. At 24 d of age, AIPD of the diets were determined using the same procedure as experiment 1 except digesta samples were collected and pooled from 8 broilers per cage. Body weight gain and FI were determined by weighing birds and feed on a per bird basis at 20 and 24 d of age.

Statistical Analyses

In both experiments, data were analyzed as repeated measures using a randomized complete block design with cage location as the blocking factor. Experiments were represented by 16 or 12 replicate cages per treatment for experiments 1 or 2, respectively, with 3 repeated measures per cage representing each DAPL. Statistical significance was established at $P \leq 0.05$, and significantly different treatment means established by a significant F-test were separated using Tukey's Honestly Significant Difference test (Tukey, 1953).

In experiment 1, analysis of variance was performed using PROC MIXED (SAS Institute, 2004) by the following mixed-effects model:

$$Y_{ijkl} = \mu... + \rho_i + \alpha_j + \beta_k + (\alpha|\beta)_{jk} + \varepsilon_{ijk}$$

where $\mu...$ is the overall mean; the ρ_i are identically and independently normally distributed random block effects with mean 0 and variance σ^2_ρ; the α_j are fixed factor level effects corresponding to the j^{th} diet type (control, 25CTD, or 75CTD) such that $\Sigma\alpha_j = 0$; the β_k are fixed factor level effects corresponding to the k^{th} DAPL (0, 24, or 72 h) such that $\Sigma\beta_k = 0$; the $(\alpha|\beta)_{jk}$ are interaction level effects corresponding to all possible permutations of either the j^{th} diet type and k^{th} DAPL such that $\Sigma(\alpha|\beta)_{jk} = 0$; and the

random error ε_{ijk} is identically and independently normally distributed with mean 0 and variance σ^2.

In experiment 2, an ANOVA was performed using PROC MIXED (SAS Institute, 2004) by the following mixed-effects model:

$$Y_{ijk} = \mu... + \rho_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk}$$

where $\mu...$ is the overall mean; the ρ_i are identically and independently normally distributed random block effects with mean 0 and variance σ^2_ρ; the α_j are fixed factor level effects corresponding to the j^{th} diet type (control, 25CTD, 75CTD, or NFD) such that $\Sigma\alpha_j = 0$; the β_k are fixed factor level effects corresponding to the k^{th} AP (24, 48, or 72 h) such that $\Sigma\beta_k = 0$; the $(\alpha\beta)_{jk}$ are interaction level effects corresponding to the j^{th} diet type and k^{th} DAPL, such that $\Sigma(\alpha\beta)_{jk} = 0$; and the random error ε_{ijkl} is identically and independently normally distributed with mean 0 and variance σ^2. Data were also analyzed using the same mixed-effects model without the inclusion of the NFD.

RESULTS AND DISCUSSION

Nutrient Analysis and Broiler Performance

Calculated and analyzed nutrient concentrations of corn sources and experimental diets are presented in Tables 3.1 and 3.2, respectively. Total P, phytate P, NPP, and Ca concentrations were similar between corn sources used for experiment 1 or 2, and did not vary substantially compared with reported values from the literature (NRC, 2010). Analyzed P and Ca values of the experimental diets varied slightly from calculated values. Due to these differences, the analyzed nutrient contents of each diet were used to calculate nutrient intake, digestibility, and retention values.

Growth performance and P intake are presented in Table 3.3. Feed and P intakes

were lowest ($P < 0.001$) for broilers fed the 25CTD and highest for birds receiving the control diet, while broilers fed the 75CTD consumed an intermediate amount in both experiments. In experiment 2, FI was lower ($P < 0.001$) for broilers fed the NFD compared with the 3 other experimental diets. Previous data have indicated that broilers consume less feed when fed P deficient diets (Driver et al., 2005). Additionally, diets formulated with purified ingredients have been reported to have low palatability when fed to broilers (Sullivan, 1999; Mutucumarana et al., 2014a,b; Shastak et al., 2014).

Reduced FI resulted in limited P intake, which likely influenced ($P < 0.001$) BWG. Broilers fed the NFD had the lowest FI and negative BWG (-4.6 g/bird/d). The lack of amino acids (**AA**) in the NFD, in combination with poor palatability of the purified ingredients, is a likely explanation for observed negative BWG. Feed intakes were higher ($P < 0.001$; 68.5, 89.4, and 105.0 g/bird/d) when diets included less purified ingredients and resulted in increased BWG ($P < 0.001$; 26.2, 48.6, or 72.7 g/bird/d) for birds fed the 25CTD, 75CTD, or control diet respectively. Only broilers receiving the control diet had BWG similar to breeder performance objectives (Ross 708 Broiler Performance Objectives, 2007).

Apparent Ileal Phosphorus Digestibility

In experiment 1, no interactions ($P > 0.05$) between the treatment diets and DAPL were detected for diet AIPD, but main effects ($P < 0.001$) of diet type and DAPL were observed (Figure 3.1). Values for AIPD were lowest for the 25CTD (1.0%), intermediate for the 75CTD (19.9%), and highest for the control diet (50.4%) (Figure 3.1). Similar effects of diet type on ($P < 0.001$) diet AIPD were observed in experiment 2 after a DAPL of 96 h except that the NFD and control diet had similar AIPD (Figure 3.2).

Values for diet AIPD were 16.6, 28.7, 52.1 or 55.4% for the 25CTD, 75CTD, control diet, or NFD, respectively.

Diet AIPD were affected ($P < 0.001$) by DAPL in experiment 1, and were highest after a DAPL of 24 h (28.4%) and lower for DAPL of 0 and 72 h (22.0 and 20.8%, respectively) (Figure 3.1). Limited data have been published on the effect of DAPL on titration diet AIPD. While a 5 d DAPL has been suggested for the determination of TPU (WPSA, 2013), most researchers determining the AIPD of titration diets have used variable DAPL, but all were longer than 5 d (Dilger et al,. 2006; Liu et al., 2013; Mutucumarana et al., 2014a, 2015). The lack of a standard DAPL between methodologies could be a potential source of the variation commonly observed in titration diet AIPD and the resulting estimates of TPU (Dilger et al,. 2006; Liu et al., 2013; Mutucumarana et al., 2014a,b, 2015).

In experiment 1, similar effects of DAPL on AIPD were observed regardless of diet type, with higher AIPD values reported after 24 h of DAPL compared with 0 and 72 h. This was an unexpected finding because broilers fed the control diet were not subjected to a dietary adaptation period because this diet had the same formulation as the common starter. Therefore, DAPL effects on AIPD may be related to factors other than differences in diet type, like changes in the lighting schedule. Broilers were not handled or moved prior to receiving the treatment diets in experiment 1. Moreover, research published by Yan et al. (2005) indicated that broilers may have an ability to adapt to low-P diets to increase P digestibility. However, improvements in AIPD with increasing DAPL were not observed in experiment 1 for broilers fed the low-P diets. This lack of an effect is likely attributable to the short DAPL utilized in the current research. In light of

these discrepancies, it is difficult to determine specific reasons for observed differences in AIPD, regardless of diet type, due to DAPL. All diets were adequate in AA, which may have limited any possible negative effect of the purified ingredients, however, additional research is warranted to better understand the effect of DAPL on P availability.

Diet type did not have an effect ($P > 0.05$) on villus to crypt height ratios of the duodenum or jejunum when measured after DAPL of 72 h (Table 3.4). Therefore, the semipurified 25CTD fed in the current experiment did not negatively affect the morphology of the small intestine, possibly explaining the lack of interaction effects between diet type and DAPL on AIPD. This is in contrast to research published by Maneewan and Yamauchi (2003), which indicated a negative effect of semipurified diets on small intestine histology. Differences may have been related to the longer DAPL before tissue sampling (24 vs. 72 h), as well as formulation differences between the semipurified diets, specifically AA content. In the current research, a DAPL of 72 h may have been adequate for the rejuvenation of any damage to the epithelial tissue of the small intestine resulting from feeding the semipurified 25CTD. Additionally, all diets in the current trial were formulated to meet the AA requirements of the bird (Ross 708 Broiler Nutrition Specification, 2007). Amino acids and CP have been suggested as the primary nutrients required for the regeneration of GIT tissue (Maneewan and Yamauchi, 2004).

Means for diet AIPD in experiment 1 for the 25CTD (1.0%) and 75CTD (19.9%) were lower than the range of values for corn titration diet AIPD (61 to 88%) in the literature (Mutucumarana et al., 2014a, 2015). These discrepancies were likely attributable to differences in dietary Ca:P ratios. It is well documented that the

concentration of Ca and P and their respective ratio to each other influence both phytate P and NPP availability (Hurwitz and Bar, 1971; Smith and Kabaija, 1985; Tamim and Angel, 2003; Plumstead et al., 2008; Selle et al., 2009). Variations in Ca:P ratios among treatments were a consequence of maintaining dietary Ca concentrations between diet types. This Ca feeding strategy was used to prevent Ca deficiencies, as maintaining dietary Ca:P ratios would have required diets to be formulated with very low Ca concentrations. Furthermore, assessing the AIPD of Ca deficient diets may lead to the overestimation of AIPD because broilers are better able to utilize phytate P when diets are Ca deficient (Tamim and Angel, 2003; Tamim et al., 2004; Selle et al., 2009). Titration diets used by Mutucumarana et al. (2014a, 2015) had Ca concentrations ranging from 0.03 to 0.48%, which possibly explains why they observed higher titration diet AIPD.

Apparent Phosphorus Retention

In experiment 2, interactive effects ($P < 0.001$) between the 4 diet types and 3 DAPL were observed for diet APR when the NFD was included in the statistical analysis (Table 3.5). The NFD had lower ($P < 0.001$) APR after 24 h (52.3%) of DAPL compared with DAPL of 48 and 72 h (64.4% and 60.5%, respectively). Conversely, APR decreased ($P < 0.001$) as DAPL increased from 24 h (25.1%) to 48 or 72 h (18.0 or 16.3%, respectively) in broilers fed the 75CTD. The control diet and 25CTD had intermediate APR values compared with the NFD and 75CTD and were not affected ($P > 0.05$) by changes in DAPL. When data were analyzed excluding the NFD, similar interaction ($P = 0.002$) effects on APR were measured for the 25CTD and 75CTD, but APR for the control diet decreased with longer DAPL (52.0, 49.1, or 46.6% for DAPL of 24, 48, or 72

h, respectively).

Measures of APR for the NFD were the highest of all diet types, which was likely attributed to the P source used when formulating this diet. The NFD contained no phytate P, with defluorinated phosphate providing NPP as the primary P source. Research conducted by Leske and Coon (2002) has demonstrated that the APR of NPP is greater compared with the APR of phytate P in broilers. Overall, values for APR of the NFD were within the reported range (41.8 to 79.0%) from the literature when inorganic P sources were the primary source of dietary P (Van der Klis and Versteegh, 1996; Leske and Coon, 2002; Shastak et al., 2012).

While the NFD had the highest APR overall, APR was lower after a DAPL of 24 compared with DAPL of 48 and 72 h. The lower APR at 24 h may have been related to purified ingredients in this diet. Purified ingredients have been reported to influence endogenous nutrient losses resulting in lower measures of nutrient digestibility (Ren et al., 2012; Kong and Adeola, 2013; Masey O'Neill et al., 2014). Research conducted by Liu et al. (2012) reported higher EPL for birds fed P-free semipurified diets for 4 h compared with birds fed a similar diet for 72 h. These researchers also suggested that dietary concentrations of AA or other nutrients may affect EPL. Furthermore, Maneewan and Yamauchi (2004) determined that GIT epithelial tissue was negatively affected by feeding semipurified, AA deficient diets. Therefore, dietary AA concentrations between the 25CTD and the NFD may explain why interaction effects were observed for the NFD and not the 25CTD. In both experiments, changes in DAPL did not affect AIPD or APR for the 25CTD, which may be due to the 25CTD being formulated with adequate AA concentrations. Therefore, we recommend that semipurified titration diets contain

adequate concentrations of AA, as changes in DAPL did not negatively affect AIPD or APR of AA adequate 25CTD in the current experiments.

In experiment 2, APR values were higher for the 25CTD compared with the 75CTD. This is in contrast to AIPD results from experiment 1, where AIPD of the 25CTD was lower than the AIPD of the 75CTD. Furthermore, AIPD was also lower for the 25CTD compared with the 75CTD in experiment 2. The 25CTD had a wider Ca:P ratio, which resulted in lower AIPD compared with the AIPD of the 75CTD. Lower APR for the 75CTD signified that P was being excreted by the kidneys, resulting in lower diet APR. This effect may have been attributed to the 75CTD having 47% more phytate P content than the 25CTD. The higher concentrations of dietary phytate P may have limited Ca digestibility (Plumstead et al., 2008), resulting in lower plasma Ca concentrations and an increase in parathyroid hormone secretion (Proszkowiec-Weglarz and Angel, 2013). Parathyroid hormone acts directly on the proximal tubules of the kidney to limit the reabsorption of P from the glomerular filtrate (Wideman et al., 1980; Wideman and Braun, 1981), which would increase P excretion resulting in lower APR values. Moreover, the limited reabsorption of P from the kidneys that results in additional P output in the excreta may have also explained why APR decreased with increasing DAPL for broilers fed the 75CTD. However, longer DAPL also resulted in reduced APR for the diet formulated as a control for DAPL. The reason for these effects remains unclear, as diets were also formulated to be adequate in Ca and additional research must be conducted to determine what specific factors related to DAPL influence measures of P availability.

In conclusion, no clear evidence was observed of broilers adapting to P deficient diets to increase AIPD or APR to maintain P homeostasis. Furthermore, the purified ingredients in the 25CTD did not appear to negatively affect GIT histology, and the AIPD and APR of the 25CTD were not affected by different DAPL when compared with other diet types. However, main effects of DAPL and diet type were observed. Significant main effects related to diet type were attributable to the diets having different Ca:P ratios as a consequence of maintaining diet Ca concentrations at 0.95%. More research is warranted to elucidate how differences in DAPL resulted in inconsistent effects for AIPD and APR, especially for the diet formulated as a control for DAPL.

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Table 3.1 Analyzed composition of corn used to determine P availability in experiments 1 and 2[1,2]

Item (%, as-fed)	Experiment 1	Experiment 2
Total P	0.276	0.284
Phytate P[1]	0.208	0.237
Non-Phytate P	0.068	0.047
Ca	0.018	0.020

[1]Analyses were performed in duplicate.
[2]Analytical procedures: Total P (method 990.08; AOAC International, 2006); Phytate-P (method K-PHYT, Megazyme International Ireland Ltd.); Non-phytate P (subtracting phytate P from total P concentrations); Ca (Fritz and Schenk, 1979)

Table 3.2 Ingredient composition of experimental diets fed from 20 to 24 (experiment 1) or 25 (experiment 2) d of age to determine changes in ileal P digestibility and P retention of diets after dietary adaptation periods ranging from 0 to 72 (experiments 1) or 24 to 96 h (experiment 2)[1]

Ingredient, % "as-fed"	Control Diet	25CTD	75CTD	Nitrogen Free Diet
Ground Corn	62.26	25.00	75.00	-
Corn Starch	-	46.34	0.74	45.34
Soybean Meal	30.57	-	-	-
Soy Protein Concentrate	-	20.83	16.60	-
Dextrose	-	1.00	1.00	38.53
SolkaFloc[2]	-	-	-	5.00
Soy Oil	1.94	1.86	1.79	5.00
Sodium Chloride	0.38	0.00	0.12	-
Sodium Bicarbonate	-	0.10	-	0.84
DL Methionine	0.31	0.40	0.33	-
Lysine HCl	0.31	0.11	0.27	-
Threonine	0.15	0.00	0.04	-
Calcium Carbonate	0.81	1.72	1.80	0.90
Dicalcium Phosphate	1.91	-	-	-
Defluorinated Phosphate	-	-	-	1.85
Choline chloride 60	0.00	0.14	0.07	0.25
Vitamin Premix[3]	0.25	0.25	0.25	0.25
Mineral Premix[4]	0.25	0.25	0.25	0.25
Magnesium Oxide	-	0.19	0.08	0.15
Potassium Sulfate	-	0.68	0.68	0.80
Potassium Chloride	-	0.25	0.10	0.34
Chromic Oxide	0.38	0.38	0.38	-
Titanium Dioxide	0.50	0.50	0.50	0.50
Calculated Analysis				
AME, kcal/kg	3,090	3,090	3,090	
Crude Protein, %	20.0	20.0	20.0	-
Phosphorus, %	0.76	0.23	0.32	0.33
Non-Phytate Phosphorus, %	0.45	0.08	0.10	0.33
Phytate Phosphorus	0.31	0.15	0.22	0.00
Calcium, %	0.95	0.95	0.95	0.95
Ca:tP	1.25	4.13	2.97	2.88
Lysine, %	1.30	1.30	1.30	-
TSAA, %	0.95	0.95	0.95	-
Threonine, %	0.90	0.90	0.90	-
Sodium	0.17	0.17	0.17	
Analyzed Composition (Exp 1)				
Ca, %	0.99	0.84	0.72	-
Phosphorus, %	0.73	0.22	0.33	-
Analyzed Composition (Exp 2)				
Ca, %	0.98	0.86	0.91	0.85
Phosphorus, %	0.75	0.26	0.32	0.33

[1]For experiment 1, broilers received 1 of 3 experimental diets (control, 25% corn [25CTD], or 75% corn [75CTD]). Ileal digesta was collected after allowing 3 adaptation periods with collection 1 (0 h) occurring at the first sight of chromic oxide in the excreta. In experiment 1, birds were either fed *ad libitum* or feed was withheld 6 h prior to the collection for 3 h to allow for the emptying of the gastrointestinal tract prior to consuming experimental diets. In experiment 1, the 3 dietary adaptation periods were 0, 24, and 72 h. For experiment 2, broilers received 1 of 4

experimental diets, and P retention was determined after 24, 48, and 72 h of dietary adaptation period. Ileal digesta was collected at 96 h for the determination of ileal P digestibility.

[2]Purified cellulose, International Fiber Corp., Tonawanda, NY.

[3]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 8,000 IU; Vitamin D (cholecalciferol), 2,000 IU; Vitamin E (DL-alpha tocopherol acetate), 8 IU; menadione (menadione sodium bisulfate complex), 2 mg; Vitamin B12 (cyanocobalamin), 0.02 mg; folacin (folic acid), 0.5 mg; D-pantothenic acid (calcium pantothenate), 15 mg; riboflavin (riboflavin), 5.4 mg; niacin (niacinamide), 45 mg; thiamin (thiamin mononitrate), 1 mg; D-biotin (biotin), 0.05 mg; and pyridoxine (pyridoxine hydrochloride), 2.2 mg; choline (choline chloride).

[4]Mineral premix include per kg of diet: Mn (manganous oxide), 65 mg; Zn (zinc oxide), 55 mg; Fe (iron sulfate monohydrate), 55 mg; Cu (copper sulfate pentahydrate), 6 mg; I (calcium iodate), 1 mg; Se (sodium selenite), 0.3 mg.

Table 3.3 Effect of dietary treatment on feed and phosphorus intake as well growth performance when fed to Ross × Ross 708 male broilers[1]

	Diet Type				Probabilities	
Measurement	Control	25CTD[2]	75CTD[2]	NFD[2]	SEM	$P \leq$
Experiment 1[3]						
Feed Intake, g/bird per d	101.6[a]	71.7[c]	94.5[b]	-	1.6	0.001
P Intake, mg/bird per d	742[a]	158[c]	312[b]	-	0.8	0.001
Experiment 2[4]						
BW Gain, g/bird per d	72.7[a]	26.6[c]	48.5[b]	-4.6[d]	1.2	0.001
Feed Intake, g/bird per d	105.0[a]	68.4[c]	89.4[b]	58.6[d]	1.7	0.001
P Intake, mg/bird per d	788[a]	178[c]	286[b]	193[c]	0.7	0.001

[1]Experimental diets were fed from 21 to 24 (experiment 1) or 25 (experiment 2) d of age.

[2]NFD = nitrogen free diet. 25CTD = 25% corn titration diet. 75CTD = 75% corn titration diet.

[3]Values represent the least-square means of 32 replicate cages containing 10 birds per cage fed either the control, 25% corn, or 75% corn diet.

[4]Values represent the least-square means of 12 replicate cages containing 10 birds per cage fed either the control, 25% corn, 75% corn, or NFD.

[abc]Means within a row for a given measurement not sharing a common superscript differ ($P \leq 0.05$).

Table 3.4 Villus to crypt height ratios of the duodenum and jejunum of Ross × Ross 708 male broilers fed either a corn-soybean meal (control), 25% corn (25CTD), or 75% corn diet (75CTD) in experiment 1[1]

	Villus to Crypt Height Ratio	
Item	Duodenum	Jejunum
Diet Type		
Control	7.12	4.96
25CTD	6.82	6.44
75CTD	7.64	5.65
SEM	0.35	0.52
Source of Variation	*Probabilities*	
Diet Type	0.27	0.07

[1]Values are least-square means of 10 replicate cages with 2 intestinal segments analyzed per cage after a dietary adaptation period of 72 h.

Table 3.5. Interactive effects of 4 diet types (corn-soybean meal control, 25% corn titration [25CTD], 75% corn titration [75CTD], and nitrogen free diet) and 3 dietary adaptation period lengths (24, 48 and 72 h) on apparent phosphorus retention for Ross × Ross 708 male broilers for experiment 2[1]

Diet Type[2]	Adaptation Period Length (h)	Apparent Phosphorus Retention[3] (%)	Apparent Phosphorus Retention[4] (%)
	24	52.0[b]	52.0[a]
Control	48	49.1[b]	49.1[ab]
	72	46.6[b]	46.6[b]
	24	32.9[c]	32.9[c]
25CTD	48	33.5[c]	33.5[c]
	72	29.5[cd]	29.5[cd]
	24	25.1[d]	25.1[d]
75CTD	48	18.0[e]	18.0[e]
	72	16.3[e]	16.3[e]
	24	52.3[b]	-
Nitrogen Free Diet	48	64.4[a]	-
	72	60.5[a]	-
SEM		1.6	1.2
Control		49.2[b]	49.2[a]
25CTD		32.0[c]	32.0[b]
75CTD		19.8[d]	19.8[c]
Nitrogen Free Diet		59.1[a]	-
SEM		1.2	1.0
	24	40.6[ab]	36.7[a]
	48	41.3[a]	33.5[b]
	72	38.2[b]	30.8[b]
	SEM	0.8	0.7

Source of Variation	*Probabilities*	
Diet Type × Adaptation Period Length	<0.001	0.002
Diet Type	<0.001	<0.001
Adaptation Period Length	<0.001	<0.001

[1]Vaules are least-square means of 12, 36, or 48 replicate cages for interactive effects, main effects of diet, or main effects of adaptation period, respectively.

[2]Experimental diets were fed from 20 to 23 d of age.

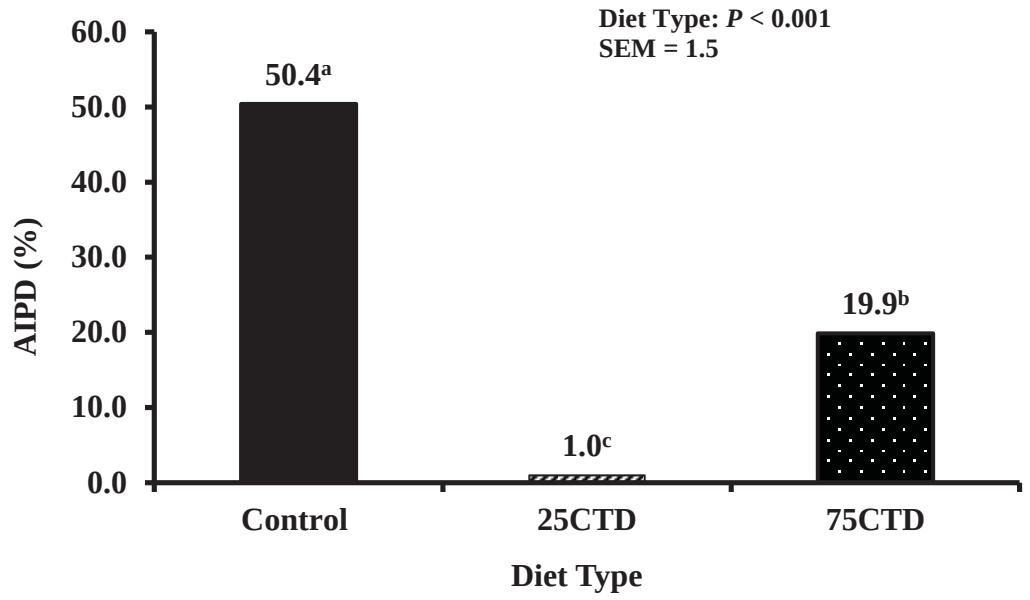
[3]For the determination of APR and ACaR, a 24 h balance assay was conducted for each collection period utilizing 10 birds per cage.

