

ABSTRACT

JOARDAR, DINABANDHU. Effect of Dietary Phosphorus and Potassium in Broiler Breeders during Onset of Lay and Effect of Dietary Limestone Particle Size, Potassium and Phytase in Broilers. (Under the direction of Dr. Adam C. Fahrenholz and Dr. Jesse L. Grimes).

This dissertation is an investigation on the effect of dietary available phosphorus (AvP) and its interaction with dietary potassium (K) on fecal moisture and egg characteristics in broiler breeders during onset of lay and the effect of dietary limestone particle size, K and phytase on physiological and nutritional aspects in broilers. In Manuscript 1, the study on broiler breeder females evaluated two levels of dietary available phosphorus (AvP; 0.3% and 0.5%) with 0.9% calcium (Ca) fed during growing period from 6 to 26 wk of age. During onset of lay, between 22 to 30 wk of age, two AvP levels (0.3% and 0.5%) were fed as layer diets, accompanied with or without added potassium (K). The effects of the diet treatments were evaluated by the fecal liquid portion (LP), fecal moisture (FM), and egg production and characteristics of the second and tenth egg laid. Two broiler breeder AvP grower diets resulted in comparable egg production and 0.3% AvP fed during growing significantly improved egg weight. During the onset of lay, 0.5% AvP layer diet with 0.2% K increased LP and FM, while the 0.3% AvP with 0.2% K reduced the LP and FM. Furthermore, in all the fed dietary treatment combinations the LP and FM generally was reduced as the breeders achieved peak egg production. The 0.3% AvP grower diet increased the egg weight and albumen weight of the second eggs. No effect of laying diet combinations relative to the 0.3% AvP to 0.5% was observed in 25-29 wk egg production.

In Manuscript 2, the study evaluated dietary limestone particle size and dietary potassium on live performance, blood physiology and wooden breast and white striping in broilers. This experiment also served as an examination of blood physiology in relationship to dietary K and limestone particle size. The increase in dietary K altered the dietary electrolyte

balance (DEB) of the diets and maintained the acid-base status and the blood gas parameters within a physiological homeostatic range. However, the added K reduced the feed intake and negatively affected live performance. The association of wooden breast with growth was attributed to dietary fine limestone.

In Manuscript 3, the study evaluated dietary limestone particle size and reduction of dietary AvP in broiler diets supplemented with 1000 and 2000 phytase units/kg on live performance, tibia bone ash and apparent ileal digestibility of Ca, P and amino acids in broilers. Coarse limestone improved feed intake and digestibility of Ca, P, Na and amino acids, while fine limestone improved feed conversion ratio (FCR) at 16 d while dietary phytase supplementation of low AvP (0.3%) diets significantly improved weight gain, feed intake, tibia bone ash.

In summary, the dietary phosphorus levels could be reduced during the onset of lay period to affect the fecal moisture without any undesirable consequences on egg production or egg characteristics, hence, adjustments in phosphorus levels may be considered during onset of lay period. In broilers, the dietary phosphorus can be reduced with addition of phytase. The addition of potassium in broiler breeders and, in broiler diets may not impact the bird homeostasis. A coarse limestone particle size can be considered in broiler diets for its nutritional effects when potential interactions with phytase were taken into consideration. It was concluded that the dietary phosphorus, potassium, limestone particle size and phytase were associated with positive physiological and nutritional effects on the birds. There were apparent paradoxical interactions between potassium and phosphorus, that should be further investigated.

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Effect of Dietary Phosphorus and Potassium in Broiler Breeders during Onset of Lay and
Effect of Dietary Limestone Particle Size, Potassium and Phytase in Broilers

by
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DEDICATION

To my Parents, Family and Dear Friends.