[4]The nitrogen free diet was removed for statistical analyses.

[a-e]Means within a column for a given measurement not sharing a common superscript differ ($P \leq 0.05$) and were separated using Tukey's Honestly Significant Difference test.

Figure 3.1 Effects on apparent ileal P digestibility (AIPD) of either **A)** 3 diet types (control, 25% corn titration [25CTD], and 75% corn titration [75CTD]) or **B)** 3 dietary adaptation period lengths of 0, 24, and 72 h for Ross × Ross 708 male broilers between 21 and 24 d of age for experiment 1. Values represent the least-square means of 32 replicate cages per diet. For the determination of AIPD, 3 birds per cage were euthanized at each sampling period for the collection of ileal digesta.

A)


Diet Type: P < 0.001
SEM = 1.5
60.0
50.0
40.0
30.0
20.0
10.0
0.0
AIPD (%)
50.4a
1.0c
19.9b
Control
25CTD
75CTD
Diet Type

B)

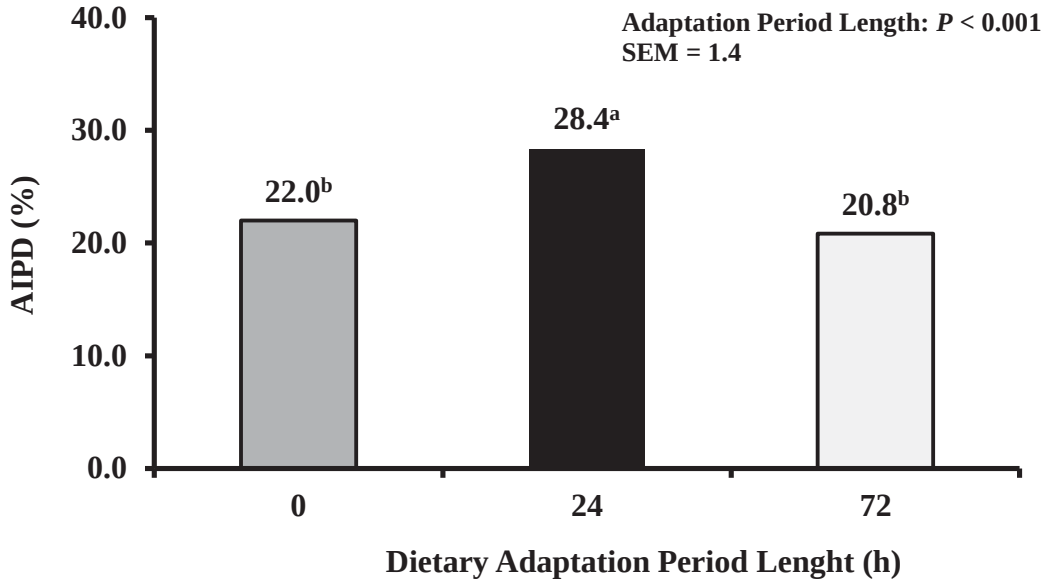
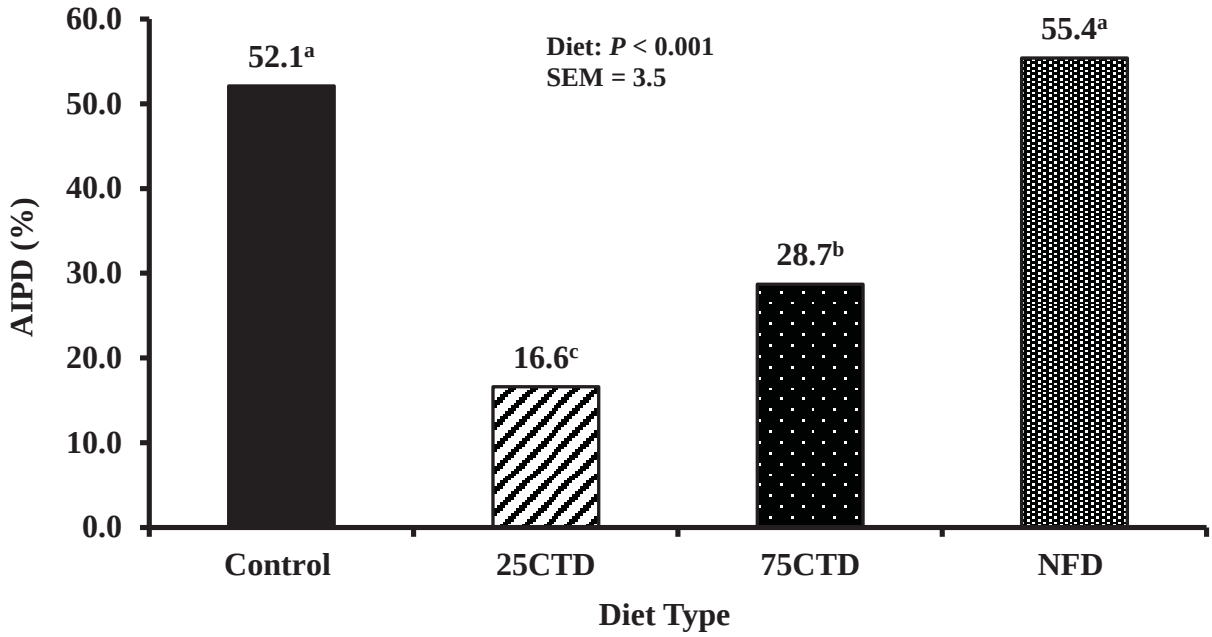

Adaptation Period Length: P < 0.001
SEM = 1.4
40.0
30.0
20.0
10.0
0.0
AIPD (%)
22.0b
28.4a
20.8b
0
24
72
Dietary Adaptation Period Lenght (h)

Figure 3.2 Apparent ileal P digestible (AIPD) values of 4 diet types (control, 25% corn titration [25CTD], 75% corn titration [75CTD], and nitrogen free diet [NFD]) after a dietary adaptation period of 96 h for Ross × Ross 708 male broilers between 20 and 24 d of age for experiment 2. Values represent the least-square means of 12 replicate cages per diet. For the determination of AIPD, 8 birds per cage were euthanized for the collection of ileal digesta. Means not sharing a common superscript differ ($P < 0.001$).


60.0
50.0
40.0
30.0
20.0
10.0
0.0
AIPD (%)
52.1a
16.6c
28.7b
55.4a
Diet: P < 0.001
SEM = 3.5
Control
25CTD
75CTD
NFD
Diet Type

IV. EFFECTS OF CALCIUM FEEDING STRATEGY ON TRUE ILEAL PHOSPHORUS DIGESTIBILITY AND TRUE PHOSPHORUS RETENTION DETERMINED WITH GROWING BROILERS

ABSTRACT

An experiment utilizing 960 Ross × Ross 708 male broilers was conducted to determine the effects of Ca feeding strategy on TIPD and TPR of corn. Experimental diets were formulated with 1 of 3 dietary Ca feeding strategies (0.95%, 0.13%, or variable Ca concentrations to maintain a 2.1:1 Ca:P ratio) and contain 0, 25, 50, or 75% corn. A practical corn-soybean meal diet (1.4:1 Ca:P ratio) was fed as a control. After receiving a common starter diet, experimental diets were fed from 19 to 26 d of age. After a 48-h dietary adaptation period, a 48-h digestibility and retention assay was conducted. At 25 and 26 d of age, ileal digesta were collected from 8 birds per cage. Broilers consuming the control diet had higher ($P < 0.001$) BW gain, feed intake, digesta P, and excreta P than broilers consuming the corn titration diets. Digesta and excreta P increased (linear, $P < 0.05$) with graded increases of corn. True ileal P digestibility and TPR were highest ($P < 0.05$) for diets with 0.13% Ca (57.3 and 69.5%, respectively) compared with diets formulated with a 2.1:1 Ca:P ratio (41.2 and 37.8%, respectively) or 0.95% Ca (25.4 and 39.0%, respectively). Values for TPR were higher ($P < 0.05$) than those for TIPD except when the dietary Ca:P ratio was fixed. Additionally, negative endogenous P losses were predicted by regression equations when TPR was

estimated for birds fed titration diets with the fixed Ca:P ratio. Changing the Ca concentration of the diets to maintain a fixed Ca:P ratio influenced ($P < 0.001$) apparent P retention, which affected the estimate for TPR due to the prediction of negative endogenous P losses. These data demonstrated that regression analysis may have limitations when estimating the TIPD or TPR of corn when formulating diets with different Ca feeding strategies. More research is necessary to elucidate the factors that contributed to regression equations predicting negative endogenous P losses.

INTRODUCTION

Phosphorus availability values for common feedstuffs vary extensively between and within laboratories (Selle et al., 2009). This variability may be associated with the multitude of methodologies employed to determine P availability (Rodehutscord, 2009). As a result, a standard method has been proposed by the WPSA Working Group 2 for the determination of P utilization using linear regression (WPSA, 2013). The WPSA Working Group recommends the TIPD method, although it is possible to report TPR and TIPD values concomitantly (Dilger and Adeola, 2006; Liu et al., 2013; Mutucumarana et al., 2014a,b). Assessing TIPD avoids the influence of post-ileal microbial fermentation while the TPR method provides data reflective of whole-body P digestion, metabolism, and excretion. Values generated by these methods should be similar if dietary P concentrations are formulated below the requirement of the animal (Shastak et al., 2012). However, TIPD and TPR data have been inconsistent (Dilger and Adeola, 2006; Liu et al., 2013).

Differences in dietary Ca concentrations could be responsible for reported variations between TIPD and TPR values of similar feedstuffs amongst laboratories (Digler and Adeola, 2006, Mutucumarana et al., 2014a,b, 2015). Additionally, differences in dietary Ca:P ratios related to Ca concentration have been reported to influence TPU of soybean meal (Liu et al., 2013). These researchers also reported negative EPL for both TIPD and TPR regardless of Ca:P ratio. Negative EPL have been a concern regarding the use of regression to determine TPU of feedstuffs (Dänner et al., 2006; Liu et al., 2013; Mutucumarana et al., 2014a,b). It is possible that differences in dietary Ca concentrations could influence EPL, but research on this topic has yet to be conducted.

To our knowledge, no data have been published on the effects of dietary Ca on the TIPD and TPR of corn. As corn is a primary feedstuff in poultry diets, possibly providing over 25% of the total P in a diet, it is critical to better understand how Ca feeding strategy affects TIPD and TPR of corn and the prediction of EPL. It is hypothesized that dietary Ca feeding strategy will affect TIPD, TPR, and predicted EPL. Therefore, an experiment was conducted to assess the effects of 3 dietary Ca feeding strategies (fixed concentrations of 0.95% or 0.13% as well as variable Ca concentrations to maintain a 2.1:1 Ca:P ratio) on the TIPD and TPR of corn using growing broilers. Changes in the estimates of EPL resulting from different Ca feeding strategies will also be assessed.

MATERIALS AND METHODS

The experimental protocol involving live birds (PRN 2013-2342) was approved by the Institutional Animal Care and Use Committee at Auburn University.

Bird Husbandry

Nine hundred sixty male Ross × Ross 708 broilers were obtained from a commercial hatchery and vaccinated for Marek's disease, Newcastle disease, and infectious bronchitis. In each experiment, broilers were placed into 96 grower battery cages (10 per cage; 0.046 m^2/bird) (Petersime, Gettysburg, OH). Cages (68 cm × 68 cm × 38 cm) were equipped with 1 trough waterer and 1 trough feeder. Battery cages were housed in 1 of 2 closed-sided research facility rooms. Each room was equipped with forced-air heaters and cooling pads to regulate room temperature. Temperatures were set to 33^{o}C at placement and were decreased gradually to 27^{o}C by the conclusion of the experiment. A 23L:1D photoperiod was used from 1 to 19 d (room 1) or 20 d of age

(room 2). After which, a 16L:8D photoperiod was utilized to ensure adequate FI in order to maximize the amount digesta available for collection. Using this lighting protocol to manipulate FI has resulted in sufficient digesta in the terminal ileum in previous research conducted in our laboratory (Perryman et al., 2013). Lighting intensity was maintained at 30 lux for the duration of the experiment.

Broilers were fed a common corn-soybean meal starter diet (AME_n, 3,090 kcal/kg; digestible Lys, 1.10%; digestible TSAA, 0.83%; digestible Thr, 0.80%; Ca, 0.95%; and NPP, 0.45%) until being fed the experimental diets. In order to address labor constraints, the timing of experimental procedures was staggered between rooms by initiating the experimental feeding period at 19 or 20 d of age for room 1 or 2, respectively. All further experimental procedures will be described in reference to the amount of time elapsed from the placement of experimental diets.

Dietary Treatments

Broilers were randomly assigned to 1 of 12 experimental diets which were formulated to assess the effects of Ca feeding strategy on TIPD and TPR of corn (Table 4.1). Diets were formulated with 1 of 4 corn concentrations (0, 25, 50, or 75%) and 1 of 3 Ca feeding strategies (0.95%, 0.13%, or variable Ca concentrations to maintain a 2.1:1 Ca:P ratio). This allowed for the creation of 3 sets of corn titration diets, with each set having a specific Ca feeding strategy. However, the formulation for the diet with 0% corn and 2.1:1 Ca:P (Diet 1) was the same as the formulation necessary to create a diet with 0% corn and a 0.13% Ca. No $CaCO_3$ was used in the formulation of this diet; therefore, a Ca:P of 2.1:1 was the lowest Ca:P ratio attainable due to Ca contributions of the protein providing ingredients and the low P content of corn. While feeding diets with a Ca:P of

2.1:1 is higher than the WPSA (2013) recommendation of 1.4:1, data published by Liu et al. (2013) demonstrated no significant differences between TIPD or TPR when feeding diets with Ca:P ratios ranging from 1.2:1 to 2.0:1.

Corn was the primary source of increasing P for the titration diets, with P concentrations of 0.06, 0.13, 0.20, or 0.27% and NPP concentrations of 0.03, 0.04, 0.05, or 0.06% for diets containing 0, 25, 50, or 75% corn, respectively. All diets, except for the control diet (0.70% P, 0.45% NPP), were deficient in P as required for digestibility and retention assays (Rodehutscord, 2009). Corn was titrated into the diets at the expense of a 50:50 blend of corn starch and dextrose. A 50:50 blend was utilized based on research reported by Kong and Adeola (2013), which demonstrated an effect of different ratios of purified ingredients on nutrient flows in the gastrointestinal tract. All experimental diets were formulated to be isocaloric (3,200 kcal AME_n/kg) and isonitrogenous (21% CP; 1.53% Lys; 1.16% TSAA; 1.08% Thr) with amino acids supplied to the titration diets by low-ash potato protein and egg protein concentrates. Potato protein contained marginal concentrations of phytate P, so the inclusion rate for this ingredient was fixed (13.6%) for all titration diets. The control diet was formulated as a corn-soybean meal diet with soybean meal as the primary amino acid source. Dicalcium phosphate was used as a source for inorganic P in the control diet to maintain a Ca:P of 1.4:1 and Ca:NPP ratio of 2.1:1 which were similar to strain nutrient recommendations (Ross 708 Broiler Nutrition Specification, 2007).

Sample Collection and Chemical Analyses

Ten subsamples of the experimental corn source were collected and pooled. A representative sample was lyophilized (Virtis Genesis Pilot Lyophilizer, SP Industries,

Warminster, PA) and ground through a cyclone mill (Cyclotec model number 1093, Foss North America, Inc., Eden Prairie, MN) equipped with a 1 mm screen to ensure a homogeneous mixture. This sample was then subjected to duplicate analyses for CP, total P, phytate P, NPP, and phytase activity. Corn CP concentrations were determined via the Dumas method (method 990.03; AOAC International, 2006) using a N analyzer (Rapid N Cube, Elementar Analysensyteme GmbH, Hanau, Germany) with CP being calculated by multiplying percentage N by 6.25.

Phosphorus concentrations of the diets, corn, digesta, and excreta samples were determined by a commercial laboratory (University of Missouri Agricultural Experiment Station Chemical Laboratory, Columbia, MO) via inductively coupled plasma optical emission spectroscopy (method 990.08; AOAC International, 2006). Phytate P content was determined by a commercial laboratory (Enzyme Service and Consultancy, Tredomen Park, United Kingdom) according to the Megazyme method (method K-PHYT, Megazyme International Ireland Ltd.). Samples were extracted in 0.66 M HCl overnight, followed by enzymatic dephosphorylation with phytase to yield myo-inositol monophosphate and 5 molecules of orthophosphate. The inositol ring was completely dephosphorylated by the action of alkaline phosphatase. Orthophosphate groups released from phytate were reacted with ammonium molybdate (5% wt/vol) to yield 12-molybdophosphoric acid. Finally, 12-molybdophosphoric acid and 1 M H_2SO_4 with ascorbic acid (10% wt/vol) reacted to form molybdenum blue. The concentration of molybdenum blue formed in the reaction was proportional to the concentration of orthophosphate in the original sample. Concentrations of orthophosphate were determined colorimetrically by measuring the increase in absorbance at 655 nm (Fiske

and Subbarrow, 1925), and phytate P content was calculated based on phytic acid having 28.2% P. Non-phytate P content of the corn was calculated as the difference between analyzed concentrations of total P and phytate P. Phytase activity of the corn was determined by Enzyme Service and Consultancy laboratory via AOAC method 2000.12 (AOAC International, 2000). Feed samples were extracted for 30 min in 25 mM borate, pH 10.0, and analyses were conducted at pH 4.5 and 60°C. Phytic acid from rice was utilized as the substrate and phytase activity was determined based on the end-point determination of phosphate using a molybdate-vanadate color system.

Broilers and feed were weighed when experimental diets were placed as well as prior to the collection of ileal digesta. These data were used to calculate daily BWG and FI over the duration of the experimental period. All broilers were allowed 48-h to adapt to the experimental diets before the commencement of a 48-h excreta collection period for the retention assay. Feed disappearance, net excreta weight, and excreta samples were collected over the 48 h collection period to calculate P intake and excretion for the calculation of APR. From the pan below each cage, 4 subsamples were collected (free from feed and feather contamination) from the total amount of excreta on the pan. Samples were homogenized and were placed in a plastic bag. Samples of feed and excreta were frozen at -20°C until later analysis. Feed and excreta samples were lyophilized and ground through a cyclone mill similar to the method previously described for the corn sample.

After 144 h of access to the experimental diets (25 or 26 d of age, for rooms 1 or 2, respectively), 8 birds per cage were randomly select and euthanized via CO_2 asphyxiation for the determination of AIPD. Digesta samples were collected by gently

flushing the contents of the ileum using deionized-distilled water. The sampled subsection of ileum was between 4 and 30 cm proximal to the ileo-cecal junction, which corresponds to the terminal two-thirds of the ileum for broilers of this BW. Digesta were pooled by cage and retained on ice before being frozen at -20°C until later analysis. Frozen samples of digesta and excreta were lyophilized and finely ground with an electric coffee grinder to avoid significant loss due to the small sample size of the collected digesta. Digesta was ground to fit through a 1mm screen.

All mineral assays were performed in quadruplicate for diet samples or in duplicate for excreta or digesta samples. Phosphorus concentrations in the diets and digesta were analyzed using the same laboratory and methodology as previously described for the corn sample. Titanium dioxide concentrations were determined by a method based on that of Leone (1973). Briefly, 0.25 g of digesta or feed were added to threaded glass test tubes and ashed at 580°C for 10 h; 0.8 g of $NaSO_4$ was added to the ashed samples, which were diluted with 5 mL of H_2SO_4 with the samples digested at 130°C for 72 h; tube contents were diluted to 50 mL with distilled deionized water and held for 12 h at 25°C; 3 mL of feed samples or 1 mL of digesta samples plus 2 mL of 1.8 M H_2SO_4 were added to glass test tubes with 150 µL of H_2O_2; and after allowing 30 min for color development, absorbance was measured on a spectrophotometer (DU 730, Beckman Coulter, Brea, CA) at 410 nm.

Calculations

Data generated from chemical analysis of excreta and ileal digesta, on a DM basis, were used for the calculation of APR, AIPD, P output on a DMI basis ($\mathbf{P_{O\text{-}DMI}}$),

true P indigestibility (**TPI**), TPR, and TIPD. Percent APR was calculated using the following equation:

$$APR = \frac{(P_{intake} - P_{excreted})}{P_{intake}} \times 100,$$

where APR represents apparent P retention (%). Phosphorus intake and excretion (g) were determined from FI and excreta output weights on a per bird basis. Percent AIPD was calculated for each diet using the following equation:

$$AIPD = \left[1 - \left(\frac{TiO_{2\ Diet,}}{TiO_{2\ Digesta}}\right) \times \left(\frac{P_{Digesta}}{P_{Diet}}\right)\right] \times 100,$$

where $P_{Digesta}$ and P_{Diet} equal analyzed percent concentrations of P in the digesta and diets, respectively, and $TiO_{2\ Digesta}$ and $TiO_{2\ Diet}$ represent the analyzed percent concentrations of TiO_2 in the digesta and diets, respectively. Phosphorus outputs in the excreta or ileal digesta on a DMI basis were calculated via the following equation:

$$P_{O-DMI} = P_{O-DMO} \times \left[\frac{TiO_{2\ Diet}}{TiO_{2\ Digesta}}\right],$$

where $P_{O\text{-}DMI}$ represents P output on a DMI basis (mg/kg) and $P_{O\text{-}DMO}$ represents P output on a DM basis of digesta samples. To calculate TIPD and TPR, P output on a DMI basis of the ileal digesta or excreta were regressed against dietary P intake as described by Dilger and Adeola (2006) using the following regression equation:

$$P_{O-DMI} = (TPI \times P_I) + EPL,$$

where TPI is representative of true P indigestibility, P_I is the P concentration of dietary intake, and EPL represented the prediction of endogenous P losses on a DMI basis. This equation represents the simple linear regression of $P_{O\text{-}DMI}$ on P_I, where TPI is the slope and EPL is the Y-intercept. True ileal P digestibility from the ileal digesta or TPR from the excreta were calculated using the following equation:

$$TIPD\ or\ TPR = 100 - (TPI\ \times 100),$$

where TIPD or TPR are the percentages of true ileal P digestibility or true P retention, respectively.

Statistical Analyses

The experiment was arranged as a randomized complete block designed. Cage location was the blocking factor and individual cages served as the experimental unit. All measurements were represented by 8 replicate cages per treatment. Regression data were analyzed by PROC REG (SAS Institute, 2004). Regression coefficients representing TPU were compared using 95% CI. Additionally, EPL were considered to be not different from zero if the point representing 0 P intake and 0 P output fell within the 95% CI. Polynomial contrasts were determined using PROC GLM (SAS Institute, 2004) to evaluate possible linear or quadratic effects of corn inclusion level on BW gain, FI, P output of the digesta and excreta, AIPD, and APR.

After excluding the control diet and the 2 diets containing 0% corn, AIPD and APR data were further analyzed as a 3 × 3 factorial with 3 dietary Ca feeding strategies and 3 corn inclusion levels. Analyses of variance were performed using PROC MIXED (SAS Institute, 2004) by the following mixed-effects model:

$$Y_{ijk} = \mu... + \rho_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk}$$

where $\mu...$ is the overall mean; the ρ_i are identically and independently normally distributed random block effects with mean 0 and variance σ^2_ρ; the α_j are the fixed effects corresponding to the j^{th} corn inclusion level (25, 50, or 75% corn) such that $\Sigma\alpha_j = 0$; the β_k are the fixed effects corresponding to the k^{th} dietary Ca feeding strategy (0.95%, 0.13%, or variable Ca concentrations to maintain a 2.1:1 Ca:P ratio) such that $\Sigma\beta_k = 0$;

the $(\alpha\beta)_{jk}$ are interaction effects corresponding to all possible permutations of either the j^{th} corn inclusion level and k^{th} dietary Ca concentration such that $\Sigma(\alpha|\beta)_{jk} = 0$; and the random error ε_{ijk} is identically and independently normally distributed with mean 0 and variance σ^2. Statistical significance was considered at $P \leq 0.05$, and significantly different treatment means established by a significant F-test were separated using Tukey's Honestly Significant Difference test (Tukey, 1953).