BIOGRAPHY

Dinabandhu Joardar is the elder of two siblings of Gopal Krishna Joardar and Bela Joardar, and, was born and raised in India. In 1992, after completion of his Bachelors' in Veterinary Science from University of Agricultural Sciences, Bangalore, India, Dina gained experience in live poultry production in India and Bangladesh where he established new projects on broiler breeder and GP stocks and designed the nutrition and feeding programs. Afterwards from 2005 he pursued his career in feed additive companies as poultry technical manager for Asia Pacific region based out of Thailand and Singapore. While actively developing poultry nutrition research collaborations with universities in Asia he developed a keen interest for further learning and decided to pursue his MSc in Poultry Science at Scottish Agricultural College, University of Glasgow. After completion of MSc in 2013, he returned to work with DSM Asia Pacific, Singapore and started to work with Dr. John Brake to provide technical services for poultry producers in SE Asia. This led Dina to pursue a PhD in poultry science. This decision was followed by acceptance into the Prestage Department of Poultry Science for his doctoral work starting in spring of 2016 under the direction of Dr. John Brake. Sadly, Dr. Brake passed away in July 2018. Under the guidance of Dr. Adam Fahrenholz and Dr. Jesse Grimes, he continued his work. After completion of his doctoral program, Dina is looking forward to work and gain experience in the US poultry industry.

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LITERATURE REVIEW

PHOSPHORUS METABOLIC FUNCTIONS

Phosphorus (P) is an essential dietary nutrient and it plays an important role in many biological processes. The P found in the body, most commonly combines with oxygen to form phosphate PO_4^{3-} (Pi) anion and its esters (Anderson, 1991). Phosphate esters participates in major metabolic pathways in the cellular metabolism which includes energy metabolism, cell signaling and regulation of protein and, nucleic acid synthesis (Biber *et al.*, 2013; Li *et al.*, 2016). Phosphorus also plays vital roles in bone mineralization, maintaining acid-base balance, and protein synthesis which is essential for growth and feed efficiency in poultry (Marks *et al.*, 2010).

Westheimer (1987), described the biological role of phosphates and discussed “why nature chose phosphates” and how the P intermediaries such as esters and anhydrides play multiple dominant roles in biochemical transformations in the living world. The negative charge on Pi is important in binding of coenzymes to the enzymes. Nicotinamide adenine dinucleotide (NAD) and key enzymes in carbohydrate metabolism fructose 1,6-bisphosphate, glucose-6-phosphate are examples of phosphate esters.

Davies (1958) described that at the physiological pH, P intermediary metabolites' ionization property gives the unique ability for the cells to trap and conserve its biomolecules within the cell membrane. This helps the metabolites to remain trapped within cells for longer durations which is essential for the stability of genetic materials. The phosphodiester form linking groups for nucleic acids of genetic materials, deoxy- and ribonucleic acids (DNA and RNA) which provides relative resistance to hydrolysis for the DNA and helps to conserve the genetic material within the cell (Fekry *et al.*, 2011).

The phospho-nucleotide nucleotide adenosine triphosphate (ATP) is a kinetically stable central carrier of cellular energy (Ross, 2006). In a cascade of reactions where the phosphoryl groups are transferred from ATP to various substrates forming adenosine di-phosphate (ADP) and adenosine mono-phosphate (AMP) thus releasing the energy required for the cellular metabolism (Pearlman *et al.*, 2011). When energy is not required immediately, ATP act as chemical energy storage. Nucleosides including guanosine triphosphate (GTP), uridine triphosphate (UTP) and cytidine triphosphate (CTP) are energy rich phosphate compounds that promote protein synthesis (Gaal *et al.*, 1997). In muscle cells phosphagen creatine phosphate high energy phosphate storage molecules provide metabolic fuel for oxidative phosphorylation.

Hardin and Knopp (2013) described that the P metabolic role includes the covalent modification resulting in phosphorylation-dephosphorylation of biochemical compounds which is the ubiquitous reversible on/off mechanism of metabolic regulation. The kinases and phosphatases are involved in the reaction which leads to activation (deactivation) of key metabolic enzymes in the regulatory biochemical reactions.