RESULTS AND DISCUSSION

Nutrient Analysis and Broiler Performance

Composition of the experimental diets as well as calculated and analyzed nutrient concentrations of the test corn source, on an as-fed basis, are presented in Tables 4.1 and 4.2, respectively. Concentrations of CP and P were similar to values reported for corn in the literature (NRC, 1994; NRC, 2010). However, values of phytate P were slightly higher and NPP concentrations slightly lower than previously reported nutrient concentrations of these sources (NRC, 1994; NRC, 2010). Due to these differences, experimental diets were formulated using the analyzed nutrient concentrations. Endogenous phytase activity of corn (< 50 FTU/kg) was similar to previously published values (Ravindran et al., 1999).

Total P, phytate, and NPP content of the experimental diets were subsequently higher with increasing concentrations of corn (Table 4.1). Analyzed values for these nutrients were in good agreement with calculated concentrations, and NPP content of the diets were below the recommended requirement of the animal at this age (Ross 708 Broiler Nutrition Specification, 2007). Dietary Ca concentrations were similar to formulated values with the exception of diets formulated with 0.13% Ca. Variability

between calculated and analyzed Ca concentrations have been previously reported (Driver et al., 20 05b; Mutucumarana et al., 2014a). To account for these differences between calculated and analyzed values, nutrient intake, digestibility, and retention values reported herein were calculated utilizing the analyzed nutrient values of each diet.

Feed intake and growth performance data are presented in Table 4.3. For broilers fed the control diet, BWG (44 g/bird/d) was lower than while FI (116 g/bird/d) was in agreement with primary breeder performance guidelines (Ross 708 Broiler Performance Objectives, 2007). Lower BWG compared with primary breeder guidelines of broilers fed nutrient sufficient control diets have been reported when using the battery cages in the same facility (Perryman et al., 2013). However, these values were greater ($P < 0.001$) than data for FI and BWG of birds fed the corn titration diets. Diets formulated with purified ingredients have been reported to have low palatability when fed to broilers (Sullivan, 1999; Mutucumarana et al., 2014a,b; Shastak et al., 2014). Corn titration diets were also deficient in P, and evidence has indicated that broilers consume less feed when fed P deficient diets (Driver et al., 2005a,b). No linear or quadratic effects were observed for FI in response to graded increases in corn. A quadratic increase ($P < 0.05$) in BWG was observed with increasing corn levels when dietary Ca was maintained at 0.13%. As P increased in these diets due to corresponding increases in corn, the ratio of Ca:P narrowed (2.1 to 0.5), and more P was likely available to the bird for growth.

Apparent Phosphorus Digestibility and Retention

Determined values for AIPD and APR are presented in Table 4.4. Apparent ileal P digestibility and APR values of diets were similar among birds fed the control or corn titration diets. Additionally, linear or quadratic effects of increasing P related to corn

inclusion concentrations were not observed for AIPD or APR, regardless of dietary Ca concentration. This finding agrees with data published by Stein et al. (2008), which demonstrated that apparent total tract P digestibility values in swine were not affected by the P concentration of the diet.

However, diet AIPD ranged from 23.9 to 61.3% and were lower than values reported for corn titration diets by Mutucumarana et al. (2014a) (60.5 to 70.4%). Apparent P retention values varied between 38.5 and 75.3% and were similar but still more variable than corn titration diet retention data (45.1 to 67.5%) published by Mutucumarana et al. (2014a). The wide range in AIPD and APR data were likely related to differences in the Ca concentrations of the diets. While 3 Ca feeding strategies were utilized in the current research, diets formulated by Mutucumarana et al. (2014a) were maintained at a fixed Ca:NPP ratio.

Interaction effects ($P < 0.001$) were detected for APR between increasing corn and dietary Ca concentration (Table 4.4). Diets with a fixed Ca:P ratio of 2.1:1 had decreased APR with increasing corn, whereas APR values were not affected by graded increases in corn for diets formulated with 0.13 or 0.95% Ca. For diets with the fixed Ca:P ratio, it was necessary to titrate $CaCO_3$ into these diets as a Ca source to maintain the ratio at 2.1:1. Therefore, fixed ratio diets with the greatest concentration of corn (75%) also had the most Ca. Even though the Ca:P ratios were fixed, the Ca:NPP ratios became progressively wider because of the differences in corn phytate P and NPP concentrations (Table 4.1). While a similar interaction effect for AIPD was not detected ($P< 0.06$), a negative effect ($P< 0.001$) of increasing dietary Ca on AIPD was observed. The effect of high (>1%) dietary Ca concentrations and wide Ca:NPP ratios on P

availability is well documented in the literature (Hurwitz and Bar, 1971, Shafey, 1993, Lei et al., 1994; Plumstead et al., 2008).

Broilers fed the control diet had greater ($P < 0.001$) P output in the excreta and ileal digesta compared with birds fed the corn titration diets. Higher concentrations of dietary P and higher FI resulted in greater P intake for broilers receiving the control diet. For all dietary Ca concentrations, supplementing corn into the diet increased (linear, $P < 0.05$) P output in both the digesta and excreta. Linear increases in P output with increasing P intake are a requirement for the application of linear regression for the determination of TPU (Fan et al., 2001; Dilger and Adeola, 2006). Additionally, a quadratic increase ($P < 0.05$) in excreta P output was observed for diets formulated with 0.95% Ca. Quadratic increases in excreta P output of broilers fed titration diets for the determination of TPR have been previously reported (Iyayi et al., 2013; Mutucumarana et al., 2014a).

True Phosphorus Utilization

Corn TIPD and TPR data are presented in Table 4.5. Values for TIPD of corn differed ($P < 0.05$) between broilers fed diets formulated with different Ca feeding strategies and were 41.2, 25.4, or 57.3% for diets formulated with a Ca:P = 2.1:1, 0.95% Ca, or 0.13% Ca, respectively. Differences in TPR were also observed when feeding broilers diets formulated with different Ca feeding strategies. Values were highest ($P < 0.05$) for diets formulated with 0.13% Ca (69.5%), while TPR data were similar between diets with a fixed Ca:P ratio (37.8%) and 0.95% Ca (39.0%). True ileal P digestibility or TPR values of corn for diets formulated with a fixed Ca:P ratio or Ca = 0.95% were lower than data published by Mutucumarana et al. (2014a)(67.6% TIPD; 63.2% TPR).

However, values reported by Mutucumarana et al. (2014a) were similar to data observed in the current study for diets formulated with 0.13% Ca (57.3% TIPD; 69.5% TPR). These similar TPU values are likely attributed to the fact that Mutucumarana et al. (2014a) maintained the Ca:NPP ratio of their diets at 2.1:1, which required dietary Ca concentrations ranging from 0.04 to 0.16%.

True P retention values were higher ($P < 0.05$) than TIPD values when Ca concentrations of the diet were fixed at either 0.95% or 0.13% (Table 4.5). These results indicated possible post-ileal absorption of P for broilers fed diets with fixed Ca concentrations. This is in contrast with evidence that little to no post-ileal P absorption occurs in monogastric animals (Biehl and Baker, 1997; Veum, 2010; Shastak et al., 2012). This effect might be attributable to the Ca concentration of the diet, as broilers consuming titration diets with a 2.1:1 Ca:P ratio had similar TIPD and TPR values. No differences between TIPD and TPR data were also reported for corn when dietary Ca:NPP ratios were fixed (Mutucumarana et al., 2014a).

Conversely, differences between TIPD and TPR have been reported when soybean meal was the test ingredient with TPR having lower values than TIPD (Dilger and Adeola, 2006). Lower TPR values were explained by these authors as a consequence of increased P excretion by the kidneys due to the feeding of highly Ca deficient diets (0.071 to 0.201%). Corroborating these findings, Van der Klis and Versteegh (1996) demonstrated that adding Ca to very deficient Ca diets improved P retention. In the current research, TPR values were not adversely affected by feeding diets with 0.13% Ca, and were actually higher than TIPD data. Methodological differences were probably responsible for the higher TPR values. A shorter dietary adaptation period (2 d) compared

with the adaptation period (6 d) used by Dilger and Adeola (2006) could have limited the time available for significant bone reabsorption and Ca excretion by the kidneys. Additionally, titration diets used by Dilger and Adeola (2006) contained no inorganic Ca sources, as SBM was the sole Ca source. Diets with 0.13% Ca in the current study were formulated with $CaCO_3$ as an inorganic Ca source or had Ca supplied by the egg protein concentrate (0.19% P; 0.89% Ca) or potato protein source (0.28% P; 0.06% Ca). Soybean meal Ca was possibly less available than the inorganic Ca or egg protein Ca provided in the current experiment. Therefore, variability in Ca source digestibility may have exacerbated differences in TPR when diets were formulated with very low Ca concentrations. Based on these data, TIPD and TPR can vary for the same ingredient depending on the Ca source and content of the diet. Furthermore, analysis of TPR may have limitations when allowing broilers a long (> 6 d) period to adapt to highly Ca deficient diets, but more research is warranted to further elucidate the effects of adaptation period length and dietary Ca content on the TIPD and TPR of corn.

Endogenous Phosphorus Losses

Estimated EPL are presented in table 4.5, and were either positive or negative but not different from 0 for broilers fed diets with 0.13% (15.9 or 2.3 mg/kg DMI for TIPD or TPR, respectively), 0.95% Ca (-44.2 or -26.2 mg/kg DMI for TIPD or TPR, respectively), or a fixed Ca:P ratio (-108 mg/kg DMI for TIPD). These values are lower than previous estimates of EPL (200 to 450 mg/kg DMI) in the literature (Rutherford et al., 2002, 2004; Dilger and Adeola, 2006). Differences between these data presented herein and previously published values for EPL are most likely related to methodology differences. Specific factors such as dietary Ca content, Ca:P ratios, and experimental P

sources have each been shown to affect EPL (Günther and al-Masri, 1988; al-Masri 1995; Dilger and Adeola, 2006).

Linear regression analysis of APR data to estimate TPR predicted negative EPL for broilers fed diets with the fixed Ca:P of 2.1:1 (Table 4.5). A primary consequence of negative EPL is the underestimation of TPU. This is a possible explanation for the lack of significant differences between TPR and TIPD data for diets formulated with the fixed Ca:P as negative EPL were almost 3 times as much for TPR (-278.8 mg/kg DMI) compared with TIPD (-108.0 mg/kg DMI). Negative EPL have been previously predicted using regression analysis (Dänner et al., 2006; Rodehutscord et al., 2012; Shastak et al., 2012; Iyayi et al., 2013; Liu et al., 2013; Mutucumarana et al., 2014a,b) and were attributed to a possible consequence of extrapolation of the regression line (Moughan et al., 1998). Errors generated by extrapolation are likely a result of variability in the influential first or last data points (Mutucumarana et al., 2014a,b). Moreover, research conducted by Liu et al. (2013, 2014) indicated that the prediction of negative EPL were a result of the differences in P indigestibility's between the basal protein source (casein) and the test P ingredient (SBM). However, in the current work, the P indigestibility of the basal protein source is likely higher than casein, as potato protein contains proportionally more phytate P (0.22) than NPP (0.06) while casein P is entirely supplied by NPP.

True P utilization values were generated by regressing P output against P intake. Apparent P availability values of titration diets with the lowest concentration of the test P source should have lower P digestibility values than titration diets with a greater amount of the test P source. In very low P diets, P output should be relatively higher compared to

P intake due to the larger contribution of EPL (Fan et al., 2001). However, when P output relative to P intake is lower (increased digestibility) for diets with less of the P source (first data points) or P output is higher (decreased digestibility) for diets with greater concentrations of the P source (last data points), the regression line will shift considerably. This alteration of the regression line would predict negative EPL and lower estimates for TPU. Apparent P retention values were 40% higher ($P < 0.001$) for titration diets with less of the P source (25% corn) compared with diets formulated with more of the P source (50 and 75% corn) when the Ca:P ratio was fixed at 2.1:1 (Table 4.4). However, linear decreases in APR were not observed ($P < 0.09$) with increasing corn. Therefore, it is not possible to directly associate decreases in P availability due to titrating corn into the experimental diets with the prediction of negative EPL without conducting more research.

Regression analysis of titration diets with a fixed Ca:P ratio has previously predicted negative EPL (Iyayi et al., 2013; Liu et al., 2013; Mutucumarana et al., 2015). These researchers also reported higher P availabilities for diets with less of the test P source compared with diets with more of the test P source. As the test P source is titrated into the diets, Ca must also be titrated into the diet to maintain the Ca:P ratio. Additional dietary Ca resulted in the Ca:NPP ratios of the titration diets to become progressively wider (Iyayi et al., 2013; Liu et al., 2013). Wide Ca:NPP ratios have been reported to lower P availability (Smith and Kabaija, 1985; al-Masri, 1995; Qian et al., 1997). Therefore, using a fixed Ca:P ratio for titration diets may have limitations when determining TPU of a feedstuff, as maintaining both the Ca:P and Ca:NPP ratios of these diets is not possible.

Additionally, positive EPL were reported when Ca:NPP ratios of titration diets were maintained below 2.2:1 (Mutucumarana et al., 2014a,b). This effect was likely due to the narrowing of the Ca:P ratios as the P source was titrated into the diet. As these ratios narrowed, P availability improved. However, formulating diets to maintain a fixed Ca:NPP ratio may also lead to overestimations of TPU than values typically found in the literature (Mutucumarana et al., 2014a,b). This is especially problematic when assessing the TPU of ingredients with very low NPP as Ca concentrations of the diets must be very low (< 0.15%). Data generated using this method may have limited use in practical formulations where dietary Ca concentrations typically exceed 0.70% (Ross 708 Broiler Nutrition Specification, 2007).

In conclusion, lower dietary Ca concentrations (0.13%) resulted in increased TIPD and TPR compared with diets formulated with high Ca concentrations (0.95%). Additionally, data indicated post-ileal retention of P except when the Ca:P ratios of the corn titration diets were fixed, which was likely attributable to estimates of EPL being negative. Based on these data, formulating diets with low Ca concentrations (0.13%) may lead to the overestimation of TPU, and the use of linear regression analysis to determine TPU may have limitations when diets have a fixed Ca:P ratio. Additional research is warranted to further determine the factors affecting the accuracy of linear regression analysis for the determination of TPU of cereal grains.

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Table 4.1 Ingredient composition, calculated nutrient analysis, and analyzed composition for select nutrients of experimental diets fed Ross 708 male broilers[1]

	Ca:P = 2.1				Ca = 0.95				C
Ingredient, % "as-fed"	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9
Ground Corn	0.00	25.00	50.00	75.00	0.00	25.00	50.00	75.00	25.00
Corn Starch	30.01	19.62	9.24	0.06	30.01	19.62	9.24	0.06	19.62
Dextrose	30.01	19.62	9.24	0.06	30.01	19.62	9.24	0.06	19.62
Soybean Meal (47.5% CP)	-	-	-	-	-	-	-	-	-
Egg Protein[2]	12.75	10.20	7.60	4.95	12.75	10.20	7.60	4.95	10.20
Potato Protein[2]	13.60	13.60	13.60	13.60	13.60	13.60	13.60	13.60	13.60
Soybean Oil	1.00	1.00	1.00	0.00	1.00	1.00	1.00	0.00	1.00
Poultry Oil	-	-	-	-	-	-	-	-	-
Solka-Floc[3]	9.57	7.47	5.39	1.92	7.43	5.68	3.94	0.83	7.82
Sodium Chloride	0.00	0.00	0.08	0.09	0.00	0.00	0.08	0.09	0.00
Sodium Bicarbonate	0.13	0.23	0.22	0.31	0.13	0.22	0.22	0.31	0.23
DL-Methionine	0.10	0.14	0.19	0.24	0.10	0.14	0.19	0.24	0.14
L-Lysine HCl	0.01	0.10	0.19	0.29	0.01	0.10	0.19	0.29	0.10
Threonine	0.00	0.00	0.04	0.06	0.00	0.02	0.04	0.06	0.02
Calcium Carbonate	0.00	0.40	0.80	1.20	2.15	2.20	2.25	2.30	0.05
Dicalcium Phosphate	-	-	-	-	-	-	-	-	-
Choline Chloride 60	0.33	0.30	0.26	0.24	0.33	0.30	0.26	0.24	0.30
Vitamin Premix[4]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral Premix[5]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Magnesium Oxide	0.30	0.25	0.20	0.15	0.30	0.25	0.20	0.15	0.25
Potassium Sulfate	1.12	0.94	0.87	0.70	1.12	0.93	0.87	0.70	0.93
Potassium Chloride	0.00	0.05	0.01	0.06	0.00	0.05	0.01	0.06	0.05
Titanium Dioxide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Biotin Blend[6]	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
Calculated Analysis									
AME, kcal/kg	3,200	3,200	3,200	3,200	3,200	3,200	3,200	3,200	3,200
Crude Protein, %	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0
Lysine, %	1.53	1.53	1.53	1.53	1.53	1.53	1.53	1.53	1.53
TSAA, %	1.16	1.16	1.16	1.16	1.16	1.16	1.16	1.16	1.16
Threonine, %	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08

Phosphorus, %	0.06	0.13	0.20	0.27	0.06	0.13	0.20	0.27	0.13
Non-Phytate Phosphorus, %	0.03	0.04	0.05	0.06	0.03	0.04	0.05	0.06	0.04
Phytate Phosphorus	0.03	0.09	0.15	0.21	0.03	0.09	0.15	0.21	0.09
Calcium, %	0.13	0.27	0.42	0.57	0.95	0.95	0.95	0.95	0.13
Ca:P	2.1	2.1	2.1	2.1	15.8	7.3	4.8	3.5	1.0
Analyzed Composition									
Phosphorus, %	0.07	0.15	0.22	0.33	0.07	0.15	0.24	0.33	0.15
Ca, %	0.10	0.35	0.54	0.76	1.03	0.93	1.04	1.22	0.12

[1]All diets were provided in mash form on an ad libitum basis.

[2]Egg protein = 85% CP; Potato protein = 74% CP.

[3]Purified cellulose, International Fiber Corp., Tonawanda, NY.

[4]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 8,000 IU; Vitamin D (cholecalciferol), 2,000 IU; Vitamin E (I
IU; menadione (menadione sodium bisulfate complex), 2 mg; Vitamin B12 (cyanocobalamin), 0.02 mg; folacin (folic acid), 0.5 mg: D-
pantothenate), 15 mg; riboflavin (riboflavin), 5.4 mg; niacin (niacinamide), 45 mg; thiamin (thiamin mononitrate), 1 mg; D-biotin (biot
(pyridoxine hydrochloride), 2.2 mg; choline (choline chloride).

[5]Mineral premix include per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30
chloride), 8 mg; I (stabilized ethylenediamine dihydriodide), 1.4 mg; Se (sodium selenite, cypress excel Se yeast), 0.3 mg.

[6]Biotin blend was composed of 13.5 g of biotin (pre-mixed with 2 kg of Solka-Floc).

Table 4.2 Analyzed nutrient composition of corn used for the determination of P availability[1]

Item (%, as-fed basis, unless otherwise noted)	Corn
Crude Protein[2]	8.18
Total P	0.274
Phytate P	0.237
Non-Phytate P[3]	0.037
Phytase activity (FTU/kg)[4,5]	< 50.0

[1]All analyses were performed in quadruplicate.
[2]Crude protein determined by AOAC International method 990.09 (2006).
[3]Calculated as the difference between total P and phytate P.
[4]Phytase units per kg of corn determined by AOAC International method 2000.12 (2000).
[5]One unit of phytase (FTU) was defined as the quantity of enzyme that releases 1 mmol of inorganic phosphorus/min from 0.00015 mol/L sodium phytate at pH 5.5 at 37°C.

Table 4.3 Growth performance and feed intake (19 to 26 d of age) of Ross × Ross 708 male broilers receiving experimental diets varying in Ca and corn inclusion concentrations[1,2]

Ca Feeding Strategy	Corn Inclusion, %	BW Gain, g/bird/d	Feed Intake, g/bird/d
Ca:P = 2.1:1	0	9.3	86.2
	25	17.6	87.2
	50	16.2	83.6
	75	11.6	79.5
Ca = 0.95%	0	6.0	82.0
	25	8.1	82.3
	50	7.8	79.4
	75	11.1	80.1
Ca = 0.13%	0	9.3	86.2
	25	18.9	88.3
	50	21.8	87.8
	75	15.5	85.2
Control		44.3	115.6
SEM		1.8	3.3
		Probabilities	
Contrasts			
Control vs. Treatment Diets		<0.001	<0.001
Ca:P = 2.1:1			
Linear		0.89	0.09
Quadratic		0.31	0.23
Ca = 0.95%			
Linear		0.09	0.21
Quadratic		0.37	0.60
Ca = 0.13%			
Linear		0.53	0.67
Quadratic		0.03	0.34

[1]Each value represents the least square mean of 8 replicate cages with 10 broilers per cage.
[2]The treatment with Ca = 0.13 and 0% corn was the same as the dietary treatment with a Ca:P = 2.1 and 0% corn.

Table 4.4 Phosphorus intake and excretion measured in digesta and excreta from Ross × Ross 708 male broilers to 26 d of age) fed experimental diets varying in Ca and corn inclusion concentrations for the determination of apparent ileal P digestibility and apparent P retention[1,2,3]

Ca Feeding Strategy	Corn, %	P_i, g/kg of DM	P_D, g/kg of DMI	P_E, g/kg of DMI	AIPD,[2] %	APR
Ca:P = 2.1:1	0	0.80	0.39	0.31	51.1	61
	25	1.60	0.75	0.57	53.1	64
	50	2.40	1.35	1.27	43.5	47
	75	3.55	1.98[b]	1.96	44.1	44
Ca = 0.95%	0	0.80	0.51	0.45	36.0	43
	25	1.65	1.34	0.98	23.9	40
	50	2.60	1.68	1.60	35.5	38
	75	3.50	2.65	2.08	24.3	40
Ca = 0.13%	0	0.80	0.39	0.31	51.1	61
	25	1.65	0.64	0.53	61.3	74
	50	2.65	1.25	0.65	52.9	75
	75	3.50	1.49	1.18	57.6	68
Control		8.10	4.75	3.13	41.3	61
SEM		-	0.12	0.07	4.3	1
Ca:P = 2.1:1		-	-	-	47.0[y]	52
Ca = 0.95%		-	-	-	27.8[z]	40
Ca = 0.13%		-	-	-	57.1[x]	72
SEM		-	-	-	2.7	1
	25	-	-	-	46.1	59
	50	-	-	-	44.0	53
	75	-	-	-	41.9	51
	SEM	-	-	-	2.8	1

			Probabilities		
Contrasts					
Control vs. Treatment Diets	-	<0.001	<0.001	0.75	0
Ca:P = 2.1:1					
Linear	-	0.003	0.010	0.20	0
Quadratic	-	0.08	0.12	0.60	0
Ca = 0.95%					
Linear	-	0.017	0.001	0.56	0
Quadratic	-	0.18	0.026	0.90	0
Ca = 0.13%					
Linear	-	0.012	0.050	0.73	0
Quadratic	-	0.15	0.20	0.89	0
ANOVA[4]					
Ca × Corn	-	-	-	0.06	<0
Ca	-	-	-	<0.001	<0
Corn	-	-	-	0.42	<0

[1]Each value represents the least square mean of 8 replicate cages with 10 broilers per cage.

[2]P_i = dietary P content; P_D = ileal P output; DMI = DM intake; P_E = excreta P output. Values were used to genera coefficients of apparent ileal P digestibility (AIPD) and apparent P retention (APR).

[3]The treatment with Ca = 0.13 and 0% corn was the same as the dietary treatment with a Ca:P = 2.1 and 0% corn

[4]ANOVA was performed on the data as a 3×3 factorial with 3 Ca feeding strategies (0.95%, 0.13%, or variable C concentrations to maintain a 2.1:1 Ca:P ratio) and 3 corn inclusion concentrations (25, 50, and 75%).

[a-d]Interaction means not sharing a common superscript differ ($P < 0.05$) using Tukey's Honestly Significant Difference test.

[x-z]Main effect means not sharing a common superscript differ ($P < 0.05$) using Tukey's Honestly Significant Difference test.

Table 4.5 Linear relationships between ileal or excreta P outputs (g/kg of DMI) and dietary P intake (g/kg of DM to contain either a Ca:P ratio = 2.1, Ca = 0.95%, or Ca = 0.13%[1]

Item	Regression equation[2]	P-value[3]	SE of the slope[4]	SE of the intercept[4]	R^2	Endo (mg
Ca:P = 2.1:1						
True ileal P digestibility	Y = 0.5880X – 0.1080	< 0.001	0.05	0.11	0.85	
True P retention	Y = 0.6222X – 0.2788	< 0.001	0.03	0.06	0.96	
Ca = 0.95%						
True ileal P digestibility	Y = 0.7465X – 0.0442	< 0.001	0.05	0.12	0.88	
True P retention	Y = 0.6096X – 0.0262	< 0.001	0.02	0.06	0.96	
Ca = 0.13%						
True ileal P digestibility	Y = 0.4266X + 0.0159	< 0.001	0.03	0.07	0.87	
True P retention	Y = 0.3055X + 0.0023	< 0.001	0.04	0.10	0.67	

[1]Equations were generated based on regressing 32 measurements of P output against 32 measures of P intake. Ea a single cage with either 10 broilers per cage (retention) or 8 broilers per cage (ileal digestibility).

[2]Regression of ileal digesta or excreta P output (g/kg of DMI) vs. dietary P intake (g/kg DM) as determined from concentrations of corn (0, 25, 50, or 75%) with either a Ca:P = 2.1:1, Ca = 0.95%, or Ca = 0.13%. The linear ter and the intercept represents endogenous P loss (g/kg of DMI).

[3]Significance of regression analysis

[4]Standard error of regression coefficient estimates (n = 32 observations).

[5]Calculated as the regression intercept ×1000. Values denoted with [t] are significantly different than 0.

[6]Values denoted with [t] are significantly different from 0.

[7]Calculated as (1 – true P indigestibility) × 100.

[a-d]Means not sharing a common superscript differed ($P < 0.05$) as compared using simultaneous 95% confidence

V. METHODOLOGY AFFECTS MEASURES OF PHOSPHORUS AVAILABILITY IN GROWING BROILERS. 1. EFFECTS OF CALCIUM FEEDING STRATEGY AND DIETARY ADAPTATION PERIOD LENGTH ON TRUE ILEAL PHOSPHORUS DIGESTIBILITY AND PREDICTED ENDOGENOUS PHOSPHORUS LOSSES

ABSTRACT

An experiment was conducted to determine the effects of Ca feeding strategy and DAPL on the AIPD and apparent ileal *myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate (**IP6**) hydrolysis (**AIIP6H**) of corn titration diets. Linear effects for AIPD of the titration diets were then analyzed to predict EPL and estimate true ileal P digestibility. Nine hundred sixty Ross × Ross 708 male broiler chicks were placed into 80 battery cages. Broilers received a common starter diet until 18 d of age and then were fed 1 of 10 experimental treatments from 19 to 21 d of age. Nine diets were formulated to evaluate 5 corn inclusion concentrations (15, 30, 45, 60, or 75%) and 2 Ca feeding strategies (fixed at 0.35% or 1.4:1 Ca:P) after 3 DAPL (0, 24, and 48 h). As a control for DAPL, 1 treatment group received the common starter diet for the duration of the experiment. Broilers consuming the control diet had higher ($P < 0.001$) BW gain and feed intake than broilers fed the corn titration diets. Apparent ileal P digestibility of the control diet was affected ($P = 0.038$) by DAPL. For diets formulated with a 1.4:1 Ca:P ratio,

negative linear effects ($P < 0.05$) on AIPD and AIIP6H were observed with increasing corn. Conversely, for diets formulated with 0.35% Ca, positive linear effects ($P < 0.05$) were measured for AIPD after a 48 DAPL while no linear or quadratic effects were observed for AIPD or AIIP6H for other DAPL. Broilers fed diets formulated with 1.4:1 Ca:P had variable ($P < 0.05$) TIPD (32.2, 15.1, and 35.4%) and negative EPL (-190, -499, and -262 mg/kg DM intake) for 0, 24, and 48 h DAPL, respectively. Conversely, TIPD values (41.2, 39.1, and 47.3%) were similar and EPL were positive (102, 197, and 154 mg/kg DM intake), regardless of DAPL, for broilers fed diets formulated with 0.35% Ca. These data demonstrated that formulating diets with a 1.4:1 Ca:P ratio affected AIPD of the titration diets, shifting the regression line resulting in the prediction of negative EPL and an underestimation of TIPD.

INTRODUCTION

Phosphorus availability data have been reported to vary among common feedstuffs (Shastak and Rodehutscord, 2013) and are likely a result, in part, of differences in experimental methodologies between laboratories (Rodehutscord, 2009). To ameliorate some of this variability, the WPSA has proposed a standard method to generate feedstuff TIPD values. Less variable P availability data generated by the TIPD method should allow nutritionists to more efficiently meet P requirements of broilers. However, researchers have reported variability in data for AIPD of titration diets resulting in regression equations that predicted negative EPL and inconsistent TIPD values (Dilger and Adeola, 2006; Iyayi et al., 2013; Liu et al., 2013, 2014; Mutucumarana et al., 2015).

Negative EPL are not only physiologically impossible, but also lead to lower estimates of TIPD compared with estimates of TIPD from regression equations that did not predict negative EPL (Mutucumarana et al., 2015). Values for TIPD should, in fact, always be higher than apparent digestibility values (Fan et al., 2001). Negative EPL were predicted when titration diet AIPD decreased linearly when the test P source was added to the experimental diets (Liu et al., 2013; Mutucumarana et al., 2014c,d, 2015). However, linear increases in AIPD should occur as the test P source is titrated into the experimental diets, as EPL contribute proportionally less to digesta P output (Fan et al., 2001).

Calcium feeding strategy has been indicated to influence measures of TPU including TIPD (Perryman et al., 2015), but a direct relationship between Ca feeding strategy and negative EPL has yet to be established. The method proposed by the WPSA recommends that titration diets are formulated with a fixed Ca:tP Ca feeding strategy.

However, this feeding strategy requires that additional Ca also be titrated into the diet with increasing P to maintain the Ca:tP ratio. Because Ca strongly influences P availability (Selle et al., 2009; Mutucumarana et al., 2014b), differences in dietary Ca could be a primary source of the variability observed in titration diet AIPD values. Research conducted by Perryman et al., (2015) demonstrated a converse effect of either high (0.95%) or low (0.13%) Ca feeding strategies on AIPD and TPU. Moreover, when a fixed Ca:tP ratio (2.1:1) feeding strategy was evaluated, negative EPL were predicted (Perryman et al., 2015). However, a Ca:tP ratio of 1.4:1 and an intermediate (0.35%) Ca feeding strategy have yet to be evaluated.

Dietary adaptation period length may also influence the AIPD of low P titration diets possibly affecting estimates of TIPD and predictions of EPL. The WPSA (2013) recommends DAPL greater than 5 d. These long DAPL may result in broilers adapting to low-P titration diets to maintain P homeostasis resulting in higher AIPD (Yan et al., 2005). Conversely, feeding semipurified P and Ca deficient diets for long DAPL may affect P metabolism due to reduced FI and growth rates (Shastak et al., 2014). Therefore, shorter DAPL may be advantageous. However, short (< 5 d) DAPL have been avoided due to the potentially negative effects of purified ingredients on GIT physiology (Maneewan and Yamauchi, 2003, 2004). Research conducted in our laboratory indicated variable results of short DAPL on titration diet P availability (Perryman et al., 2013).

Limited research has been conducted to examine the effects of different Ca feeding strategies and short (≤ 48 h) DAPL on TIPD and the possible prediction of negative EPL. Therefore, an experiment was conducted to assess 3 DAPL (0, 24, or 48 h) and 2 Ca feeding strategies (0.35% Ca or fixed Ca:tP ratio of 1.4:1) on dietary AIPD and

corn TIPD in growing broilers. Corn was selected as the experimental P source due to its importance as a primary feedstuff in the poultry industry. Because corn P is primarily stored as IP6, AIIP6H was also assessed. It was hypothesized that Ca feeding strategy and different DAPL would affect diet AIPD, altering both predictions of EPL and estimates of TIPD.

MATERIALS AND METHODS

The experimental protocol involving live birds (PRN 2013-2342) was approved by the Institutional Animal Care and Use Committee at Auburn University.

Bird Husbandry

Nine hundred sixty Ross × Ross 708 male broilers were obtained from a commercial hatchery and vaccinated for Marek's disease, Newcastle disease, and infectious bronchitis. Broilers were placed into 80 grower battery cages (12 per cage; 0.039 m^2/bird; Petersime, Gettysburg, OH) in a close-sided research facility. Battery cages (68 cm × 68 cm × 38 cm) contained 1 trough waterer and 1 trough feeder. Rooms were equipped with cooling pads and forced-air furnaces to maintain temperature set points. Temperatures were set to 33°C at placement and were decreased gradually to 27°C by the conclusion of the experiment. Lighting intensity was maintained at 30 lux, and a 23L:1D photoperiod was used from 1 to 18 d of age. From 19 to 21 d of age, a 16L:8D photoperiod was used, with lights turning off 11 h prior to collections to ensure adequate FI for the sampling of ileal digesta. Broilers were fed a common corn-SBM starter diet (AME_n, 3,200 kcal/kg; Lys, 1.50%; TSAA, 1.15%; Thr, 1.00%; Ca, 1.09%; and NPP, 0.50%) until receiving experimental diets at 19 d of age. Birds and feed were weighed on

a per cage basis at the beginning and at the termination of the experimental period for the determination of FI and BWG.

Dietary Treatments

Broilers were randomly assigned to 1 of 10 experimental diets. Experimental diets were formulated to assess the effects of Ca concentration on TIPD of the corn source. Crude protein, tP, phytate P, and NPP concentrations of the test corn source are presented in Table 5.1. Diets were formulated with 1 of 5 corn concentrations (15, 30, 45, 60, or 75%) and 1 of 2 Ca feeding strategies (fixed at 0.35% or variable Ca concentrations to maintain a 1.4:1 Ca:tP ratio). Five corn inclusion concentrations were used to increase the power of analysis, as improving the fit of the regression line may reduce previously observed variability in the prediction of EPL (Mutucumarana et al., 2014c,d, 2015). The treatment formulated with 75% corn and 1.4:1 Ca:tP ratio (Diet 5) had 0.35% Ca. Therefore, this Ca concentration was selected for the set of titration diets with a fixed Ca inclusion level. Therefore, the diet with a fixed Ca:tP ratio and 0.35% Ca was also utilized as the summit diet (75% corn) for the set of diets formulated with 0.35% Ca. This allowed regression analysis of 2 sets of titration diets with 5 corn concentrations each.

Increases in dietary P content were accomplished by titrating corn into the experimental diets. Calculated tP concentrations were 0.10, 0.14, 0.17, 0.21, or 0.25%, and NPP concentrations were 0.035, 0.038, 0.041, 0.044, or 0.047% for diets containing 15, 30, 45, 60, or 75% corn, respectively. All diets, except for the control diet (0.78% tP, 0.50% NPP) were deficient in P as required for the determination of TIPD (WPSA, 2013). Corn was titrated into the diets at the expense of a 50:50 blend of corn starch and

dextrose (Kong and Adeola 2013). All experimental diets were formulated to be isocaloric (3,200 kcal AME_n/kg) and isonitrogenous (21% CP; 1.50% Lys; 1.15% TSAA; 1.00% Thr) with low-ash potato protein and egg protein concentrates as the primary CP/amino acid source. The use of basal ingredients that provided P only as NPP (casein) has been cited as a possible reason for negative EPL (Liu et al., 2013, 2014). In order to address this concern, a protein source with phytate P (low-ash potato protein) was utilized in dietary formulations.

A control diet was formulated with SBM as the primary amino acid source and had the same formulation as the common starter diet except for the addition of the inert markers. This diet served as a control for DAPL as broilers assigned to this dietary treatment were fed the same diet for the entire experiment except for the addition of inert markers. The control diet contained dicalcium phosphate as a source of inorganic P to maintain a Ca:tP ratio of 1.4:1 and Ca:NPP ratio of 2.2:1, which were based on nutrient recommendations for the broiler strain utilized in this experiment (Ross 708 Broiler Nutrition Specification, 2014). All diets contained TiO_2 and Cr_2O_3 as digestibility and visual markers, respectively.

Ileal Digesta Collections

Broilers were fed the experimental diets on d 19. Digesta collection for the 0 h DAPL occurred at the first observation of Cr_2O_3 in excreta of the broilers in the first cage being sampled. The first appearance of Cr_2O_3 in the excreta occurred approximately 3 h after broilers were fed the experimental diets. It can be assumed that the first observation of Cr_2O_3 in the excreta signified the earliest possible collection time for which ileal digesta content originated from the experimental feed and not from the common starter

diet. Subsequent digesta collections occurred at 24 and 48 h after the 0 h collection. After each DAPL, 4 birds were randomly selected from each cage and euthanized via CO_2 asphyxiation for the determination of diet AIPD. Digesta samples were collected by gently flushing out the contents of the terminal ileum using deionized-distilled water. The sampled subsection of ileum was between 4 and 30 cm proximal to the ileo-cecal junction, which corresponded to the terminal two-thirds of the ileum for broilers of this BW. Digesta were pooled by cage and retained on ice before being frozen at -20°C until later analysis. Frozen samples of digesta were lyophilized and ground sufficiently to fit through a 1 mm screen with an electric coffee grinder to avoid significant loss due to the small sample size.

Chemical Analyses

Ten subsamples of the experimental corn source were collected and pooled. A representative sample was lyophilized (Virtis Genesis Pilot Lyophilizer, SP Industries, Warminster, PA) and ground through a cyclone mill (Cyclotec model number 1093, Foss North America, Inc., Eden Prairie, MN) equipped with a 1 mm screen to ensure a homogeneous mixture. This sample was then subjected to duplicate analyses for CP, tP, phytate P, and Ca concentrations (Table 5.1). Corn CP concentrations were determined via the Dumas method (method 990.03; AOAC International, 2006) using a N analyzer (Rapid N Cube, Elementar Analysensysteme GmbH, Hanau, Germany) with CP calculated by multiplying percentage N by a 6.25.

Phosphorus and Ca concentrations of the corn sample were determined by a commercial laboratory (University of Arkansas Central Analytical Laboratory, Fayetteville, AR) via inductively coupled plasma optical emission spectroscopy (method

990.08; AOAC International, 2006). Concentrations of IP6 were determined using high-performance ion chromatography according to the methods of Blaabjerg et al. (2010). Phytate P concentrations were calculated assuming that IP6 is 28.2% P. Analyzed NPP contents of the corn were calculated as the difference between analyzed tP and phytate P concentrations. All mineral assays were performed in quadruplicate for diet samples or in duplicate for excreta and digesta samples. Phosphorus and Ca concentrations in the diets and P concentrations in the digesta were analyzed using the same laboratory and methodology as previously described for the test corn sample. Eight replicate cages were used for calculating AIPD while only 4 replicate cages were used for calculating AIIP6H.

Titanium dioxide concentrations were determined by a method based on that of Leone (1973). Briefly, 0.25 g of digesta or feed were added to threaded glass test tubes and ashed at 580°C for 10 h; 0.8 g of $NaSO_4$ was added to the ashed samples, which were digested in 5 mL of H_2SO_4 and then heated at 130°C for 72 h; tube contents were diluted to 50 mL with distilled deionized water and held for 12 h at 25°C; 3 mL of feed sample or 1 mL of digesta sample digestate plus 2 mL of 1.8 M H_2SO_4 were added to glass test tubes with 150 µL of H_2O_2; and after allowing 30 min for color development, absorbance was measured on a spectrophotometer (DU 730, Beckman Coulter, Brea, CA) at 410 nm. Accuracy of this method was verified by assaying the TiO_2 of the diets (formulated with 0.50%), which resulted in values between 0.48 and 0.51%.

Calculations

Data generated from chemical analyses of ileal digesta were used for the calculation of AIPD, P output on a dry-matter intake basis ($\mathbf{P_{O\text{-}DMI}}$), TPI, and TIPD. Percent AIPD was calculated for each treatment using the following equation:

$$AIPD = \left[1 - \left(\frac{TiO_{2\ Diet,}}{TiO_{2\ Digesta}}\right) \times \left(\frac{P_{Digesta}}{P_{Diet}}\right)\right] * 100$$

where $P_{Digesta}$ and P_{Diet} equal analyzed percent concentrations of P in the digesta and diets, respectively, and $TiO_{2\ Digesta}$ and $TiO_{2\ Diet}$ represent the analyzed percent concentrations of TiO_2 in the digesta and diets, respectively. This same equation was utilized to calculate AIIP6H with IP6 concentrations replacing P concentrations of the digesta and diet. Phosphorus output from the ileum on a DMI basis was calculated via the following equation:

$$P_{O-DMI} = P_{O-DMO} \times \left[\frac{TiO_{2\ Diet}}{TiO_{2\ Digesta}}\right]$$

where $P_{O\text{-}DMI}$ represents P output on a DMI basis (mg/kg) and $P_{O\text{-}DMO}$ represents P output on a DM basis of the digesta samples. To calculate TIPD, P output on a DMI basis of the ileal digesta were regressed against dietary P intake as described by Dilger and Adeola (2006) using the following regression equation:

$$P_{O-DMI} = (TPI \times P_I) + EPL$$

where TPI is representative of true P indigestibility, P_I is P intake, and EPL is endogenous P losses on a DMI basis. This equation represents the simple linear regression of $P_{O\text{-}DMI}$ on P_I, where TPI is the slope and EPL is the Y-intercept. True ileal P digestibility was calculated using the following equation:

$$TPUi = 100 - (TPI \times 100),$$

where TIPD is the percentage of true ileal P digestibility.

Statistical Analyses

The experiment was arranged as a randomized complete block design. Cage location was the blocking factor and individual cages were the experimental unit. All

measurements were represented by 8 replicate cages per treatment, except for IP6 analysis, which represented 4 replicate cages per treatment. Regression analysis was conducted using PROC REG (SAS Institute, 2004). Regression coefficients of the slope (true P indigestibility) and Y-intercept (endogenous P losses) were compared using 95% CI. Additionally, EPL were not considered to be different from zero if the point representing 0 P intake fell within the 95% CI for the Y-intercept. Polynomial contrasts for the determination of linear and quadratic effects of corn inclusion on BW gain, FI, P output of the digesta, AIPD, and AIIP6H were analyzed using PROC GLM (SAS Institute, 2004). Differences between broilers consuming the control diet or treatment diets were compared using preplanned orthogonal contrasts in PROC GLM (SAS Institute, 2004).

When the 75% corn diet was excluded, AIPD data could be further analyzed as a 2 × 3 factorial with 2 dietary Ca feeding strategies and 3 DAPL treated as repeated measures. This analysis was used to understand how changes in Ca feeding strategy or DAPL affected the prediction of EPL and TIPD estimates compared with AIPD data. Analysis of variance was performed using PROC MIXED (SAS Institute, 2004) by the following mixed-effects model:

$$Y_{ijk} = \mu... + \rho_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk}$$

where $\mu...$ is the overall mean; the ρ_i are identically and independently normally distributed random block effects with mean 0 and variance σ^2_ρ; the α_j are fixed factor level effects corresponding to the j^{th} dietary Ca feeding strategy (fixed at 0.35% or variable Ca concentrations to maintain a 1.4:1 Ca:tP ratio) such that $\Sigma\alpha_j = 0$; the β_k are fixed factor level effects corresponding to the k^{th} DAPL (0, 24, or 48 h) such that $\Sigma\beta_k = 0$;

the $(\alpha\beta)_{jk}$ are interaction level effects corresponding to all possible permutations of either the j^{th} Ca feeding strategy effect and k^{th} DAPL such that $\Sigma(\alpha|\beta)_{jk} = 0$; and the random error ε_{ijk} is identically and independently normally distributed with mean 0 and variance σ^2. Statistical significance was considered at $P \leq 0.05$, and significantly different treatment means established by a significant overall F-test were further separated using Tukey's Honestly Significant Difference test (Tukey, 1953).

RESULTS AND DISCUSSION

Nutrient Analysis and Broiler Performance

Analyzed nutrient concentrations of the test corn source are presented in Table 5.1. Concentrations of CP (7.87%) and P (0.27%) were similar to values reported for corn grain by the National Research Council (National Research Council, 2010). Phytate P (0.23%), NPP (0.04%), and Ca (0.02%) concentrations were also similar to data published data (NRC, 2010). Experimental diets were formulated using analyzed nutrient concentrations. As corn was titrated into the experimental diets, tP, phytate P, and NPP content increased (Table 5.2). Analyzed nutrient concentrations of the diet were utilized when calculating nutrient intake and digestibility data.

Broilers consuming the corn titration diets had lower ($P < 0.001$) FI compared with broilers fed the control diet (Table 5.3). Additionally, FI increased (linear, $P < 0.001$) with increasing dietary P content as corn replaced the 50:50 blend of corn starch and dextrose, independent of Ca concentration. Previous data have indicated that broilers consumed less feed when fed P deficient diets (Driver et al., 2005). Reductions in FI have also been reported when researchers fed broilers diets containing purified ingredients (Sullivan, 1999; Mutucumarana et al., 2014c,d; Shastak et al., 2014). In the present

experiment, broilers fed the titration diets consumed deficient quantities of P and Ca, which resulted in lower ($P = 0.04$) BWG for these broilers compared with birds fed the control diet. However, no linear or quadratic effects were observed for BWG in response to graded increases in P from added corn. The random removal of 4 birds per pen for each sampling time inherently increased the observed variability of BWG in this experiment.

Apparent Ileal Digestibility

The AIPD of titration diets were affected differently depending on dietary Ca feeding strategy and DAPL (Table 5.4). When titration diets were formulated with 0.35% Ca, linear and quadratic ($P = 0.003$) increases in AIPD were observed with added corn after a 48 h DAPL. However, no linear effects of increasing corn on AIPD were observed after DAPL of 0 or 24 h. Similarly, DAPL did not affect AIIP6H when corn titration diets were formulated with 0.35% Ca. Conversely, when the Ca:tP ratio was maintained at 1.4:1, linear decreases ($P < 0.05$) were observed for AIPD and AIIP6H, regardless of DAPL. Additionally, broilers fed the control diet had higher ($P < 0.001$) AIPD values (49.8%) compared with the corn titration diets (39.4%). The opposite effect was observed for AIIP6H, which was negative (-5.9%) and lower ($P < 0.001$) for the control diet compared with AIIP6H data (21%) observed for the corn titration diets.

Apparent ileal P digestibilities of the corn titration diets (18 to 57%) were similar to the range (25 to 57%) previously reported in our laboratory when feeding comparable diets (Perryman et al., 2015). However, these values were lower than previously reported data for the AIPD of corn titration diets (60.5 to 70.4%, Mutucumarana et al., 2014c; 66.1 to 88.2%, Mutucumarana et al., 2015). Dietary concentrations of Ca, CP, and P, as well

as the source of protein (casein or egg protein and potato protein) and DAPL (7 d or ≤ 2 d) differed between the current research and that published by Mutucumarana et al. (2014c; 2015).

In titration diets formulated with a fixed Ca:tP ratio, low Ca concentrations of diets with low P resulted in high estimates for AIPD and AIIP6H. Diets with low Ca concentrations (< 0.15%) have been reported to increase phytate disappearance (Tamim and Angel, 2003; Tamim et al., 2004). When the Ca concentration of the diet was titrated higher (0.13 to 0.36%) in order to maintain the Ca:tP ratio, both AIPD and AIIP6H were negatively affected. Similar linear decreases in AIPD were observed by Liu et al. (2013) when SBM and $CaCO_3$ were titrated into diets to maintain fixed Ca:tP ratios. Adding $CaCO_3$ to diets resulted in progressively wider Ca:NPP ratios (4.0 to 7.4 Ca:NPP). Wide Ca:NPP ratios have been reported to decrease P availability (Hurwitz and Bar, 1971, Smith and Kabaija, 1984; Shafey, 1993; Lei et al., 1994; Plumstead et al., 2008). This problem is unavoidable when maintaining a fixed Ca:tP ratio due to inherent differences in NPP and phytate P concentrations of many common feedstuffs (Eeckhout and de Paepe, 1994).

Titrating corn P into diets formulated with 0.35% Ca generally did not affect AIPD or AIIP6H with the exception of AIPD after a DAPL of 48 h. These data indicate that a fixed dietary Ca concentration may limit variability when assessing P availability. This is logical as differences in dietary Ca concentration have been reported to have a strong influence on both AIPD and AIIP6H (Selle et al., 2009). The linear increase in diet AIPD due to increasing corn P after a DAPL of 48 h may have been attributable to broilers adapting to differences in the Ca:tP ratios of the titration diet. The Ca:tP ratio of

the 15% corn diet was widest (3.3) and likely responsible for the low AIPD (18.0%) value measured at 48 h. Wide Ca:tP ratios have been reported to negatively affect P availability (Qian et al., 1997; Liu et al., 2013). However, the wide Ca:tP ratio did not seem to result in lower AIPD for the 15% corn diet at the 0 h (32.4%) collection. Perhaps the wide Ca:tP ratio of the 15% corn diet resulted in broilers becoming less able to digest and absorb P as DAPL increased. Therefore, a possible minimal P concentration similar to that provided by the 30% corn (0.13% P, 2.8 Ca:tP ratio) diet may be required when assessing the AIPD of diets with 0.35% Ca when utilizing short DAPL. Moreover, the lack of significant linear effects for AIPD after DAPL of 0 and 24 h may have been related to wide variability between measures of diet AIPD. Similar variability associated with measures of P availability of corn titration diets after short DAPL have been previously reported (Perryman et al., 2013).