Phosphorus plays an important role in the secondary hormonal messenger biochemical compounds such as cyclic AMP, cyclic guanine mono-phosphate (c-GMP) and inositol polyphosphates. Phosphorus is an active component of 2, 3-bisphosphoglycerate (BPG) which is derived from the glycolysis intermediate, 3-phosphoglycerate that helps in controlling the binding of oxygen to hemoglobin and provides a direct link between status of glucose metabolism and extent of oxygen uptake in the cells (Hardin and Knopp, 2013).

In lipid metabolism, P participates in fatty acid transportation via formation of phospholipids and the physiological effects of dietary P on fatty acid metabolism have been demonstrated in broilers (Li *et al.*, 2016). However, the molecular mechanisms are not well

established in birds. Thus, P is necessary for growth, cellular membrane function, energy metabolism.

Additionally, phosphate buffer functions to maintain acid-base balance at physiological pH (Danisi and Murer, 1991). It is an important buffer in the renal tubular fluids where it is available as a result of bone resorption during egg shell calcification. The phosphate binds H^+ to form H_2PO_4 when compared to excretion of H^+ via the bicarbonate pathway producing water (H_2O) molecules.

PHOSPHORUS ABSORPTION AND HOMEOSTASIS

Primary phosphorus (Pi) absorption in the chicken small intestine has been studied extensively (Hurwitz and Bar, 1970; 1971; Bar and Hurwitz, 1979; Yan *et al.*, 2007). Dietary P is hydrolyzed into Pi by phytase enzyme, phospholipase C and alkaline phosphatase to release P from bound forms (Ravindran *et al.*, 1995). Inorganic Pi exists in two forms in the intestinal lumen: negatively charged monovalent $H_2PO_4^-$ and divalent HPO_4^{2-} ions (Anderson, 1991) which is often abbreviated as Pi (Biber *et al.*, 2013). The pH is directly found to modify the intestinal Pi transport (Danisi *et al.*, 1984). At low luminal Pi concentrations, the simple diffusion across intestinal mucosa through paracellular space is limited due to the negative charge at the tight junctions (occludins and claudins) and inside of cells (Manghat *et al.*, 2014). The transcellular active transport mechanism using the sodium-dependent phosphate transporters are involved in the uptake of Pi across the intestinal brush border membranes (Danisi and Murer, 1991). The apical entry of Pi into epithelial cells of intestine and kidney have been studied extensively, however little information is known about the enterocyte basolateral phosphate uptake and its regulation (Marks *et al.*, 2010). The P absorption in the small intestines includes; paracellular concentration gradient-dependent passive transport system which is quantitatively important mode of P uptake. The sodium dependent active

transport mechanisms in the brush border membrane of small intestines (Berner *et al.*, 1976) largely depends on the dietary phosphate load (Giral *et al.*, 2009). The active intra-cellular transport of Pi is mediated via the specific proteins; the type II sodium-phosphate co-transporters (type II Na-Pi) are expressed in the brush border membranes. The type IIb Na-Pi co-transporters that are primarily expressed in the duodenum, jejunum, and ileum are considered to be the most important Pi transporter against its concentration gradient in small intestines of chickens (Giral *et al.*, 2009; Yan *et al.*, 2007). In the chicken, duodenum has a greater expression of Na-Pi cotransporter proteins, however its short length limits the Pi absorption. Therefore, jejunum is considered to be the major site of Pi absorption where the Na-Pi cotransporters are found in both villi and crypts (Hurwitz and Bar, 1970, Yan *et al.*, 2007). Further, Huber *et al.* (2015) reported that these Pi transporters are the key modulators expressed in response to the luminal P concentrations in the jejunum of chickens for facilitation of Pi uptake.