The length of the dietary adaptation period also affected ($P = 0.038$) AIPD for broilers fed the diet serving as a control for DAPL (Figure 5.1). Additionally, DAPL differences for corn titration diets with different Ca feeding strategies did not appear to be the result of physiological adaptations to improve P digestibility of the low-P titration diets. If broilers were adapting to the lowest P diets to increase P digestibility, increasing DAPL would result in measurements of linear decreases in diet AIPD when titrating corn into the diets due to higher AIPD values for the low-P diets. However, these effects were not observed in the current experiment. Conversely, a linear increases in AIPD were measured after a 48 DAPL for diets formulated with 0.35%, while linear decreases were observed for diets formulated with a fixed Ca:tP ratio regardless of DAPL. The DAPL in the current research were possibly too short to elicit an improvement in P digestibility of

low-P diets to maintain P homeostasis. Previous evidence of broilers adapting to low-P diets required several weeks of dietary exposure (Yan et al., 2005). These data indicated that DAPL effects on P availability were likely related to factors other than differences in dietary P concentrations. As no clear effects of diet type were observed because of differences in DAPL, a recommendation for DAPL when assessing the AIPD of titration diets cannot be made at this time. However, because differences in DAPL affected AIPD, a standard DAPL should be established for future TIPD protocols. Additional research on this topic is warranted.

The control diet had higher AIPD compared with the corn titration diets because an inorganic P source was formulated into the control diet. This resulted in a lower Ca:NPP ratio (2.1:1) for the control diet compared with the corn titration diets (> 4.0). Conversely, control diet AIIP6H was lower than corn titration diet AIIP6H because the control diet had more dietary Ca (1.38 or 0.31%, respectively) and P (0.76 or 0.16%, respectively) than the corn titration diets. Excess analyzed Ca in the control diet likely originated from SBM (Edwards, 1993). Increased dietary Ca concentrations have been reported to decrease phytate P disappearance in broilers (Nelson and Kirby, 1987; Applegate et al., 2003; Tamim and Angel, 2003; Plumstead et al., 2008) as have higher NPP concentrations (Abudabos, 2012; Shastak et al., 2014)

True Ileal Phosphorus Digestibility

True ileal P digestibility was affected differently depending on Ca feeding strategy and DAPL (Table 5.5). Different DAPL did not affect TIPD (37.9 to 47.3%) or EPL (102 to 167 mg/kg DMI) when diets were formulated with 0.35% Ca. Conversely, EPL were negative (-190, -524, and -262 mg/kg DMI) and varied based on 95%

confidence intervals for diets formulated with a fixed Ca:tP ratio. True ileal P digestibility of corn were affected ($P < 0.05$) by DAPL for diets formulated with a fixed Ca:tP ratio. Values for corn TIPD were 32.2, 14.2, and 35.4% after DAPL of 0, 24, and 48 h, respectively. The lowest TIPD estimate (14.2%) corresponded with the most negative predicted value for EPL (-524 mg/kg DMI) and the most significant linear decrease in AIPD as a result of increasing corn inclusion concentrations. More research is warranted to elucidate the reasons why this strong linear decrease in AIPD, which resulted in the prediction of the most negative EPL, occurred after a 24 h DAPL for diets formulated with a fixed Ca:tP ratio.

Estimates for TIPD of corn in the present experiment (14.2 to 47.3%) were lower compared with previously published corn TIPD (67.6%, Mutucumarana et al., 2014c; 42.6 and 72.8%, Mutucumarana et al., 2015). Higher corn TIPD determined by Mutucumarana et al. (2014c, 2015) were likely related to differences in Ca feeding strategy, as diets utilized by these researchers contained less Ca (< 0.16%). Corn TIPD values have been reported to be influenced by the Ca concentration of the titration diets (Mutucumarana et al., 2015; Perryman et al., 2015). Additionally, the corn TIPD value of 42.6% reported by Mutucumarana et al. (2015) was lower than the other two estimates determined by their laboratory (Mutucumarana et al., 2014c) due to the prediction of negative EPL (-1,016 mg/kg DMI) for that specific regression equation.

Negative Endogenous Phosphorus Losses

The prediction of negative EPL influences the estimation of feedstuff TIPD. Negative EPL have been reported when applying the regression method to estimate the TPU of feedstuffs in poultry (Dänner et al., 2006; Iyayi et al., 2013; Liu et al., 2013;

Mutucumarana et al., 2014c,d, 2015; Perryman et al., 2015). Researchers have indicated that the prediction of negative EPL could be a result of inherent limitations associated with the extrapolation of the regression line (Mutucumarana et al., 2014c,d). To improve the fit of our regression line, 5 corn titration diets were utilized per Ca feeding strategy. However, negative EPL were still predicted by regression analysis of titration diet AIPD for diets formulated with a fixed Ca:tP ratio.

Additionally, research conducted by Liu et al. (2013, 2014) indicated that the prediction of negative EPL were a result of titrating SBM into the diet while subsequently reducing the basal protein source (casein). When SBM was titrated into the diets, the observed linear decreases in titration diet AIPD were attributed to phytate P from the test P source replacing the highly digestible NPP from casein (Liu et al., 2013, 2014). In the current study, a blend of 2 protein sources (egg white protein and low-ash potato protein) was utilized to provide a relatively equal mix of both phytate P and NPP in the basal diet.

Although a protein blend with less digestible P was used in the current experiment, linear decreases ($P < 0.05$) in AIPD were still observed when corn was titrated into diets with a Ca:tP ratio of 1.4:1. Regression analysis of these AIPD data predicted negative EPL. Conversely, AIPD increased when corn was titrated into diets with a fixed Ca concentration of 0.35%. This occurred despite the replacement of protein source P with corn P in the exact same manner as diets formulated with a 1.4:1 Ca:tP ratio. Regression analysis of AIPD data for diets formulated with 0.35% Ca predicted positive EPL. Therefore, these data indicated that the Ca feeding strategy used in the determination of TIPD is a contributor to the prediction of negative EPL.

Different Ca feeding strategies have generated different estimates of EPL for the same feedstuff (Dilger and Adeola, 2006; Liu et al., 2013; Mutucumarana et al., 2014c,d, 2015). When diets had a fixed Ca:tP ratio, negative EPL were predicted for corn (Mutucumarana et al., 2015) and SBM (Liu et al., 2013; Mutucumarana et al., 2015). Conversely, when dietary Ca:NPP ratios were fixed, positive EPL were predicted for corn (Mutucumarana et al., 2014c) and SBM (Mutucumarana et al., 2014d). Consequently, maintaining fixed Ca:NPP ratios required diets to be formulated with very low Ca concentrations for both corn- (0.04 to 0.16%, Mutucumarana et al., 2014c) and SBM- (0.06 to 0.24%, Mutucumarana et al., 2014d) based diets. However, TIPD may be overestimated when feeding titration diets with low Ca concentrations. Estimates for the TIPD of SBM ranged from 79.8% (Mutucumarana et al., 2014d) to 93.9% (Dilger and Adeola, 2006). Non-phytate P comprised between 33 and 43% percent of the tP for these test SBM, thus indicating substantial phytate P utilization in these low Ca diets. Low dietary Ca concentrations (0.18%) have increased phytate P disappearance in broilers compared with diets formulated with 0.68% Ca (Tamim and Angel, 2003). However, poultry diets are typically formulated with over 0.70% Ca, so TIPD data generated using diets formulated with low Ca concentrations may have limitations in practical feeding applications (Ross 708 Broiler Nutrition Specification, 2014).

Theoretically, the AIPD of titration diets for the determination of TIPD should be lowest when diet P is very low due to the influential contribution of actual EPL to digesta P (Fan et al., 2001). Therefore, diet AIPD should increase when the test P source is titrated into the diets. However, in most instances when regression analysis predicted negative EPL, the lowest P containing diets had the highest AIPD, and the highest P

containing diets had the lowest AIPD (Iyayi et al., 2013; Liu et al., 2013; Mutucumarana et al., 2015). In these studies, the titration diets were formulated with a fixed Ca:tP ratio, so diets with the highest P also had the highest Ca. High dietary Ca concentrations may increase actual EPL due to Ca chelating endogenous phosphate, limiting potential reabsorption of endogenous P. Rutherfurd et al. (2002, 2004) measured EPL ranging from 227 to 446 mg/kg DMI when feeding very low-P (0.02%) diets with 0.78% Ca. Conversely, lower EPL (104 mg/kg DMI) were reported by Mutucumarana (2014a) when feeding diets with 0.08% Ca. These data indicated that feeding different Ca concentrations may influence actual EPL, altering P output in the digesta and affecting AIPD.

Regression equations which predicted negative EPL resulted in lower estimates for TIPD compared with AIPD values for those diets with a fixed Ca:tP ratio (Table 5.5). Apparent ileal P digestibilities were lower ($P < 0.05$) for diets with 0.35% Ca than diets with the fixed Ca:tP ratio (0.27% average Ca), likely due to the strong negative effects of dietary Ca on P availability (Selle et al., 2009). Conversely, TIPD values were higher ($P < 0.05$) for diets with 0.35% Ca compared with diets formulated to maintain a fixed Ca:tP ratio after DAPL of 0 or 24 h. This was a result of AIPD for diets with the fixed Ca:tP ratio being corrected downwards rather than upwards when estimating TIPD as a result of the predicted negative EPL. Researchers have reported similar effects on TIPD values compared with apparent digestibility or retention data when regression equations predicted negative EPL (Iyayi et al., 2013; Liu et al., 2013; Mutucumarana et al., 2014c,d, 2015). These lower TIPD estimates are contrary to the accepted understanding that true digestibility values should be higher than apparent digestibility values (Fan et

al., 2001). Therefore, regression equations that predict negative EPL likely underestimate TIPD and indeed must be considered as suspect.

When diets were formulated with a fixed Ca concentration of 0.35%, positive EPL were predicted and TIPD estimates were corrected to values greater than those of AIPD. Estimates for TIPD of corn (37.9 to 47.3%) were similar to reported standardized P retention values (37.6 to 40.2%) for which AIPD values were corrected using directly measured, rather than predicted EPL (Liu et al. 2014). Results of the current study indicated that formulating diets with a fixed Ca concentration may be a more suitable method for the determination of feedstuff TIPD.

In conclusion, these data presented herein demonstrated that Ca feeding strategies and DAPL affect TIPD and the prediction of EPL when diets were formulated to maintain a Ca:tP ratio. Apparent ileal P digestibility for these diets decreased linearly with increasing corn P, resulting in the prediction of negative EPL and an underestimation of TIPD. Endogenous P losses were positive and no effects of DAPL were observed for TIPD when the dietary Ca concentrations were 0.35%. Therefore, regression analysis of the AIPD of titration diets with a fixed Ca:tP ratio may have limitations in generating accurate estimates of TIPD.

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Table 5.1 Analyzed nutrient composition of the experimental corn source

Item (%, as-fed basis)	Corn
Crude Protein[1]	7.87
Total P[2]	0.27
Phytate P[3,4]	0.23
Non-Phytate P[5]	0.04
Calcium	0.02

[1]Crude protein determined by AOAC International method 990.09 (2006).
[2]Mineral analyses determined via inductively coupled plasma optical emission spectroscopy (method 990.08; AOAC International, 2006).
[3]Determined using high-performance ion chromatography using the methods of Blaabjerg et al. (2010).
[4]Phytate P content was calculated based on IP6 having 28.2% P.
[4]Calculated as the difference between total P and phytate P.

Table 5.2 Ingredient composition, calculated nutrient analysis, and analyzed composition for select nutrients of experimental diets fed broilers from 19 to 26 d of age[1]

	Ca:tP = 1.4:1					Ca = 0.35		
Ingredient, % "as-fed"	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet
Ground Corn	15.00	30.00	45.00	60.00	75.00	15.00	30.00	45.0
Corn Starch	24.40	18.16	11.90	5.65	0.00	24.40	18.16	11.9
Dextrose	24.40	18.16	11.90	5.65	0.00	24.40	18.16	11.9
Soybean Meal (47.5%)	-	-	-	-	-	-	-	-
Egg Protein[2]	12.00	10.50	9.00	7.50	6.00	12.00	10.50	9.0
Potato Protein[2]	11.50	11.50	11.50	11.50	11.50	11.50	11.50	11.5
Soybean Oil	1.00	1.00	1.00	1.00	0.50	1.00	1.00	1.0
Solka-Floc[3]	8.00	6.80	5.62	4.41	2.52	7.43	6.36	5.3
Sodium Chloride	0.00	0.01	0.03	0.03	0.05	0.00	0.01	0.0
Sodium Bicarbonate	0.12	0.16	0.20	0.25	0.28	0.12	0.16	0.2
DL-Methionine	0.130	0.155	0.180	0.205	0.235	0.130	0.155	0.1
L-Lysine HCl	0.136	0.190	0.244	0.298	0.352	0.136	0.190	0.2
Threonine	0.005	0.012	0.022	0.032	0.042	0.005	0.012	0.0
Calcium Carbonate	0.020	0.190	0.360	0.530	0.705	0.585	0.616	0.6
Dicalcium Phosphate	-	-	-	-	-	-	-	-
Choline Chloride 60	0.350	0.330	0.310	0.290	0.270	0.350	0.330	0.3
Vitamin Premix[4]	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.1
Mineral Premix[5]	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.1
Magnesium Oxide	0.255	0.228	0.200	0.168	0.140	0.255	0.228	0.2
Potassium Sulfate	1.740	1.650	1.560	1.470	1.380	1.740	1.650	1.5
Potassium Chloride	-	0.015	0.034	0.062	0.070	-	0.015	0.0
Titanium Dioxide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.5
Chromic Oxide	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.2
Biotin Blend[6]	0.20	0. 20	0.20	0.20	0.20	0.20	0.20	0.2
Calculated Analysis								
AME, kcal/kg	3,200	3,200	3,200	3,200	3,200	3,200	3,200	3,200
Crude Protein, %	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0
Lysine, %	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.5

TSAA, %	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.1
Threonine, %	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.0
Phosphorus, %	0.10	0.14	0.17	0.21	0.25	0.10	0.14	0.1
Non-Phytate Phosphorus, %	0.035	0.038	0.041	0.044	0.047	0.035	0.038	0.0
Phytate Phosphorus	0.06	0.10	0.13	0.17	0.20	0.06	0.10	0.1
Calcium, %	0.14	0.19	0.24	0.29	0.35	0.35	0.35	0.3
Ca:P	1.4	1.4	1.4	1.4	1.4	3.6	2.6	2.0
Analyzed Composition								
Phosphorus, %	0.101	0.142	0.181	0.195	0.246	0.090	0.128	0.1
Ca, %	0.129	0.207	0.303	0.362	0.360	0.300	0.355	0.3
Ca:P	1.3	1.5	1.7	1.9	1.5	3.3	2.8	2.4

[1]All diets were provided in mash form on an ad libitum basis.

[2]Egg protein = 85% CP; Potato protein = 74% CP

[3]Purified cellulose, International Fiber Corp., Tonawanda, NY.

[4]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 8,000 IU; Vitamin D (cholecalciferol), 2,000 IU; Vitamin E (
IU; menadione (menadione sodium bisulfate complex), 2 mg; Vitamin B12 (cyanocobalamin), 0.02 mg; folacin (folic acid), 0.5 mg; D-
pantothenate), 15 mg; riboflavin (riboflavin), 5.4 mg; niacin (niacinamide), 45 mg; thiamin (thiamin mononitrate), 1 mg; D-biotin (biot
(pyridoxine hydrochloride), 2.2 mg; choline (choline chloride).

[5]Mineral premix includes per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30
chloride), 8 mg; I (stabilized ethylenediamine dihydriodide), 1.4 mg; Se (sodium selenite, cypress excel Se yeast), 0.3 mg.

[6]Biotin blend was composed of 13.5 g of biotin pre-mixed with 2 kg of Solka-floc.

Table 5.3 Growth performance and feed intake (19 to 21 d of age) of Ross × Ross 708 male broilers receiving experimental diets varying in Ca and corn inclusion concentrations[1,2]

Ca Feeding Strategy	Corn Inclusion, %	BW Gain, g/bird/d	Feed Intake, g/bird/d
Ca:P = 1.4:1	15	0.9	61.6
	30	6.3	65.1
	45	5.0	70.2
	60	6.0	69.8
	75	6.3	69.4
Ca = 0.35%	15	0.4	62.7
	30	4.7	67.9
	45	3.2	66.7
	60	8.3	67.5
	75	6.3	69.4
Control Diet[3]		43.3	127.0
SEM		8.5	1.9

Contrasts	***Probabilities***	
Control vs. Titration Diets	0.04	<0.001
Ca:P = 1.4:1		
Linear	0.72	0.032
Quadratic	0.82	0.55
Ca = 0.95%		
Linear	0.65	0.041
Quadratic	0.76	0.68

[1]Values represent the least square mean of 8 replicate cages with 12 broilers per cage. Four birds per cage were removed for the 3 adaptation periods and final BW was determined on the 4 remaining birds per cage. These weights was used to calculate BW gain per d per bird.
[2]The treatment with Ca = 0.35% and 75% corn is the same as the dietary treatment with a Ca:P = 1.4:1 and 75% corn.
[3]The control diet was a corn-soybean meal diet with a 1.4:1 Ca:total P ratio and was the same formulation of the common starter diet except for the addition of inert markers (TiO_2 and Cr_2O_3.

Table 5.4 Linear and quadratic effects of graded increases in dietary corn on phosphorus intake, ileal dig
phosphorus output, apparent ileal phosphorus digestibility (AIPD), and apparent ileal phytate hydrolysis (
measured using Ross × Ross 708 male broilers (19 to 21 d of age)[1,2,3]

Ca Feeding Strategy	Adaptation Period, h	Corn, %	P_i, g/kg of DM	P_D, g/kg of DMI	AIPD, %
Ca:tP = 1.4:1	0	15	1.080	0.606	44.2
		30	1.530	0.814	46.8
		45	1.940	1.079	44.4
		60	2.100	1.246	40.7
		75	2.650	1.635	38.3
	24	15	1.080	0.477	55.9
		30	1.530	0.779	48.9
		45	1.940	0.954	50.8
		60	2.100	1.340	36.2
		75	2.650	1.816	31.5
	48	15	1.080	0.566	47.6
		30	1.530	0.672	56.1
		45	1.940	0.834	57.0
		60	2.100	1.039	50.5
		75	2.650	1.589	40.0
Ca = 0.35%	0	15	0.970	0.652	32.4
		30	1.320	0.859	34.9
		45	1.650	1.108	32.8
		60	2.140	1.380	35.5
		75	2.650	1.635	38.3
	24	15	0.970	0.795	18.1
		30	1.320	0.875	33.6
		45	1.650	1.331	19.1
		60	2.140	1.459	31.8

		75	2.650	1.816	31.5
		15	0.970	0.796	18.0
		30	1.320	0.685	47.1
	48	45	1.650	1.025	37.9
		60	2.140	1.272	40.6
		75	2.650	1.589	40.0
Control			8.200	4.113	49.8
SEM			-	0.089	4.0
Contrasts					
Control vs. Titration Diets			-	<0.001	<0.001
Ca:P = 1.4:1					
0 h Linear			-	<0.001	0.046
Quadratic			-	0.17	0.33
24 h Linear			-	<0.001	<0.001
Quadratic			-	0.017	0.26
48 h Linear			-	<0.001	0.044
Quadratic			-	0.001	0.005
Ca:P = 0.35					
0 h Linear			-	<0.001	0.22
Quadratic			-	0.42	0.70
24 h Linear			-	<0.001	0.06
Quadratic			-	0.34	0.72
48 h Linear			-	<0.001	0.003
Quadratic			-	0.004	0.003

[1]Each value represents the least square mean of 8 replicate cages with 4 broilers sampled per cage per ada
[2]P_i = dietary P content; P_D = ileal digesta P output; DMI = DM intake
[3]The treatment with Ca = 0.35% and 75% corn was the same as the dietary treatment with a Ca:P = 1.4:1

Table 5.5 Effects of Ca feeding strategy and adaptation period length on estimates of corn true ileal P digestibili endogenous P losses of corn titration diets fed to growing Ross × Ross 708 male broilers[1]

Item	Regression equation[2]	P-Value[3]	SE of the slope[4]	SE of the intercept[4]	R^2	Endogenous P loss[5] (mg/kg of DMI)	Tru dige
Ca:total P = 1.4							
0 h	Y = 0.678X – 0.190	< 0.001	0.04	0.08	88.4	-190[b]	
24 h	Y = 0.858X – 0.524	< 0.001	0.06	0.11	85.3	-524[c]	
48 h	Y = 0.646X – 0.262	< 0.001	0.06	0.12	74.5	-262[b]	
Ca = 0.35%							
0 h	Y = 0.588X + 0.102	< 0.001	0.03	0.06	89.7	102[a]	
24 h	Y = 0.621X + 0.167	< 0.001	0.05	0.09	81.3	167[a]	
48 h	Y = 0.527X + 0.154	< 0.001	0.05	0.10	70.6	154[a]	

[1]Values represent the regression of apparent ileal P digestibility data for 5 corn titration diets.

[2]Regression of ileal digesta P output (g/kg of DMI) vs. dietary P intake (g/kg DM) as determined from feeding b concentrations of corn (15, 30, 45, 60, or 75%) and diets formulated with either a Ca:total P = 1.4:1 or Ca = 0.3 true ileal P indigestibility, and the intercept represents endogenous P loss (g/kg of DMI). Corn was the test P so utilization.

[3]Significance of regression.

[4]Standard error of the slope representing true P indigestibility and standard error of the intercept representing en

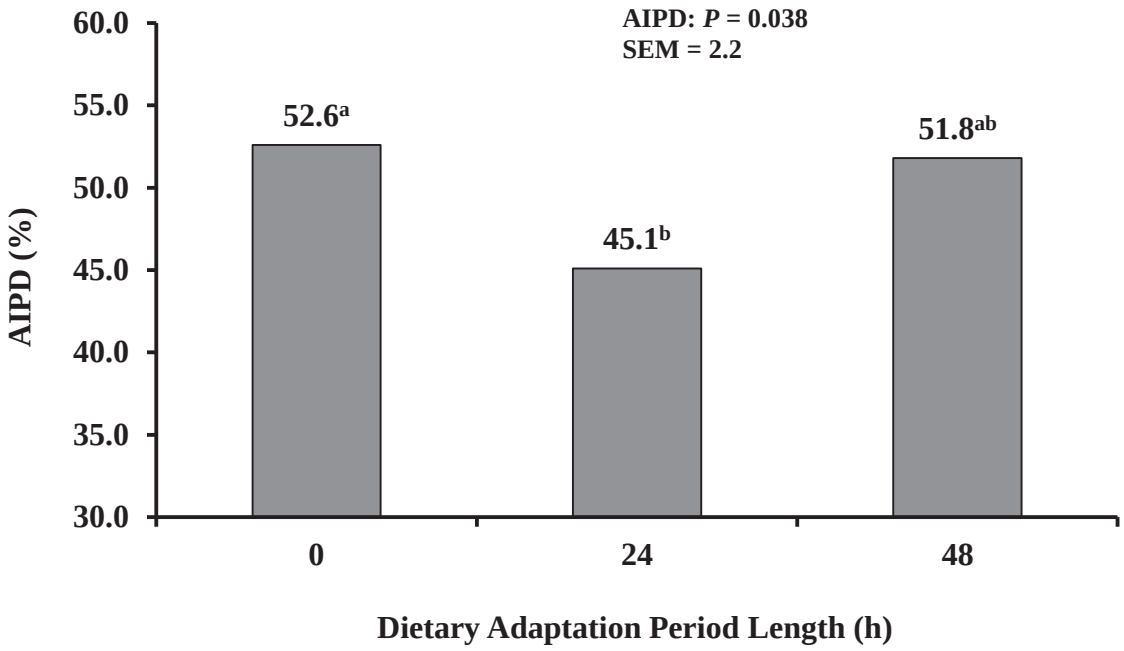
[5]Calculated as the regression intercept ×1000.

[6]Calculated as (1 – true P indigestibility) × 100.

[a-e]Estimates not sharing a common superscript differed ($P < 0.05$) as compared using 95% confidence intervals.

[w-z]Least square means not sharing a common superscript differed ($P = 0.001$; SEM = 2.4) as compared using Tu Significantly Different test (Tukey, 1953)

Figure 5.1 Effects of differences in dietary adaptation period length on the apparent ileal P digestibility (AIPD) of a corn-soybean meal control diet fed to Ross × Ross 708 male broilers from 19 to 21 d of age.


AIPD: P = 0.038
SEM = 2.2
60.0
55.0
50.0
45.0
40.0
35.0
30.0
AIPD (%)
52.6a
45.1b
51.8ab
0
24
48
Dietary Adaptation Period Length (h)

VI. METHODOLOGY AFFECTS MEASURES OF PHOSPHORUS AVAILABILITY IN GROWING BROILERS. 2. EFFECTS OF CALCIUM FEEDING STRATEGY AND DIETARY ADAPTATION PERIOD LENGTH ON PHYTATE HYDROLYSIS AT DIFFERENT LOCATIONS IN THE GASTROINTESTINAL TRACT

ABSTRACT

An experiment was conducted to determine the effects of dietary adaptation period length (DAPL; 0, 24, and 48 h) and Ca feeding strategy (0.35% or 1.4:1 Ca:P ratio) on apparent phytate P (*myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate; IP6) hydrolysis (AIP6H) and apparent digestibility (A∑IPD) of the sum of all inositol phosphate esters (∑IP) of corn-titration diets at 3 locations (proventriculus/gizzard [Pro/Giz], jejunum, and distal ileum) in the gastrointestinal tract (GIT). Four hundred thirty two Ross × Ross 708 male broilers were placed into 36 battery cages and fed a common starter diet until 18 d of age. Eight semipurified diets and a control diet for DAPL were fed from 19 to 21 d of age. Digesta were collected at each location from 4 birds per pen after each DAPL. Diets formulated with a 1.4:1 Ca:P ratio had higher ($P < 0.001$) AIP6H and A∑IPD when measured in the jejunum and ileum, but no differences were observed in the Pro/Giz. No interaction effects between DAPL and sampling location were observed for AIP6H and A∑IPD of the control diet. Conversely, interactive effects ($P < 0.05$) were measured for AIP6H and A∑IPD of the corn-titration diets. The

highest values for both AIP6H (73.9%) and A∑IPD (80.7%) were measured in the Pro/Giz after 24 h. Phytate hydrolysis and A∑IPD were similar regardless of DAPL when sampled from the distal ileum. Concentrations of TiO_2, IP6 and ∑IP also varied ($P < 0.05$) in response to DAPL and sampling location. Variability was likely due to inconsistencies in the flow of inositol phosphate esters and TiO_2 through the GIT, specifically the Pro/Giz. Therefore, the use of TiO_2 as an inert marker may have limitations when determining the digestibility of phytate esters. Additional research is warranted to further elucidate the effects related to DAPL because both AIP6H and A∑IPD of the control and corn-titration diets were influenced by differences in DAPL.

INTRODUCTION

Formulation of broiler diets with true ileal P digestibility (**TIPD**) values may be a more advantageous method than formulating diets on a non-phytate P (**NPP**) basis because feedstuff TIPD better account for potential phytate P (*myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate) hydrolysis when meeting broiler P requirements (Rodehutscord, 2009). Studies have indicated that broilers have some capacity to hydrolyze IP6 into lower inositol phosphate (**IP**) esters (Leytem et al., 2008; Shastak et al., 2014; Zeller et al., 2015c). However, hydrolysis of IP6 is likely dependent on several methodological factors (Angel et al., 2002), which may have been a source of the variability reported among TIPD estimates in the companion manuscript (Perryman et al., 2015b).

Perryman et al. (2015b) determined that corn TIPD varied (14.2 to 35.4%) when broilers were fed diets formulated with a fixed Ca:tP ratio and DAPL of 0, 24, or 48 h. The proposed WPSA (2013) method for TIPD determination recommends the use of DAPL greater than 5 d and test diets formulated with a Ca:P ratio fixed at 1.4:1. However, prolonged exposure to P deficient diets may negatively affect P metabolism (Shastak et al., 2014) or possibly stimulate adaptive mechanisms resulting in increased hydrolysis and absorption of phosphate from IP6 in order to maintain P homeostasis (Yan et al., 2005). The use of shorter DAPL ($\leq$ 2 d) may limit these potential effects. However, the use of shorter DAPL when assessing phytate hydrolysis has yet to be evaluated.

In addition to the knowledge gaps regarding DAPL effects on phytate hydrolysis, formulating corn titration diets with a fixed Ca:tP ratio resulted in regression equations that predicted negative EPL (Perryman et al., 2015a,b). Consequences of negative EPL

include possible underestimations of TIPD and true P retention values (Perryman et al., 2015a,b). It is likely that differences in Ca feeding strategy affected titration diet apparent ileal P digestibility. However, the effect of different Ca feeding strategies on phytate hydrolysis of these diets has yet to be determined.

Considering that phytate P comprises over 70% of the tP in corn (Eeckhout and De Paepe, 1994), assessing apparent IP6 hydrolysis (**AIP6H**) and apparent digestibility (**A∑IPD)** of the summation of all inositol phosphate esters (**∑IP**) of corn-titration diets may be advantageous in further elucidating sources of variability when determining the TIPD of primary feedstuffs. Perryman et al. (2015b) measured wide variations in AIP6H (-10.5 to 51.9%) at the terminal ileum when determining TIPD. Moreover, AIP6H have been reported to be variable when measured at different locations in the GIT (Sooncharernying and Edwards, Jr., 1993; Truong et al., 2014; Walk et al., 2014). These inconsistencies may be related to differences in experimental methodologies among studies.

Therefore, an experiment was conducted to determine the effects of 3 DAPL (0, 24, and 48 h) and 2 Ca feeding strategies (0.35% Ca or fixed Ca:tP ratio) on AIP6H and A∑IPD of corn-titration diets when sampled from 3 locations (proventriculus/gizzard [**Pro/Giz**], jejunum, and terminal ileum) in the GIT of growing broilers. It was hypothesized that Ca feeding strategy and DAPL may interact to affect AIP6H and A∑IPD differently when measured from different segments of the GIT. These results may lead to the adoption of improved methodologies aimed at reducing the variation currently associated with the determination of TIPD.

MATERIALS AND METHODS

The experimental protocol involving live birds (PRN 2013-2342) was approved by the Institutional Animal Care and Use Committee at Auburn University.

Bird Husbandry

Four hundred thirty two Ross × Ross 708 male broilers were obtained from a commercial hatchery and vaccinated for Marek's disease, Newcastle disease, and infectious bronchitis. Broilers were placed into 36 grower battery cages (12 broilers per cage; 68 cm × 68 cm × 38 cm; 0.039 m^2/bird; Petersime, Gettysburg, OH) and were housed in a closed-sided research facility. Battery cages were furnished with 1 trough waterer and 1 trough feeder. Feed and water were provided *ad libitum*. The room was equipped with cooling pads and forced-air furnaces to regulate room temperature. Temperatures were set to 33^{o}C at placement and were decreased gradually to 27^{o}C by the conclusion of the experiment. Lighting intensity was maintained at 30 lux and a 23L:1D photoperiod was used from 1 to 18 d of age. From 19 to 21 d of age, a 16L:8D photoperiod was utilized to ensure adequate FI in order to maximize the amount digesta available for collections. A similar lighting protocol was effectively utilized by our laboratory to maximize digesta collections (Perryman et al., 2013). Broilers were allotted a common corn-soybean meal (**SBM**) starter diet (AME_n, 3,200 kcal/kg; Lys, 1.50%; TSAA, 1.15%; Thr, 1.00%; Ca, 1.09%; and NPP, 0.50%) until receiving experimental diets at 19 d of age.

Dietary Treatments

Crude protein, tP, phytate P, and NPP of the test corn source are presented in Table 6.1. These analyzed nutrient concentrations were used to formulate the

experimental diets (Table 6.2). Broilers were randomly assigned to 1 of 9 experimental diets. Diets included 8 semipurified corn-titration diets. These diets were formulated with 1 of 4 corn concentrations (15, 30, 45, or 60%) and 1 of 2 Ca feeding strategies (fixed at 0.35% or variable Ca concentrations to maintain a 1.4:1 Ca:tP ratio). A control treatment was also formulated as a corn-SBM diet with the same formulation as the common starter diet except for the addition of inert markers (Cr_2O_3 and TiO_2). Therefore, the control diet served as a control for DAPL.

All diets, except for the control diet (0.78% tP, 0.50% NPP), were deficient in P as required for diets used to determine TIPD (WPSA, 2013). Experimental diets were formulated to be isocaloric (3,200 kcal AME_n/kg) and isonitrogenous (21% CP; 1.50% Lys; 1.15% TSAA; 1.00% Thr; Table 6.2). The primary sources of CP/amino acids in the treatment diets were low-ash potato protein and egg protein concentrate, and SBM was the primary source of AA in the control diet. All diets contained TiO_2 and Cr_2O_3 as digestibility and visual markers, respectively. Diets were not supplemented with an exogenous phytase source.

Digesta Collection

Broilers received the experimental diets on d 19. The collection representing the 0 h DAPL began with the first observation of Cr_2O_3 in the excreta of the first cage sampled. This occurred approximately 3 h after broilers were transitioned from the common starter diet to the experimental diets. Chromic oxide was included in the diets as a visual marker so it can be assumed that the first observation of Cr_2O_3 in the excreta was the earliest possible collection time for which digesta contents at the 3 GIT locations originated from

the experimental diets and not the common starter. Subsequent digesta collections occurred at 24 and 48 h after the 0 h collection.

At the culmination of each DAPL, 4 birds were randomly selected from each cage and euthanized via CO_2 asphyxiation for the determination of AIP6H and A∑IPD. From each bird, the Pro/Giz, a segment representing the middle third of the jejunum, and the terminal ileum were excised for sampling. The entire jejunum (distal end of duodenal loop to the Meckel's diverticulum) was removed and folded into thirds with digesta being flushed from the middle third. The sampled subsection of ileum was between 4 and 30 cm proximal to the ileo-cecal junction, which corresponds to the terminal two-thirds of the ileum for broilers of this BW. Digesta from the Pro/Giz, jejunum, and ileum were collected by gently flushing out the contents of each sample using deionized-distilled water. Digesta were pooled by cage and location and retained on ice before being frozen at -20°C until later analysis. Frozen samples of digesta were lyophilized and ground with an electric coffee grinder to fit through a 1 mm screen to avoid significant loss due to the small sample size.

Chemical Analyses

Ten subsamples of the experimental corn source were collected and pooled. An aliquot was collected from the larger sample representing the10 subsamples and was lyophilized (Virtis Genesis Pilot Lyophilizer, SP Industries, Warminster, PA) and ground through a cyclone mill (Cyclotec model number 1093, Foss North America, Inc., Eden Prairie, MN) equipped with a 1 mm screen to ensure a homogeneous mixture. This sample was then subjected to duplicate analyses for CP, tP, Ca, and IPE concentrations. Corn CP concentrations were determined via the Dumas method (method 990.03; AOAC

International, 2006) using a N analyzer (Rapid N Cube, Elementar Analysensysteme GmbH, Hanau, Germany) with CP calculated by multiplying percentage N by 6.25. Phosphorus and Ca concentrations of the corn sample were determined by a commercial laboratory (University of Arkansas Central Analytical Laboratory, Fayetteville, AR) via inductively coupled plasma optical emission spectroscopy (method 990.08; AOAC International, 2006).

Concentrations of IP including IP6, inositol penta-phosphate (**IP5**), inositol tetra-phosphate (**IP4**), inositol tri-phosphate (**IP3**), inositol di-phosphate (**IP2**)] and *myo*-inositol were determined using high-performance ion chromatography using the methods of Blaabjerg et al. (2010). Phytate P content was calculated based on IP6 having 28.2% P. Analyzed NPP content of the corn was calculated as the difference between analyzed tP and phytate P concentrations. All mineral assays were performed in quadruplicate for diet samples or in duplicate for excreta or digesta samples. Phosphorus and Ca concentrations in the diets and digesta P contents were analyzed using the same laboratory and methodology as previously described for the test corn sample.

Titanium dioxide concentrations were determined by a method based on that of Leone (1973). Briefly, 0.25 g of digesta or feed were added to threaded glass test tubes and ashed at 580°C for 10 h; 0.8 g of $NaSO_4$ was added to the ashed samples, which were digested in 5 mL of H_2SO_4 and then heated at 130°C for 72 h; tube contents were diluted to 50 mL with distilled deionized water and held for 12 h at 25°C; 3 mL of feed samples or 1 mL of digesta samples plus 2 mL of 1.8 M H_2SO_4 were added to glass test tubes with 150 μL of H_2O_2; and after allowing 30 min for color development, absorbance was measured on a spectrophotometer (DU 730, Beckman Coulter, Brea, CA) at 410 nm.

Accuracy of this method was verified by assaying the TiO_2 of the diets (formulated with 0.50%), which resulted in values between 0.48 and 0.51%.

Calculations

Data generated from chemical analysis of the digesta at each location, on a DM basis, were used for the calculation of AIP6H, A∑IPD, and IP6 and ∑IP output on a DM intake basis. Percent AIP6H were calculated for each treatment using the following equation:

$$AIP6H = \left[1 - \left(\frac{TiO_{2\ Diet,}}{TiO_{2\ Digesta}}\right) \times \left(\frac{IP6_{Digesta}}{IP6_{Diet}}\right)\right] * 100$$

where $IP6_{Digesta}$ and $IP6_{Diet}$ equal analyzed percent concentrations of IP6 in the digesta and diets, respectively, $TiO_{2\ Digesta}$ and $TiO_{2\ Diet}$ represent the analyzed percent concentrations of TiO_2 in the digesta and diets, respectively. A similar equation was utilized to calculated A∑IPD:

$$\text{A}\sum\text{IPD} = \left[1 - \left(\frac{TiO_{2\ Diet,}}{TiO_{2\ Digesta}}\right) \times \left(\frac{\sum \text{IP}_{Digesta}}{\sum \text{IP}_{Diet}}\right)\right] * 100$$

where $\sum IP_{\ Digesta}$ and $\sum IP_{\ Diet}$ represent analyzed percent concentrations of ∑IP in the digesta and diets, respectively. The variables$TiO_{2\ Digesta}$ and $TiO_{2\ Diet}$ represent the analyzed percent concentrations of TiO_2 in the digesta and diets, respectively. Outputs of IP and ∑IP in the digesta at each location on a DM intake (**DMI**) basis were calculated via the following equation:

$$IP_{O-DMI} = IP_{O-DMO} \times \left[\frac{TiO_{2\ Diet}}{TiO_{2\ Digesta}}\right]$$

where $IP_{O\text{-}DMI}$ represented either IP6 or ∑IP output on a DMI basis (mg/kg) and $IP_{O\text{-}DMO}$ represents the measured output of either IP6 or ∑IP on a DM basis of each digesta sample at each location.

Statistical Analyses

The experiment was arranged as a randomized complete block design. Cage location was the blocking factor and individual cages were the experimental unit. All measurements were represented by 4 replicate cages per treatment. Corn-titration data were analyzed as a 3 × 3 × 2 factorial with 3 DAPL analyzed as repeated measures, 3 sampling locations, and 2 dietary Ca concentrations. Analysis of variance was performed as using PROC MIXED (SAS Institute, 2004) by the following mixed-effects model:

$$Y_{ijkl} = \mu... + \rho_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \tau_l + (\alpha\tau)_{jl} + (\beta\tau)_{kl} + (\alpha\beta\tau)_{jkl} + \varepsilon_{ijkl}$$

where $\mu...$ is the overall mean; the ρ_i are identically and independently normally distributed random block effects with mean 0 and variance σ^2_ρ; the α_j are fixed factor effects corresponding to the j^{th} DAPL (0, 24, or 48 h) such that $\Sigma\alpha_j = 0$; the β_k are fixed factor effects corresponding to the k^{th} sampling location (Pro/Giz, jejunum, or ileum) such that $\Sigma\beta_k = 0$; and the τ_l are fixed factor effects corresponding to the l^{th} dietary Ca feeding strategy (fixed at 0.35% or variable Ca concentrations to maintain a 1.4:1 Ca:tP ratio) such that $\Sigma\tau_l = 0$; $(\alpha\beta)_{jk}$, $(\alpha\tau)_{jl}$, $(\beta\tau)_{kl}$, and $(\alpha\beta\tau)_{jkl}$ are interaction effects corresponding to all possible permutations of the j^{th} DAPL, k^{th} sampling location, and l^{th} dietary Ca feeding strategy such that $\Sigma(\alpha|\beta|\tau)_{jkl} = 0$; and the random error ε_{ijkl} is identically and independently normally distributed with mean 0 and variance σ^2.

The control diet was fed to determine the effects of DAPL on AIP6H and A∑IPD when broilers were fed the same diet as the common starter diet. Therefore, data obtained

from broilers fed the control diet were analyzed separately as there was no Ca feeding strategy effect. These data were analyzed as a 3 × 3 factorial with 3 DAPL analyzed as repeated measures and 3 sampling locations. Analysis of variance was performed as using PROC MIXED (SAS Institute, 2004) by the following mixed-effects model:

$$Y_{ijkl} = \mu... + \rho_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk}$$

where $\mu...$ is the overall mean; the ρ_i are identically and independently normally distributed random block effects with mean 0 and variance σ^2_ρ; the α_j are fixed factor effects corresponding to the j^{th} DAPL (0, 24, or 48 h) such that $\Sigma\alpha_j = 0$; β_k are fixed factor effects corresponding to the k^{th} sampling location (Pro/Giz, jejunum, or ileum) such that $\Sigma\beta_k = 0$; $(\alpha\beta)_{jk}$, are interaction effects corresponding to all possible permutations of the j^{th} DAPL and k^{th} sampling location such that $\Sigma(\alpha|\beta)_{jk} = 0$; and the random error ε_{ijk} is identically and independently normally distributed with mean 0 and variance σ^2. Statistical significance was considered at $P \leq 0.05$, and significantly different treatment means established by a significant F-test were further separated using Tukey's Honestly Significant Difference test (Tukey, 1953).

RESULTS AND DISCUSSION

Nutrient Analysis and Growth Performance

Analyzed nutrient concentrations of the test corn source are presented in Table 6.1. Concentrations of CP (7.87%), tP (0.27%), phytate P (0.23%), NPP (0.04%), and Ca (0.02%) were similar to values reported for corn grain by the National Research Council (NRC, 2010). Phytate P comprised 85% of the tP content in the test corn source, which exceeded previously reported data (68%) from Eeckhout and de Paepe (1994). Concentrations of IP6 were highest (7,483 nmol/g), followed by *myo*-inositol (704

nmol/g), IP2 (665 nmol/g), IP5 (383 nmol/g), and IP3 (84 nmol/g). Measurable amounts of IP4 were not detected in this corn sample. Although data are limited, similar corn IP concentrations have been reported with IP6 comprising the majority of IP and minimal concentrations of IP3 and IP4 (Mendoza et al., 1998). Diet formulations and analyzed nutrient concentrations are presented in Table 6.2. The corn-SBM control diet had higher concentrations of IP6 (8,399 nmol/g) and ∑IP (11,197 nmol/g) than the average concentrations for the 8 corn-titration diets (2,721 and 5,469 nmol/g, respectively, for IP6 and ∑IP).

Growth performance and FI data for the current research have been previously reported in the companion paper and will not be presented herein (Perryman et al., 2015b). No 3-way interactions between DAPL, sampling location, and Ca feeding strategy occurred for broilers fed the corn-titration diets. Furthermore, no interactive effects were measured between Ca feeding strategy and DAPL. Thus, the following discussion is focused on the interactive effects between Ca feeding strategy and sampling location as well as DAPL and sampling location for broilers fed the corn-titration diets. Additionally, main effects are presented for DAPL and sampling location for broilers fed the control diet. However, DAPL and sampling location effects for the control diet were not compared directly with results from feeding the corn-titration diets because Ca feeding strategy effects were not assessed for broilers fed the control diet.

Phytate Hydrolysis and Total Inositol Phosphate Digestibility

Sampling Location and Ca Feeding Strategy. Interactive effects ($P < 0.001$) were observed between sampling location and Ca feeding strategy for AIP6H and A∑IPD of the corn-titration diets (Figure 6.1). Moreover, sampling location and Ca feeding

strategy interacted to affect ($P < 0.001$) IP6 and ∑IP concentrations (Table 6.3). The highest AIP6H and A∑IPD and lowest concentrations of IP6 and ∑IP were observed in the Pro/Giz, and Ca feeding strategy did not affect these response criteria at this location. Conversely, different Ca feeding strategies did affect AIP6H and A∑IPD as well as concentrations of IP6 and ∑IP when sampling occurred in the jejunum and terminal ileum. In the jejunum and ileum, diets formulated with a fixed Ca:tP ratio of 1.4:1 had higher AIP6H and A∑IPD compared with diets formulated with 0.35% Ca. Higher AIP6H and A∑IPD values corresponded with lower concentrations of IP6 and ∑IP. Additionally, AIP6H decreased while A∑IPD increased for diets formulated with the same Ca feeding strategy between the jejunum and ileum.

The observed higher concentrations of ∑IP, and concomitantly lower values for A∑IPD, between the jejunum and ileum for diets formulated with the same Ca feeding strategy were due to increased concentrations of lower IP esters in the jejunum. Increased concentrations of lower IP esters in the jejunum vs. the ileum were due to the higher AIP6H measured in the jejunum when compared with AIP6H measured in the ileal digesta. Phytate hydrolysis in the anterior small intestine was likely a result of endogenous phytase enzymes in duodenum (Maenz and Classen, 1998). It is probable that Ca feeding strategy also influenced endogenous phytase activity because increased AIP6H were observed for diets formulated with a 1.4:1 Ca:tP ratio (0.24% Ca), which had lower Ca than diets formulated with 0.35% Ca. However, a corresponding increase in ∑IP concentrations as a result of increased AIP6H was not observed in broilers fed diets with the fixed Ca:tP ratio, which was in contrast with broilers fed diets formulated to contain 0.35% Ca. This finding indicated that a portion of the lower IP esters responsible

for higher concentrations of ∑IP measured for diets formulated with 0.35% in the jejunum may originate from another source besides those formed from the hydrolysis of orthophosphate from IP6. It is possible that the lower IP esters measured in the small intestine may be of endogenous origin because *myo*-inositol and lower IP esters are ubiquitous in eukaryotic cells (Eagle et al., 1957). Additional research is warranted to establish the potential sources of these IP esters.

Hydrolysis of IP6 and A∑IPD in the jejunum and ileum were affected by differences in dietary Ca feeding strategy. Overall, AIP6H and A∑IPD were lower and concentrations of IP6 and ∑IP were higher in diets formulated with 0.35% Ca. The 4 titration diets formulated with a fixed Ca:tP ratio had an average analyzed Ca concentration of 0.25%, which was less than diets formulated with 0.35% Ca. Increasing the Ca concentration of the diet has been reported to limit AIP6H (Tamim and Angel, 2003; Tamim et al., 2004; Selle et al., 2009). Higher dietary Ca concentrations increase the pH of the digesta (Shafey et al., 1991; Angel et al., 2002), which creates a more favorable luminal environment for the formation of insoluble Ca-phytate chelates (Grynspan and Cheryan, 1983; Selle et al., 2009). Dietary Ca has also been shown to reduce the efficacy of mucosal phytase (McCuaig et al., 1972; Applegate et al., 2003).

Additionally, negative values for both AIP6H and A∑IPD (-9.3 and -5.9%, respectively) measured from the distal ileum of broilers fed the control diet (Figure 6.2) provide further evidence that Ca concentration exerts a strong influence on the dynamics of phytate hydrolysis. The analyzed Ca concentration was 1.38% for the control diet with excess Ca likely originating from SBM (Edwards, 1993). Furthermore, the concentration of NPP (0.50%) contributed by dicalcium phosphate in the control diet was 10 fold

higher than any of the corn-titration diets. Non-phytate P has been previously reported to reduce AIP6H in broilers (Abudabos, 2012; Shastak et al., 2014). Therefore, the high concentration of Ca and NPP in the control diet likely resulted in the observed negative data.