A novel result was found by Hu *et al.* (2018), where a study was conducted on broilers that demonstrated greater mRNA expression for inorganic phosphate transporter 1 (PiT-1) in jejunum with low (0.66%) dietary P at 7 d of age. The same study showed that the concentration of P in plasma in the hepatic portal vein was increased as the dietary P was increased from 0.21% to 0.44%. Most broilers studies have reported a low P digestibility at ileal level which suggested a low efficiency of Pi absorption in the small intestines of chicken. The apparent ileal absorption of P was reported to be in the range of 40% to 50% in broilers that were fed diets containing optimum concentrations of P (Ravindran *et al.*, 2000; Tamim *et al.*, 2004; Yan *et al.*, 2007). Recently, experiments conducted in broilers by Zeller *et al.* (2015, 2016) reported a significant clearance of Pi measured at the crop and distal ileum, when fed both low and high dietary P. Olukosi *et al.* (2007) reported that the total gastrointestinal tract

P absorption generally increased with age observed from 7 to 21 d of age in broiler chickens. The gene expression data from broiler studies reported that increasing the dietary P reduced the expression of Na-Pi IIB co transporters in the small intestine (Yan *et al.*, 2007; Li *et al.*, 2012; Hu *et al.*, 2018). Olukosi *et al.* (2011) reported that the NaPi-IIB gene expression was upregulated in the jejunum and ileum of broiler chicks on a diet that was severely deficient in P (~2.5g of non-phytate P, (NPP)) relative to those chicks that were fed adequate or excess NPP diets. In chickens the type II Na-Pi cotransporters are involved in regulating both intestinal absorption and renal Pi reabsorption for maintaining P homeostasis.

Given the importance of P in the cellular functions major mechanisms exist to maintain P homeostasis and the plasma P concentrations are held within a relative narrow range and the homeostatic mechanisms include; altering the efficiency of Pi absorption in the small intestine or its reabsorption or excretion by the kidneys (Marks *et al.*, 2006, 2010). Three major mechanisms that are responsible for the maintenance of systemic Pi homeostasis are: mainly the modulation of intestinal uptake of P, the retention or release from the bone, and the renal reabsorption and excretion (Xu *et al.*, 2002; Kiela and Ghishan, 2009). When animals are fed low P diets this results in decreased serum P concentration. Which reciprocally is associated with increased plasma Ca concentrations leading to inhibition of parathyroid hormone (PTH), which reduces the renal Pi excretion. Additionally, the hydroxylation of 25-hydroxyvitamin D which increases $1\alpha,25$ -hydroxyvitamin D synthesis leading to the expression of Na-Pi IIA cotransporters in proximal renal tubules, which results in increasing the jejunal Pi absorption via active transport mechanism (Berndt and Kumar, 2009; Marks *et al.*, 2006;). It was also demonstrated in the knock out mice studies that the intestinal cells and renal cells can respond directly to changes in dietary Pi content which has the capacity to increase the phosphate uptake that are independent of $1,25$ -dihydroxyvitamin pathway (Segawa *et al.*, 2004). The

above studies demonstrated that the Pi transporters play important roles in the adaptation of Pi transport in low-P diets by up regulation of Na-Pi and PiT-1 cotransporters throughout the small intestine. Homeostasis is maintained by kidney by reabsorption of Pi which is mediated via the Na-Pi-IIa cotransporters. However, an important question regarding P homeostasis was discussed by Berndt and Kumar (2009) and how the “body senses” changes in the Pi concentrations and adjusts the metabolic processes to adapt to these changes to regulate P homeostasis. Berndt *et al.* (2005) cited evidences which suggested that individual cells and unicellular organisms have specific phosphate sensors known as phosphatonins (Kumar, 1997) that are capable of altering intracellular protein metabolism. The studies conducted with knockout mice (thyro-Para thyroidectomized) confirmed that the phosphatonins; fibroblast growth factor-23 (FGF-23) and sFRP-4 were found in similar concentration in serum and the FGF-23 protein was associated with increased Pi excretion and decreased plasma Pi concentrations (Miyamoto *et al.*, 2005; Berndt and Kumar, 2007). The reported studies described that the phosphatonins were associated with Vitamin D endocrine system, resulting in similar effects like PTH, and PTH is involved in the regulation of P in the P homeostasis.

Phosphorus and Calcium Homeostasis

The physiological roles of P and Ca are known to be intricately linked to each other. The P and Ca homeostasis have been well reported in chickens. The plasma Ca and P concentrations, dietary P and, calcitriol mediates intestinal absorption, and the inter-relationship between dietary P and Ca concentrations on P absorption is well known (Quamme and Shapiro, 1987). Morrissey and Wasserman (1971) reported greater Ca absorption resulted when 17 d of age chicks were fed P deficient (0.25% P) and Ca adequate (1.20% Ca) diets. Hurwitz and Bar (1971) reported that a quantitative relationship exists between Ca and P resulting from the chemical association in the intestinal lumen rather than from an interaction

at the site of absorption. The same authors reported that a wider Ca to P ratio in diets negatively influenced the utilization of both P and Ca. Hormonal regulation of P and Ca homeostasis is characterized by the common mechanisms (Hurwitz, 1989) that are mediated by action of PTH (in intestinal Ca transport), calcitonin and $1\alpha, 25\text{-dihydroxyvitamin D}$ ($1\alpha, 25\text{-(OH)}_2\text{D}_3$). The calcium-sensing receptor (CaR) is known to be a key factor in Ca homeostasis in the extracellular Ca homeostasis (Diaz *et al.*, 1997; Hofer and Brown, 2003) and Ca binding protein (CaBP) associated with the intestinal transport of Ca was shown to respond to changes in dietary Ca levels (Morrissey and Wasserman, 1971), upregulated when fed diets are deficient in Ca. The PTH, the main Ca regulating hormone has a direct action on the bone Ca flow and has an indirect action on the intestinal Ca absorption through regulation of the synthesis of $1, 25\text{-dihydroxycholecalceferol}$ (vitamin D3). The renal $1\alpha\text{-hydroxylase}$ activation depends on Ca concentration and is additionally regulated by PTH and calcitonin (Anderson, 1991). This increases Ca absorption by opening up Ca channels in the intestinal mucosa and stimulates the Ca binding protein, calbindin (Favus and Tembe, 1992) which also decreases urinary excretion of Ca. The hormone calcitonin is released when the serum calcium level is high (Talmage *et al.*, 1982). It decreases the osteoclast activity while increasing osteoblast activity to build bones and lowering the serum Ca. Its expression is regulated by vitamin D3 (Anderson, 1991). The homeostatic control of Ca concentration is controlled within a narrow physiological range through the feedback mechanism between PTH, vitamin D3 and calcitonin (Hurwitz, 1989).

PHOSPHORUS AND CALCIUM HOMEOSTASIS DURING LAY

On an average, an avian egg contains 80 to 120 mg of P (Romanoff and Romanoff, 1949). Phosvitin, a phosphoprotein, is the second largest macromolecule in the egg yolk. Phosphate is required for the formation of phosvitin which serves as reservoir of organic

phosphate for the embryo. The phosvitin contains high proportion of ionizable phosphate groups that function as polyelectrolyte that primarily chelates iron in the egg, which acts as an antioxidant and plays an important function of storing iron for the embryo (Romanoff and Romanoff, 1949). Thus, the stored P plays an important role in the metabolic functions required for the growth of the avian embryo.

The onset of lay is characterized by increase in the plasma Ca levels (Hertelendy and Taylor, 1961), retention and formation of medullary bone in response to the gonadal steroids (Dacke *et al.*, 1993) which usually occurs fourteen to sixteen days prior to onset of egg production (Hurwitz, 1964). During the egg shell formation, Ca and P are mainly regulated by the net flow out of the medullary bones (Hurwitz and Bar, 1965). The hydroxyapatite structure dissociation releases Ca and P, from which the Ca is transported to the uterine glands and the excess phosphate is excreted (Kerschnitzki *et al.*, 2014). Smith *et al.* (1954) reported that the calcification stops at appearance of phosphates in the lumen of uterus. Therefore, consistently high plasma levels of P high were correlated with decline in egg shell quality (Leeson and Summers, 1991). Normally, the avian kidney reabsorbs more than 98% Ca and excretes 60% phosphate. As mentioned elsewhere the dual action of PTH on reabsorption and excretion of Ca and P aids in maintaining an appropriate Ca/P ratio. Martindale (1973) studied the effect of parathyroid extract (PTE) on P excretion and reported that the PTE increased the P excretion by inhibiting both reabsorption and secretion of P by the kidneys. During lay period the P homeostasis is governed by PTH which stimulates urinary P excretion and prevents accumulation of P in the blood (Wideman, 1987). Choi *et al.* (1979) reported that laying hen efficiently maintains a normal level of blood P with adequate or higher concentrations of dietary P (0.37% and 1.40%), however, would decrease the blood P when fed P deficient