These findings demonstrated that differences in dietary Ca concentrations exert a significant influence on AIP6H and A∑IPD. Moreover, these data are consistent with similar effects of Ca feeding strategy on apparent ileal P digestibilities of titration diets measured when estimating corn TIPD in the companion manuscript (Perryman et al., 2015b). Therefore, it can be concluded that a lower dietary Ca concentration may result in overestimations of titration diet P availability due to increased AIP6H and A∑IPD when measured from the terminal ileum. This is especially critical when titration diets used to estimate TIPD are typically formulated with less than 0.60% Ca (Dilger and Adeola, 2006; Iyayi et al., 2013; Mutucumarana et al., 2014a,b, 2015) while diets commonly fed in commercial broiler production are formulated with more than 0.70% Ca (Ross 708 Broiler Nutrition Specification, 2014). Therefore, careful consideration must be taken as to which Ca feeding strategy is most appropriate when estimating feedstuff TIPD.

Sampling Location and Dietary Adaptation Period Length. Interaction effects between sampling location and DAPL for AIP6H and A∑IPD were observed ($P < 0.001$) in broilers fed the corn-titration diets (Figure 6.3). Measurements of diet AIP6H (73.9%) and A∑IPD (80.7%) were highest in the Pro/Giz of broilers after a DAPL of 24 h. Values of AIP6H were intermediate in the jejunum (43.2 to 50.3%) and lowest in the ileum (15.3 to 26.0%) and were not affected by DAPL. The highest AIP6H and A∑IPD corresponded

to the lowest concentrations of IP6 and ∑IP in the Pro/Giz. Additionally, A∑IPD varied depending on DAPL in the jejunum and were lowest after a DAPL of 48 h (33.2, 38.4, and 22.5% for 0, 24, and 48 h, respectively). However, ∑IP concentrations in the jejunum (3,630; 3,434; and 4,304 nmol/g) after DAPL of 0, 24, and 48 h were not significantly different. At the present time, limited data exist on the concentrations of IP6, ∑IP, and the resulting AIP6H and A∑IPD in broilers fed the corn-titration diets for the determination of TIPD. These data provide a foundation for additional research regarding the dynamics of phytate hydrolysis in the GIT of broilers.

No significant interaction effects between DAPL and sampling location were observed for AIP6H and A∑IPD of the control diet. However, main effects ($P < 0.05$) of DAPL were observed for AIP6H and A∑IPD (Figure 6.2). Apparent IP6 hydrolyses were similar among broilers after 0 or 24 h (34.8 or 32.2%) but lower (19.2%) after a DAPL of 48 h. Results for A∑IPD were similar to AIP6H and were highest after 24 h (30.2%), intermediate after 0 h (26.6%), and lowest after 48 h (16.5%).

Concentrations of IP6 were highest in the digesta ($P < 0.05$) after 48 h (6,783 nmol/g) compared with DAPL of 0 and 24 h (5,476 and 5,698 nmol/g, respectively) while ∑IP concentrations were highest at 48 h (9,346 nmol/g), intermediate at 0 h (8,216 nmol/g), and lowest at 24 h (7,818 nmol/g) (Table 6.4). Although broilers have demonstrated an ability to adapt to P deficient diets (Yan et al., 2005), adequate dietary P and Ca concentrations of the control diet may have resulted in the down-regulation of mechanisms critical to the digestion and absorption of phytate P. However, more research should be conducted to further assess the effects of differences in DAPL on AIP6H and A∑IPD with broilers fed a diet adequate in Ca and P.

When the control diet was fed to broilers, both AIP6H ($P < 0.001$) and A∑IPD ($P < 0.001$) varied depending on sampling location (Figure 6.2). Values for AIP6H decreased ($P < 0.001$) as digesta transited the GIT (69.9, 25.6, and -9.3% for the Pro/Giz, jejunum, and terminal ileum, respectively). Similar effects ($P < 0.001$) were observed for A∑IPD, which were highest in the Pro/Giz (65.4%) and subsequently decreased when measured in the jejunum (13.8%) and ileum (-5.9%). Conversely, Zeller et al. (2015b) measured increases in AIP6H as digesta transited from the crop to the distal ileum when feeding broilers a corn-SBM diet. Inconsistencies in AIP6H between studies may have been related to methodological differences. Broilers in the current study were allowed feed for 3 h prior to the collections, whereas Zeller et al. (2015b) exposed broilers to feed for only 1 h prior to digesta collections. This period of time may have been an insufficient duration for feed to pass into the distal parts of the small intestine (Dänicke et al., 1999). Additional research is required to elucidate why AIP6H varied dramatically between studies when measured in the same GIT segment of broilers fed similar corn-SBM diets.

Concentrations of IP6 (2,526; 6,251; and 9,180 nmol/g) and ∑IP (3,873; 9,654; and 11,853 nmol/g) increased in digesta sampled from the Pro/Giz, jejunum, and ileum, respectively (Table 6.4). Walk et al. (2014) measured a similar IP6 concentrations (2,850 nmol/g on a DM basis) in the gizzard. As with broilers fed the corn-titration diets, high AIP6H (69.9%) and A∑IPD (65.4%) corresponded with the lowest concentrations of IP6 and ∑IP in the Pro/Giz. Conversely, concentrations of IP6 and ∑IP were highest in the ileal digesta and corresponded with negative values for AIP6H and A∑IPD. Negative coefficients for IP6 retention have been previously measured in the small intestine for

birds fed corn-SBM diets (Elliot and Edwards, 1991a,b; Sooncharernying and Edwards, Jr., 1993). Truong et al. (2014) also reported lower AIP6H for digesta sampled at the terminal ileum compared with digesta sampled from the proximal jejunum when feeding corn-SBM diets not supplemented with an exogenous phytase source. These authors attributed the low AIP6H in the ileum to the increased relative concentrations of IP6 resulting from the absorption of other nutrients (Sooncharernying and Edwards, Jr., 1993; Truong et al., 2014). However, in the current research, IP6 and IP concentrations were corrected using TiO_2 and reported on a DMI basis. This may indicate that IP6 accumulates more rapidly in the distal GIT relative to the marker due to different transit times between IP6 and the inert marker in the proximal GIT (Zeller, 2015a).

Titanium Dioxide Concentrations

For broilers fed the control or corn-titration diets, TiO_2 concentrations increased as nutrients were absorbed with the progression of digesta distally through the GIT (Figure 6.4). Moreover, TiO_2 concentrations varied ($P < 0.001$) depending on DAPL when sampled from the Pro/Giz of broilers fed the control diet. Concentrations of TiO_2 were highest after 24 h (0.77%), intermediate at 0 h (0.45%), and lowest at 48 h (0.26%). For broilers fed the corn-titration diets, digesta TiO_2 concentrations varied ($P < 0.001$) depending on DAPL when sampled from the Pro/Giz and ileum. Quantities of TiO_2 were highest (1.02%) after a DAPL of 24 h, but were not different after 0 or 48 h when measured from the Pro/Giz. Additionally, DAPL affected TiO_2 concentrations in the ileum with a lower value observed at 0 h (1.78%) compared with 24 h (2.08%) or 48 h (2.18%).

These effects of DAPL on TiO_2 concentrations at specifically sampled locations

may partially explain the differences observed for AIP6H and A∑IPD because high concentrations of TiO_2 corresponded with high AIP6H and A∑IPD. While putatively inert (Sales and Janssens, 2003), the addition of TiO_2 to the diet may have affected the measure of AIP6H and A∑IPD. Evidence proposed by Wilson et al. (2015) indicated that TiO_2 may be able to bind IP in highly acidic conditions (pH < 1). However, it is currently unknown whether this is possible as these conditions are not probable in broilers because TiO_2 is not soluble in hydrochloric acid (Sales and Janssens, 2003) and the pH of the Pro/Giz has been measured to range from 3 to 4 (Svihus, 2011). More research is necessary to validate the efficacy of TiO_2 as an inert marker for measuring AIP6H and A∑IPD, especially in the anterior GIT.

Dietary Adaptation Period Length

Dietary adaptation periods longer than 5 d were recommended for the determination of TIPD (WPSA, 2013). However, low FI and broiler growth have been observed when feeding P and Ca semipurified diets for 8 d, which may affect P metabolism (Shastak et al., 2014). Broilers also have been reported to adapt to nutrient deficient diets to maintain P homeostasis (Yan et al., 2005). Therefore, shorter DAPL (< 2 d) were evaluated in the current research to determine if adaptation effects related to feeding P and Ca deficient diets could be minimized. However, interactions were still observed between DAPL and sampling location for AIP6H, A∑IPD, and IP6, ∑IP, and TiO_2 concentrations when broilers were fed the corn-titration diets. The reasons for these effects are not readily discernable as significant differences in AIP6H, A∑IPD, and IP6, ∑IP, and TiO_2 concentrations due to changes in DAPL were also observed for broilers fed the diet serving as a control for DAPL.

The manipulation of the photoperiod to ensure adequate FI prior to sample collections may have contributed to the DAPL effects on AIP6H and A∑IPD for broilers fed the control diet and corn-titration diets. Although data are limited, changes in lighting schedules have been demonstrated to influence phytate digestibility in broilers (Leslie, 2006). Changes in photoperiod likely resulted in changes in FI and could have influenced feed retention in the crop (Savory, 1985; Svihus, 2014). Differing feed retention time in the crop may have affected AIP6H and A∑IPD, but the effects of transitioning broilers between lighting programs on nutrient digestibility have yet to be fully established. However, when using a similar lighting and collection schedule, variable apparent ileal P digestibilities for corn-titration diets and a control diet were observed as a result of different DAPL (Perryman et al., 2013, 2015a).

Phytate Hydrolysis in the Proventriculus and Gizzard

High coefficients of AIP6H in the Pro/Giz were unexpected for both diet types because an exogenous phytase was not supplemented to these diets. Therefore, endogenous phytase activity proximal to or within the Pro/Giz may have been responsible for any hydrolysis of IP6. Although phytase activity from microbial or endogenous sources has previously been measured in the crop (Denbow et al., 2000; Kerr et al., 2000) and the Pro/Giz (Józefiak et al., 2006; Rehman et al., 2007), the rapid transit of feed through the anterior GIT in broilers likely limits the action of such phytases (Svihus, 2014). Therefore, it is unlikely that endogenous phytase activity alone could result in the substantial AIP6H (> 50%) observed in the Pro/Giz in the current experiment.

Additionally, concentrations of ∑IP were lowest in the Pro/Giz, which resulted in the highest measurements of A∑IPD. For the digestibility of IP esters to occur, IP6 must

first be dephosphorylated to a lower IP or *myo*-inositol and then subsequently absorbed. While this mechanism has been suggested in rats (Sakamoto et al., 1993), evidence for the digestion and absorption of IP6 in the anterior GIT has yet to be demonstrated in broilers. Furthermore, the primary location for phytate hydrolysis (Maenz and Classen, 1998) and *myo*-inositol absorption is in the small intestine via Na-dependent glucose transporters (Weigensberg et al., 1990). Based on these findings, dephosphorylation of IP6 and absorption of lower IP or *myo*-inositol in the Pro/Giz can be rejected as a plausible explanation for the disappearance of ∑IP.

Inositol Phosphate and Marker Flows

Unexpectedly high AIP6H in the Pro/Giz may have occurred because of differences in IP and TiO_2 transit rates through the GIT. It is possible that IP6 and lower IP were either retained in the crop or were forced out of the Pro/Giz faster than the TiO_2. When feeding corn-SBM diets without supplemental phytase, Zeller (2015a) reported negative AIP6H in the crop indicating possible retention of IP6 in the crop as TiO_2 flowed into the Pro/Giz. This could potentially explain the high AIP6H measured in the Pro/Giz. The highest concentration of TiO_2 in the Pro/Giz was measured after a 24 h DAPL. Additionally, the lowest concentration of IP6 was observed after the 24 h DAPL, which corresponded with the highest measurement of AIP6H. Retention time in the gizzard can vary depending on particle size and density (Svihus et al., 2002) as well as nutrient solubility (Vergara et al., 1989). Phytate is highly soluble in the low pH environment of the Pro/Giz (Grynspan and Cheryan, 1983) while TiO_2 is not (Sales and Janssens, 2003), so muscular action of the gizzard likely affected the motility of TiO_2 and IP differently. Moreover, decreasing AIP6H and increasing concentrations of IP6

measured in the posterior sections of the GIT also support the possibility that soluble IP6 was forced out of the Pro/Giz ahead of the insoluble TiO_2.

In conclusion, a 1.4:1 fixed Ca:tP feeding strategy resulted in higher AIP6H and A∑IPD in the jejunum and ileum compared with broilers fed corn-titration diets formulated with 0.35% Ca. Changes in DAPL also affected AIP6H and A∑IPD regardless of whether broilers were fed the corn-titration diets or control diet. Values of AIP6H and A∑IPD were higher in the Pro/Giz than when measured from distal sections of the GIT of broilers independent of diet type. This is most likely due to the inconsistent flow of TiO_2 and IP into or out of the Pro/Giz. Additional research aimed at better understanding methodological factors affecting the flow of IP and TiO_2 through the broiler GIT is warranted to aid in the establishment of standard protocols to determine the TIPD of feedstuffs.

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Table 6.1 Analyzed nutrient composition of the experimental corn source

Item (%, as-fed basis, unless otherwise noted)	Corn
Crude Protein[1]	7.87
Total P[2]	0.27
Phytate P[3]	0.23
Non-Phytate P[4]	0.04
Calcium	0.02
Inositol Phosphate Esters[5,6] (nmol/g)	
IP6	7,484
IP5	383
IP4	0
IP3	84
IP2	665
Myo-Inositol	704

[1]Crude protein determined by AOAC International method 990.09 (2006).
[2]Mineral analyses determined via inductively coupled plasma optical emission spectroscopy (method 990.08; AOAC International, 2006).
[3]Phytate P content was calculated based on IP6 having 28.2% P
[4]Calculated as the difference between total P and phytate P.
[5]Inositol phosphate esters include: Inositol hexa-phosphate (IP6), inositol penta-phosphate (IP5), inositol tetra-phosphate (IP4), inositol tri-phosphate (IP3), and inositol di-phosphate (IP2).
[6]Determined using high-performance ion chromatography using the methods of Blaabjerg et al. (2010)

Table 6.2 Ingredient composition, calculated nutrient analysis, and analyzed composition for select nutrients of experimental diets fed 19 to 26 d of age[1]

	Ca:tP = 1.4:1				Ca = 0.35		
Ingredient, % "as-fed"	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet
Ground Corn	15.00	30.00	45.00	60.00	15.00	30.00	45.0
Corn Starch	24.40	18.16	11.90	5.65	24.40	18.16	11.9
Dextrose	24.40	18.16	11.90	5.65	24.40	18.16	11.9
Soybean Meal (47.5%)	-	-	-	-	-	-	-
Egg Protein[2]	12.00	10.50	9.00	7.50	12.00	10.50	9.0
Potato Protein[2]	11.50	11.50	11.50	11.50	11.50	11.50	11.5
Soybean Oil	1.00	1.00	1.00	1.00	1.00	1.00	1.0
Solka-Floc[3]	8.00	6.80	5.62	4.41	7.43	6.36	5.3
Sodium Chloride	0.00	0.01	0.03	0.03	0.00	0.01	0.0
Sodium Bicarbonate	0.12	0.16	0.20	0.25	0.12	0.16	0.2
DL-Methionine	0.130	0.155	0.180	0.205	0.130	0.155	0.1
L-Lysine HCl	0.136	0.190	0.244	0.298	0.136	0.190	0.2
Threonine	0.005	0.012	0.022	0.032	0.005	0.012	0.0
Calcium Carbonate	0.020	0.190	0.360	0.530	0.585	0.616	0.6
Dicalcium Phosphate	-	-	-	-	-	-	-
Choline Chloride 60	0.350	0.330	0.310	0.290	0.350	0.330	0.3
Vitamin Premix[4]	0.100	0.100	0.100	0.100	0.100	0.100	0.1
Mineral Premix[5]	0.100	0.100	0.100	0.100	0.100	0.100	0.1
Magnesium Oxide	0.255	0.228	0.200	0.168	0.255	0.228	0.2
Potassium Sulfate	1.740	1.650	1.560	1.470	1.740	1.650	1.5
Potassium Chloride	-	0.015	0.034	0.062	-	0.015	0.0
Titanium Dioxide	0.50	0.50	0.50	0.50	0.50	0.50	0.5
Chromic Oxide	0.25	0.25	0.25	0.25	0.25	0.25	0.2
Biotin Blend[6]	0.20	0.20	0.20	0.20	0.20	0.20	0.2
Calculated Analysis							
AME, kcal/kg	3,200	3,200	3,200	3,200	3,200	3,200	3,200
Crude Protein, %	21.0	21.0	21.0	21.0	21.0	21.0	21.0
Lysine, %	1.50	1.50	1.50	1.50	1.50	1.50	1.5
TSAA, %	1.15	1.15	1.15	1.15	1.15	1.15	1.1
Threonine, %	1.00	1.00	1.00	1.00	1.00	1.00	1.0
Phosphorus, %	0.10	0.14	0.17	0.21	0.10	0.14	0.1

Non-Phytate Phosphorus, %	0.035	0.038	0.041	0.044	0.035	0.038	0.0
Phytate Phosphorus, %	0.06	0.10	0.13	0.17	0.06	0.10	0.1
Calcium, %	0.14	0.19	0.24	0.29	0.35	0.35	0.3
Ca:P	1.4	1.4	1.4	1.4	3.6	2.6	2.0
Analyzed Composition							
Phosphorus, %	0.101	0.142	0.181	0.195	0.090	0.128	0.1
Ca, %	0.129	0.207	0.303	0.362	0.300	0.355	0.3
Ca:P	1.3	1.5	1.7	1.9	3.3	2.8	2.4
Inositol hexa-phosphate (IP6), nmol/g	1,247	2,335	3,220	4,510	1,300	1,963	3,067
∑Inositol phosphate esters[7], nmol/g	3,574	4,906	5,939	8,027	3,728	4,357	6,039

[1]All diets were provided in mash form on an ad libitum basis.

[2]Egg protein = 85% CP; Potato protein = 74% CP

[3]Purified cellulose, International Fiber Corp., Tonawanda, NY.

[4]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 8,000 IU; Vitamin D (cholecalciferol), 2,000 IU; Vitamin E (
menadione (menadione sodium bisulfate complex), 2 mg; Vitamin B12 (cyanocobalamin), 0.02 mg; folacin (folic acid), 0.5 mg; D-pan
15 mg; riboflavin (riboflavin), 5.4 mg; niacin (niacinamide), 45 mg; thiamin (thiamin mononitrate), 1 mg; D-biotin (biotin), 0.05 mg; a
hydrochloride), 2.2 mg; choline (choline chloride).

[5]Mineral premix includes per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30
mg; I (stabilized ethylenediamine dihydriodide), 1.4 mg; Se (sodium selenite, cypress excel Se yeast), 0.3 mg.

[6]Biotin blend was composed of 13.5 g of biotin pre-mixed with 2 kg of Solka-floc.

[7] Inositol phosphate esters included: inositol hexa-phosphate (IP6), inositol penta-phosphate (IP5), inositol tetra-phosphate (IP4), inosit
phosphate (IP2) and were determined using high-performance ion chromatography using the methods of Blaabjerg et al. (2010).

Table 6.3. Digesta concentrations of inositol phosphate esters on a dry matter intake basis at 3 locations when feeding corn-titration diets with 2 Ca feeding strategies (fixed at 0.35% or variable to maintain a 1.4:1 Ca:P ratio) to Ross × Ross 708 male broilers (19 to 21 d of age)[1,2,3]

Location	Ca Feeding Strategy	IP6	∑IP
		nmol/g	
Pro/Giz	0.35%	1,164[d]	1,879[d]
	1.4 Ca:tP Ratio	1,150[d]	1,889[d]
Jejunum	0.35%	1,634[c]	3,951[a]
	1.4 Ca:tP Ratio	1,287[d]	3,624[b]
Ileum	0.35%	2,413[a]	3,375[b]
	1.4 Ca:tP Ratio	1,929[b]	2,893[c]
	SEM	53.7	83.8
Pro/Giz		1,157[c]	1,884[c]
Jejunum		1,460[b]	3,788[a]
Ileum		2,171[a]	3,134[b]
SEM		36.8	60.8
	0.35%	1,737	3,068
	1.4 Ca:tP Ratio	1,455	2,802
	SEM	29.6	49.0
Source of Variation		***Probabilities***	
Location × Ca Feeding Strategy		< 0.001	< 0.001
Location		< 0.001	< 0.001
Ca Feeding Strategy		< 0.001	< 0.001

[1]Interaction effect values represent the least square mean of 42 replicate cages with 4 broilers sampled per cage per adaptation period.
[2]Inositol phosphate esters were determined using high-performance ion chromatography using the methods of Blaabjerg et al. (2010) and include: inositol hexa-phosphate (IP6) and the summation of all measured inositol phosphate esters (∑IP).
[3]Concentrations are on a DM intake basis.
[a-d]Least square means not sharing a common superscript differed as compared using Tukey's Honestly Significant Difference test (Tukey, 1953).

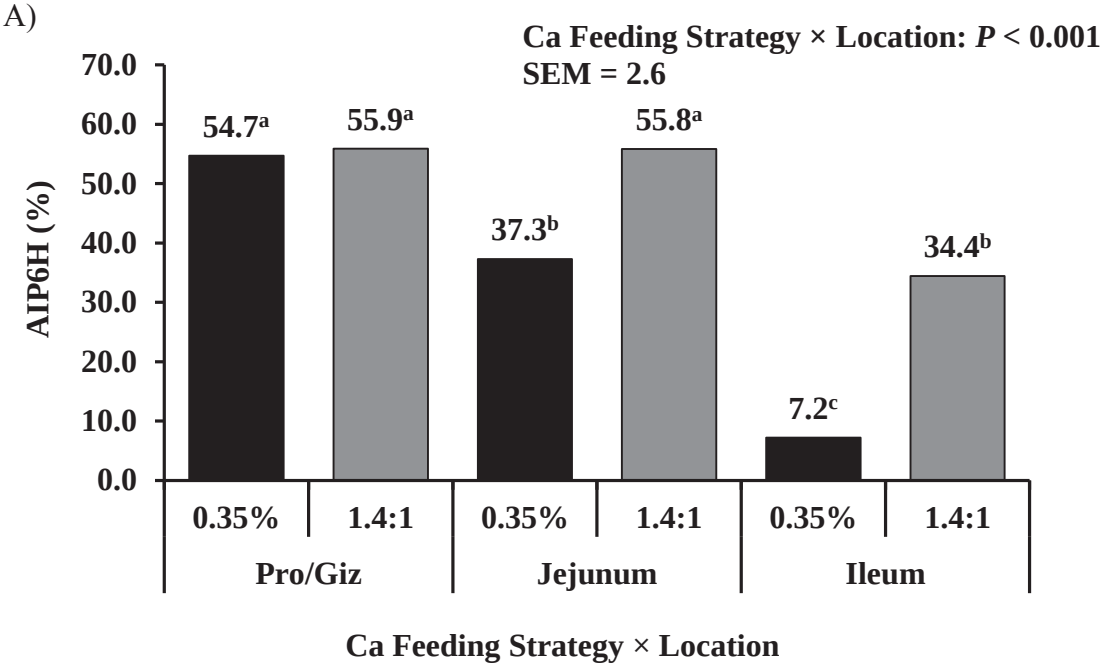
Table 6.4 Digesta concentrations of inositol phosphate esters on a dry matter intake basis at 3 locations after dietary adaptation period lengths (DAPL) of 0, 24, and 48 h when feeding corn-soybean meal control diet to Ross × Ross 708 male broilers (19 to 21 d of age)[1,2,3]

		Corn Titration Diets		Control Diet	
Location	DAPL, h	IP6	∑IP	IP6	∑IP
		nmol/g			
	0	1,327[d]	2,172[d]	2,543	3,950
Pro/Giz	24	656[e]	1,032[e]	1,467	2,152
	48	1,489[cd]	2,447[cd]	3,567	5,518
	0	1,341[d]	3,630[ab]	5,281	9,449
Jejunum	24	1,492[cd]	3,434[ab]	5,953	8,823
	48	1,566[bcd]	4,304[a]	7,520	10,691
	0	2,217[ab]	3,229[bc]	8,603	11,249
Ileum	24	2,306[a]	3,250[bc]	9,675	12,480
	48	2,023[abc]	2,929[cd]	9,262	11,832
	SEM	215.5	304.9	703.2	916.5
Pro/Giz		1,157[c]	1,883[c]	2,526[c]	3,873[c]
Jejunum		1,466[b]	3,789[a]	6,251[b]	9,654[b]
Ileum		2,182[a]	3,136[b]	9,180[a]	11,853[a]
SEM		120.8	173.2	406.1	529.3
	0	1,628	3,010[a]	5,476[b]	8,216[ab]
	24	1,485	2,572[b]	5,698[b]	7,818[b]
	48	1,693	3,226[a]	6,783[a]	9,346[a]
	SEM	120.8	173.2	406.1	529.3
Source of Variation		***Probabilities***			
Location × DAPL		0.001	0.001	0.06	0.054
Location		0.001	0.001	0.001	0.001
DAPL		0.21	0.001	0.010	0.047

[1]Each value represents the least square mean of 4 replicate cages with 4 broilers sampled per cage per adaptation period.
[2]Inositiol phosphate esters were determined using high-performance ion chromatography using the methods of Blaabjerg et al. (2010) and include: inositol hexa-phosphate (IP6) and the summation of all inositol phosphate esters (∑IP).
[3]Concentrations are on a DM intake basis.
[a-d]Least square means not sharing a common superscript differed as compared using Tukey's Honestly Significant Difference test (Tukey, 1953).