(0.22%) diets. Thus, modern broilers are capable of maintaining a normal blood P over a wide range of dietary P concentration.

PHOSPHORUS EXCRETION DURING LAY

Prashad and Edwards (1973) reported that in the last 8 or 10 h of shell formation there is a considerable increase in the urinary excretion of P. The accurate P excretion is difficult to measure in chickens because the urine is mixed with digesta rather than being directly eliminated. However, the difficulty can be somewhat circumvented by performing a colostomy procedure for short-term separate urine collection (Manangi and Coon, 2007).

Earlier studies by Hurwitz and Griminger (1961) measured P excretion using colostomized laying hens and reported that the P excretion in urine was 62% of the total excreted P and concluded that extra P mobilized from bone resorption led to increased urinary P excretion and low P retention. Taylor and Kirkley (1967) reported an increased P excretion in urine on egg laying days with colostomized hens. Mongin and Sauveur (1979) demonstrated a peak in plasma P occurred in night when medullary bone activity is greatest and noted that most of the P is excreted in the urine, since there is no immediate metabolic need.

Recently, Manangi *et al.* (2018) measured urinary P, excretion of urinary P and plasma inorganic P in colostomized and non-colostomized broiler breeder hens. It was reported that in the colostomized hens at 6 wk of age the total volume of urine collected with two dietary sources of limestone was not different. The total P excreted, respectively, was 80.4 mg and 90.2 mg. The urinary P excretion was found suddenly increased to maximum (~50 mg/dL) during 11 to 20 h post oviposition. The plasma P was in the range of ~5 to 7 mg/dL, with peak values at 18 to 24 h post oviposition. However, in laying hens Rao and Roland (1990) reported that the serum P peak was during 11 and 14 h. The authors noted that bone is mobilized during egg production for both laying hens and broiler breeders.

It was suggested by studies that the bone mobilization physiology may be different in broiler breeders and laying hens associated with P handling by the renal system.

DIETARY ELECTROLYTE BALANCE

The monovalent, Na^+ , K^+ and Cl^- , are the fixed bioavailable ions that cannot be further metabolized (Hooze, 2003). In the diets the electrolyte balance between the cation, Na^+ , K^+ and the anion Cl^- can be expressed as a formula $\text{Na}^+\text{K}^+\text{Cl}^-$ mEq/kg is known as dietary electrolyte balance (DEB) (Scott *et al.*, 2001). In relation to acid-base homeostasis the DEB, which is the net charge associated with combined intake of dietary Na, K and Cl, is known to affect the acid-base status of an animal. These electrolytes play major role in osmotic regulation, cell physiology, animal metabolism and body fluid acid-base balance, mainly the water balance (Leeson and Summers, 1991). Potassium is 90% intracellular and 10% extracellular, Na is mostly present outside the cell and Cl ion represents 65% of total extracellular anion concentration provides exchange counter balance for each other which constitutes the birds' electrolyte physiology (Suhail, 2010). The dietary sodium intake affects post renal water absorption in the colon region and the water absorption is dependent on efficiency of Na absorption (Van der Klis *et al.*, 1993). However, the role of K, though not well understood, is suggested that its secretion aids in maintaining membrane potential via the sodium pump (Lind *et al.*, 1980).

Mongin (1981) and coworkers described dietary electrolyte balance (DEB) state as in which animal utilizes dietary ions to maintain an acid-base homeostasis (Mongin and Sauveur, 1977). The DEB is described as a formula of dietary $\text{Na}^+\text{K}^+\text{Cl}^-$, which is expressed as mEq/kg. Mongin (1981) and suggested the optimal requirement of DEB for broilers as 250 mEq/kg. However, recent studies reported that DEB ~300 mEq/kg was necessary in heat stress conditions to achieve optimal live performance (Borges *et al.*, 2004, 2007). This increased

DEB was affected due to increased water intake associated with increased plasma volume. Rondon *et al.* (2001) reported that DEB of 319 mEq/kg was optimum for growth performance in broilers when the dietary DEB was varied with supplementation K in the diet.

Determination of DEB optimum is often challenging because of the interaction among dietary electrolytes and macro-minerals affected by varied environmental conditions which alters acid-base balance, metabolism and changes in feed consumption.

OSMOREGULATION DURING LAY

The osmoregulation can be defined as turnover and homeostasis of the major electrolytes sodium (Na) and chloride (Cl) of the plasma and in the extracellular fluids, potassium (K) (Scott *et al.*, 1982). Physiologically birds do not produce hyperosmotic urine i.e., more than five times plasma osmotic concentration since they lack loop structure of henle and cannot excrete hyperosmotic urine (Sturkie, 1976). Skadhauge (1981) reported that the urine is mixed with digesta rather than directly eliminated, hence the avian gut plays a dual role in digestion and osmoregulation. The avian hind gut comprises of coprodeum, colon and ceca, an important function of the hindgut is sodium reabsorption and solute linked water reabsorption (Lavery & Skadhauge, 1999), these physiological processes modify both the composition and volume of refluxed urine. Though the epithelial permeability decreases along the length of the intestine however, the transport of NaCl and water across the hindgut can happen by hormonal regulation via aldosterone, particularly within the cloaca, suggesting local control of reflexing action (Braun, 2003).

In preparation for the egg production laying hen and broiler breeder pullets undergo major changes in its mineral metabolism during the pre-lay period. Transitioning into sexual maturity is mainly characterized by bone remodeling that takes place approximately between 24-26 wk of age in the broiler breeder pullets (Ekmay *et al.*, 2012). However, all individual

birds in a flock do not start laying in the same week but are fed the same amount of dietary Ca and P which may alter the flock status of mineral metabolism, electrolyte balance and individual bird homeostasis. This period may extend over several weeks and the bird coming into egg production and until attaining peak egg production are affected by dietary Ca and P concentration (Hurwitz and Bar, 1970) which can alter the acid-base homeostasis. Jack and Lake (1967) described that K, Na, Mg and Ca are the major electrolytes in the avian uterine fluid and their flux differs during plumping, prior to maximal shell deposition and oviposition. These interactions suggest that there may be an altered dietary requirement of Ca and P during this particular period. The egg shell carbonate is derived from the metabolic carbon dioxide of the shell gland and that in the process of this transformation considerable amounts of hydrogen ions are produced (Hodges & Lorcher, 1967) which results in altered acid-base balance. Hodges (1969) reported that the development of the large arterio-venous differences in pH would therefore appear to be mainly associated with the production of hydrogen ions by the shell gland, and the changes that occur in the systemic pH, pCO₂ and bicarbonate during the shell formation cycle would appear to be caused by the introduction of abnormally large amounts of H ions into the blood circulation causing transient metabolic acidosis. The same authors reported that the metabolic acidosis continues to develop until the point of lowest pH (approximately 17 h of shell formation; 3 h before lay). And after this point the process is reversed, and the pH returns towards normal along the line of a metabolic alkalosis indicating that there was an active compensation by the kidneys. When the bone Ca is mobilized the excess P is primarily excreted via urine (Manangi *et al.*, 2018). These compensatory mechanisms to maintain acid-base homeostasis often results in watery droppings.