Figure 6.1. Interaction effects of 3 sampling locations (proventriculus/gizzard [Pro/Giz], jejunum, and ileum) and 2 Ca feeding strategies (Fixed at 0.35% or variable Ca concentration to maintain a 1.4:1 Ca:P ratio) for Ross × Ross 708 male broilers fed the corn-titration diets on **A)** apparent *myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate (IP6) hydrolysis (AIP6H) or **B)** apparent digestibility of the summation of measured *myo*-inositol phosphate esters (A∑IPD). Measured *myo*-inositol phosphate esters included IP6, inositol penta-phosphate (IP5), inositol tetra-phosphate (IP4), inositol tri-phosphate (IP3), and inositol di-phosphate (IP2). Least square means represent 48 replicate cages and means not sharing a common superscript differed as compared using Tukey's Honestly Significant Difference test (Tukey, 1953).

A)


Ca Feeding Strategy × Location: P < 0.001
SEM = 2.6
AIP6H (%)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
54.7a
55.9a
37.3b
55.8a
7.2c
34.4b
0.35%
1.4:1
0.35%
1.4:1
0.35%
1.4:1
Pro/Giz
Jejunum
Ileum
Ca Feeding Strategy × Location

B)

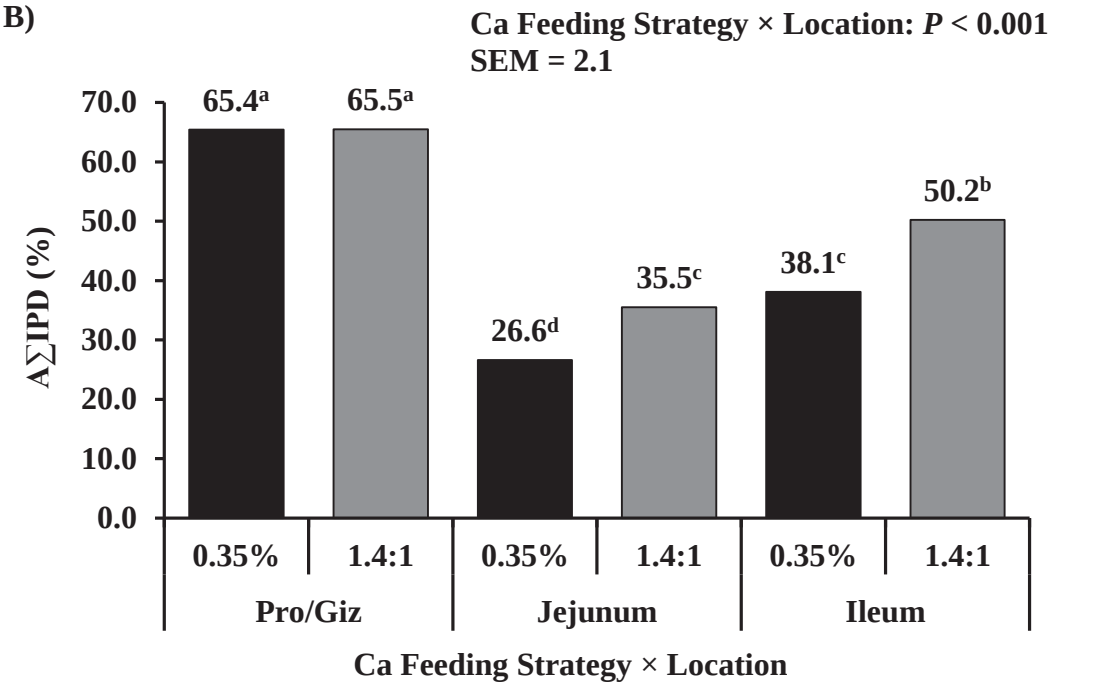

Ca Feeding Strategy × Location: P < 0.001
SEM = 2.1
AΣIPD (%)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
65.4a
65.5a
26.6d
35.5c
38.1c
50.2b
0.35%
1.4:1
0.35%
1.4:1
0.35%
1.4:1
Pro/Giz
Jejunum
Ileum
Ca Feeding Strategy × Location

Figure 6.2 Main effects of sampling location and dietary adaptation period length (DAPL) for Ross × Ross 708 male broilers fed a corn-SBM control diet on **A)** apparent *myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate (IP6) hydrolysis (AIP6H) or **B)** apparent digestibility of the summation of measured *myo*-inositol phosphate esters (A∑IPD). Measured *myo*-inositol phosphate esters included IP6, inositol penta-phosphate (IP5), inositol tetra-phosphate (IP4), inositol tri-phosphate (IP3), and inositol di-phosphate (IP2). Least square means not sharing a common superscript differed as compared using Tukey's Honestly Significant Difference test (Tukey, 1953).

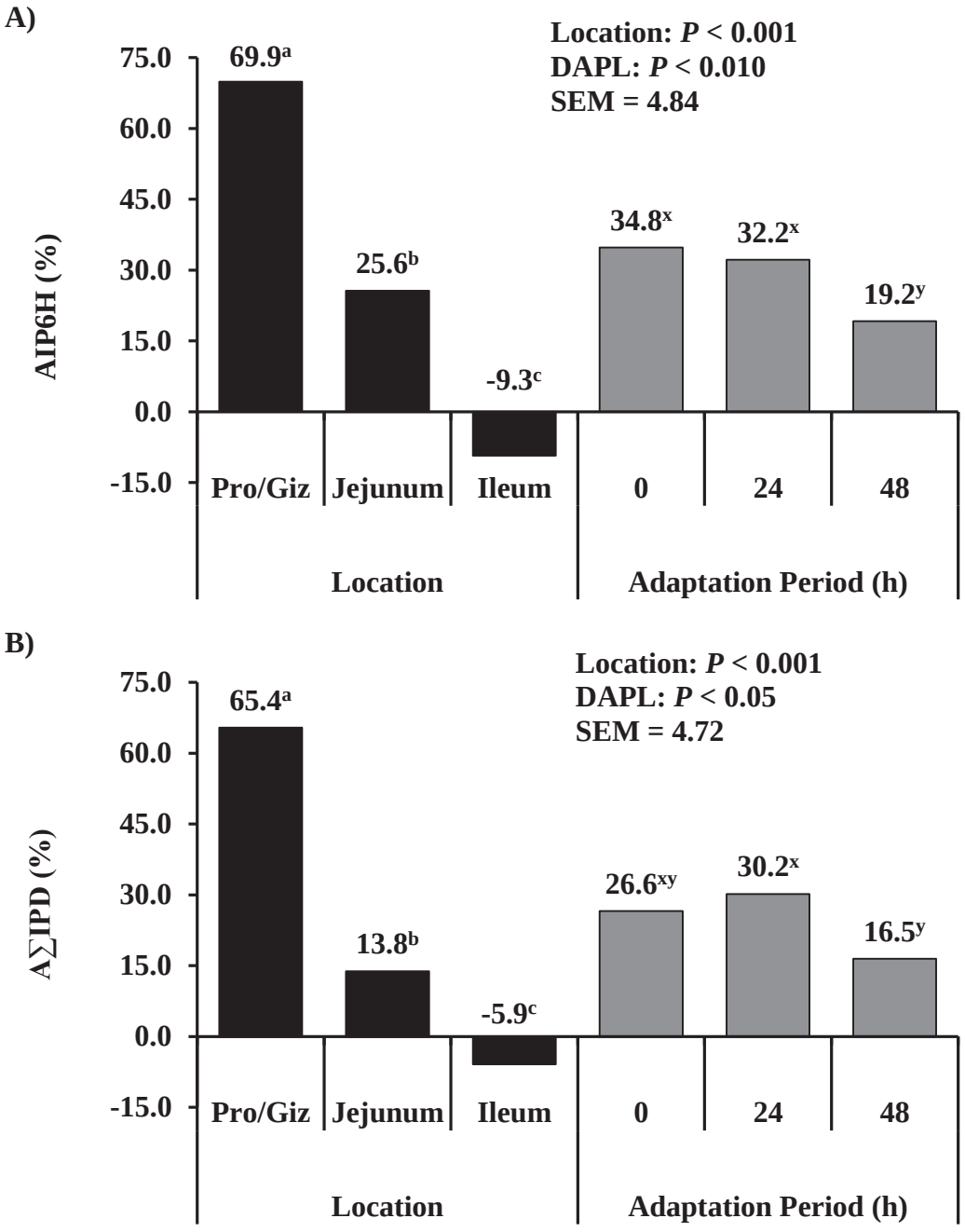
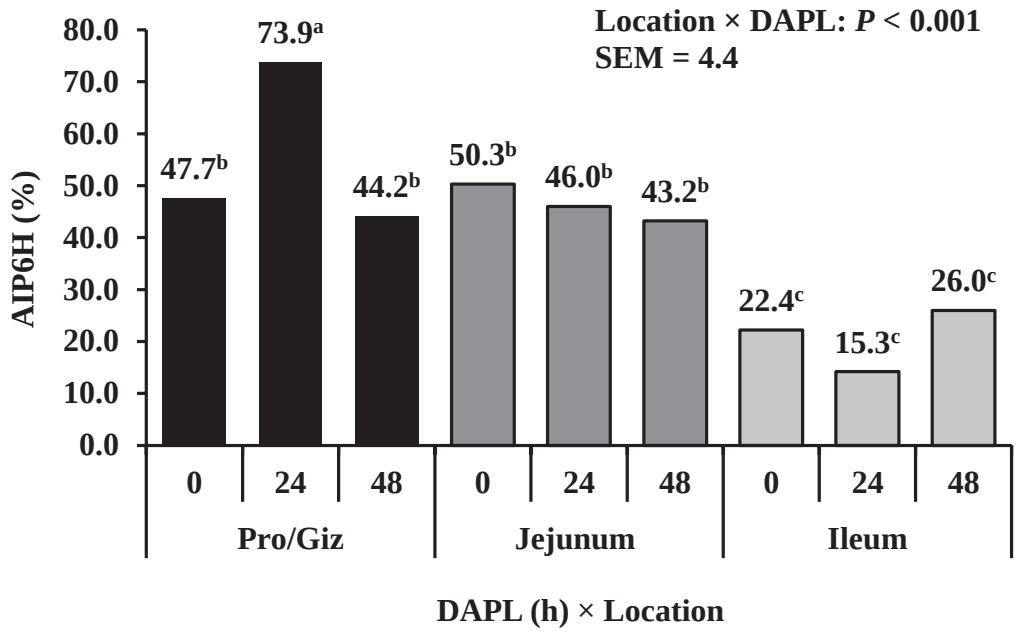

A)
Location: P < 0.001
DAPL: P < 0.010
SEM = 4.84
AIP6H (%)
75.0
60.0
45.0
30.0
15.0
0.0
-15.0
69.9a
25.6b
-9.3c
34.8x
32.2x
19.2y
Pro/Giz
Jejunum
Ileum
0
24
48
Location
Adaptation Period (h)
B)
Location: P < 0.001
DAPL: P < 0.05
SEM = 4.72
AΣIPD (%)
75.0
60.0
45.0
30.0
15.0
0.0
-15.0
65.4a
13.8b
-5.9c
26.6xy
30.2x
16.5y
Pro/Giz
Jejunum
Ileum
0
24
48
Location
Adaptation Period (h)

Figure 6.3 Interaction effects of sampling location and dietary adaptation period length (DAPL) for Ross × Ross 708 male broilers fed the corn titration diets on **A)** apparent *myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate (IP6) hydrolysis (AIP6H) or **B)** apparent digestibility of the summation of measured *myo*-inositol phosphate esters (A∑IPD). Measured *myo*-inositol phosphate esters included IP6, inositol penta-phosphate (IP5), inositol tetra-phosphate (IP4), inositol tri-phosphate (IP3), and inositol di-phosphate (IP2). Least square means not sharing a common superscript differed as compared using Tukey's Honestly Significant Difference test (Tukey, 1953).

A)


Location × DAPL: P < 0.001
SEM = 4.4
AIP6H (%)
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
47.7b
73.9a
44.2b
50.3b
46.0b
43.2b
22.4c
15.3c
26.0c
0
24
48
0
24
48
0
24
48
Pro/Giz
Jejunum
Ileum
DAPL (h) × Location

B)

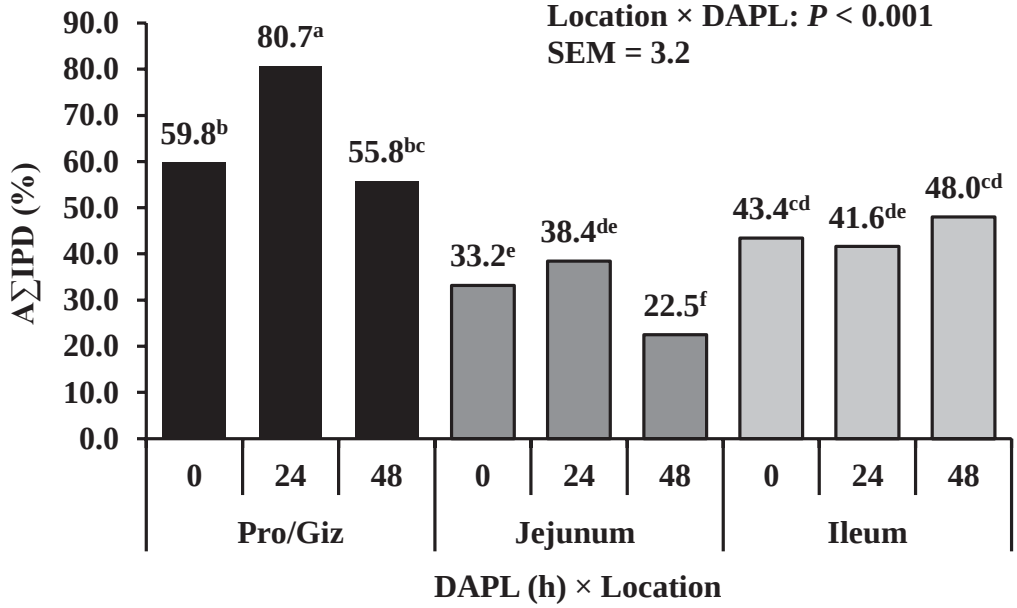
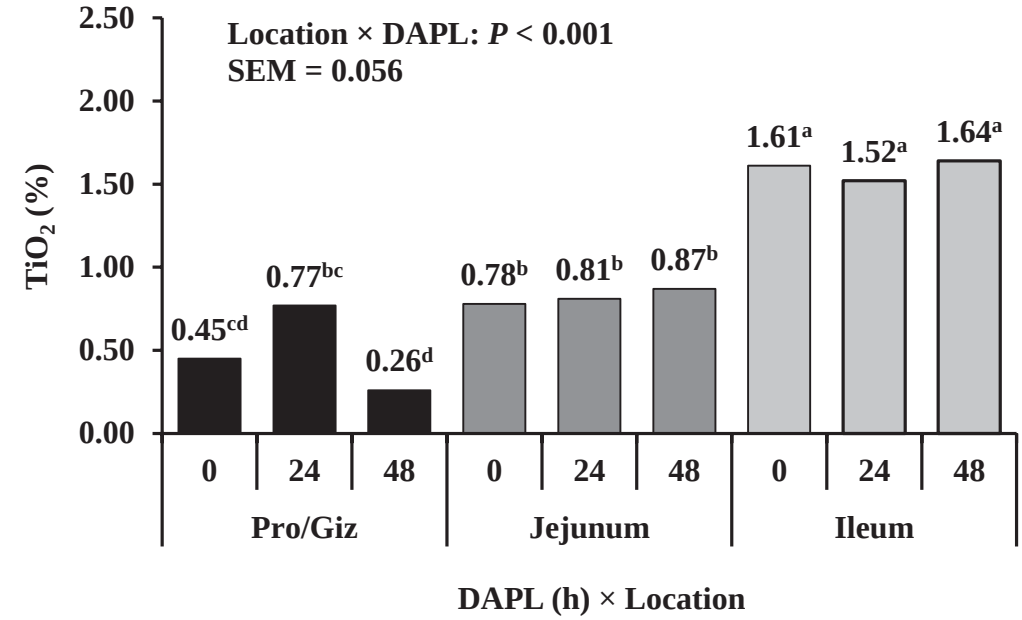
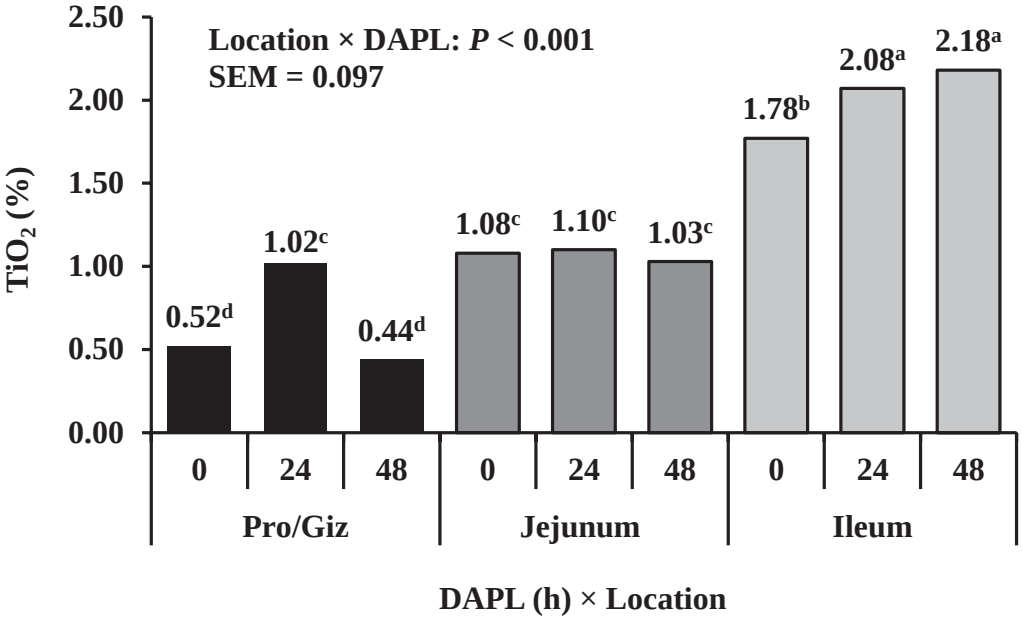

Location × DAPL: P < 0.001
SEM = 3.2
AΣIPD (%)
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
59.8b
80.7a
55.8bc
33.2e
38.4de
22.5f
43.4cd
41.6de
48.0cd
0
24
48
0
24
48
0
24
48
Pro/Giz
Jejunum
Ileum
DAPL (h) × Location

Figure 6.4. Interaction effects of sampling location and dietary adaptation period length (DAPL) on titanium dioxide (TiO_2) concentrations for broilers fed either **A)** corn-soybean meal control diet or **B)** corn titration diets. Least square means not sharing a common superscript differed as compared using Tukey's Honestly Significantly Different test (Tukey, 1953).

A)


Location × DAPL: P < 0.001
SEM = 0.056
TiO2 (%)
2.50
2.00
1.50
1.00
0.50
0.00
0.45cd
0.77bc
0.26d
0.78b
0.81b
0.87b
1.61a
1.52a
1.64a
0
24
48
0
24
48
0
24
48
Pro/Giz
Jejunum
Ileum
DAPL (h) × Location

B)


Location × DAPL: P < 0.001
SEM = 0.097
TiO2 (%)
2.50
2.00
1.50
1.00
0.50
0.00
0.52d
1.02c
0.44d
1.08c
1.10c
1.03c
1.78b
2.08a
2.18a
0
24
48
0
24
48
0
24
48
Pro/Giz
Jejunum
Ileum
DAPL (h) × Location

VII. CONCLUSIONS

Proper growth and bone development is contingent on an adequate supply of dietary P. However, current formulation methods require the use of safety margins due to variability in P concentrations and availability of feedstuff P. To more efficiently meet the P requirements of broilers, improved procedures have been proposed for the assessment of TPU of common feedstuffs. However, TPU estimates have varied between and within laboratories for similar feedstuffs. For diets to be formulated utilizing TPU data, methodologies must be refined and standardized in order to develop an accurate feedstuff TPU database. Therefore, 4 experiments were conducted to assess different factors that may contribute to the variation observed in published estimates of TPU.

Experiments 1 and 2 were designed to determine the effects of differences in diet type on AIPD and APR of diets fed to broilers subjected to different DAPL. Differences in DAPL affected the APR of titration diets for the determination of TPU. However, interaction effects were not observed for AIPD and APR of the 25% corn diet, likely because it was formulated to be adequate in AA. Furthermore, changes in DAPL also resulted in differences in APR for the diet serving as a control for DAPL. This result indicated that other factors, in addition to diet type, may be responsible for the differences observed for AIPD and APR after different DAPL. Main effects of diet type on AIPD and APR were most likely related to differences in dietary Ca:P ratios. As a result, both DAPL and Ca feeding strategy should be standardized when assessing the P availability of titration diets for the determination of TPU.

Experiment 3 examined the effects of different Ca feeding strategies on titration diet AIPD and APR and estimates of TPU and predicted EPL based on the regression analysis of diet apparent P digestibility data. Lower dietary Ca concentrations (0.13%) resulted in increased TIPD and TPR compared with diets formulated at higher Ca concentrations (0.95%). However, formulating diets with low Ca concentrations may result in the overestimation of TPU. Conversely, the prediction of negative EPL for diets formulated with a fixed Ca:tP ratio may have resulted in an underestimation of TPU. Therefore, the use of linear regression analysis to determine TPU may have limitations when diets are formulated with a fixed Ca:tP ratio or very low Ca concentrations.

Experiment 4 was conducted to determine the effects of Ca feeding strategy and DAPL on AIPD and AIIP6H of corn titration diets used to determine feedstuff TIPD. These data demonstrated that DAPL and Ca feeding strategies affected TIPD and the prediction of EPL when diets were formulated to maintain a Ca:tP ratio. Due to changing Ca concentrations of the titration diets, AIPD for these diets decreased linearly resulting in the prediction of negative EPL and an underestimation of TIPD. When titration diet Ca concentrations were fixed at 0.35%, EPL were positive and no effects of DAPL were observed for TIPD. These data further support that regression analysis may have limitations when applied to AIPD data of titration diets with a fixed Ca:tP ratio.

Additional digesta samples were collected from broilers in experiment 4 to assess the effects of different DAPL on AIP6H and A∑IPD at 3 locations (Pro/Giz, jejunum, and terminal ileum) in the GIT. Apparent IP6 hydrolysis and A∑IPD in the Pro/Giz were affected by DAPL when feeding the corn titration diets or the control diet for DAPL. This observation was likely attributable to inconsistent flows of TiO_2 and IP into or out of the

Pro/Giz. These inconsistent flows for IP or TiO_2 resulted in higher values for AIP6H in the Pro/Giz but lower values for AIP6H in the distal ileum for both the corn titration diets and the control diet.

Collectively, these data confirmed that several common methodological differences are potential contributions to the variability associated with TPU estimates. Differences in DAPL affected measures of P availability but the mechanism behind this finding remains unresolved. However, this supports the idea that a standard DAPL must be established to limit variability when assessing P availability. Among the procedural differences examined, dietary Ca feeding strategy appeared to have the largest impact on variability in TPU estimates and the prediction of negative EPL. Estimates for TPU were particularly affected by different DAPL when a constant Ca:tP ratio was maintained. Additional research is warranted to better elucidate the influence of different Ca feeding strategies on measures of P availability for the determination of TPU. Moreover, the use of TiO_2 as an inert marker may have limitations as it does not appear to transit the anterior GIT at the same rate as inositol phosphate esters. However, additional research on this subject is warranted to determine to what extent this finding impacts the measurement of AIP6H and P availability in the posterior GIT.