Smith et al. (2000) investigated effects of dietary Na, K, Ca, P on laying hens water intake and fecal moisture, observed that each 1 g/kg increase in dietary Na, K, Ca, P, increased

excreta moisture content by 9.04, 11.95, 5.59 g/kg, respectively. However, increasing dietary Ca did not affect the excreta moisture.

Leeson and Summers (1987) investigated effects of dietary Ca on water balance of laying hens at the time of sexual maturity and reported that 4% Ca introduced at 16 wk or 19 wk increased excreta moisture content at 21 wk and persisted up to 35 wk of age. The authors noted that there is a transitory effect of dietary Ca concentration on water intake and excreta moisture content.

The egg shell quality assessed in Japanese quails by Costa *et al.* (2011) reported that egg shell quality was improved with the supplementation of dietary K (from 0.42% to 0.45%). The study found reduction in the absolute weight of the shell with increasing K levels. Nevertheless, despite the reduction in shell weight, the increased K levels did not influence the specific gravity or shell thickness, and it could be inferred that the external quality of quail eggs was not affected by the different levels of K. Therefore, the supply of diet balanced in electrolytes such as K enhances the physiological acidity buffering, resulting in better performance in egg quality, however the effect on the fecal moisture is unexplored.

Jonchere *et al.* (2012), reported molecular expression of 3 coding K channel genes thus the role of K was evident in maintaining the cell membrane potential and the resulting mechanisms induced K recycling during eggshell calcification. Earlier study by Hopkinson *et al.* (1990) reported occurrence of physiological depression in plasma K concentrations during onset of lay and suggested that there might be a greater requirement of K during eggshell formation.

POTASSIUM AND BLOOD ACID-BASE

Potassium as an essential electrolyte in animal diets was demonstrated as early as in the 19th century. Potassium plays an important role in regulation of acid-base balance and the

maximum concentration of K is found in the muscles (Ward, 1966). During normal metabolic processes the body constantly produces H^+ , CO_2 , and HCO_3^- ions. The H ion concentration is typically maintained in a narrow range through interaction of multiple buffer systems. Bicarbonate ions produced via carbonic anhydrase enzyme system, are essential in the regulation of acid-base balance that affects the H ion movement, excretion and re-absorption. Skadhauge (1983) demonstrated that every mole of metabolic CO_2 produces equivalent amount of acid that is compensated for by the 95% of CO_2 excreted via lungs. In turn, bicarbonate buffer system lowers the concentration of carbonic acid (H_2CO_3) with the production of H ions (Teeter *et al.*, 1985). The role of K in regulation of acid-base balance has been investigated by many researchers in relation to heat stress conditions in broilers (Teeter *et al.*, 1996). Belay *et al.* (1992) reported that heat-distressed broilers exhibited an increased urinary excretion of K. The respiratory alkalosis resulting due to heat stress has been related to negative balance of K due to increased excretion of K. Borges *et al.* (2003) reported occurrence of respiratory alkalosis in broilers when supplemented K as potassium bicarbonate.

Smith and Teeter (1987) reported improved thermo-tolerance of broilers when supplemented K salts (KCl) in drinking water. Rao *et al.* (2002) reported K intake of 1.8 to 2.3 g/day for optimum BW gain in broilers in heat stress conditions. Ait-Boulahsen *et al.* (1995) reported increased BW gain and feed intake when dietary K was supplemented 0.6% KCl in heat stressed birds.

Blood biochemical parameters measures are optimum indicators to detect the state of acid-base balance and the blood variables involved in the assessment of acid-base disturbances are pH, partial pressure of CO_2 (pCO_2) and plasma HCO_3^- , and hemoglobin concentration (Bottje and Harrison 1985; Ait-Boulahsen *et al.*, 1989).

Raup and Bottje (1990) reported that in broilers decrease in $p\text{CO}_2$ results in changes in acid-base balance leading to respiratory alkalosis.

Ait-Boulahsen *et al.* (1995) reported decrease in blood pH during heat stress when chickens drank electrolyte solution rich in K, supplemented as 0.6% KCl. Similarly, Ahmed *et al.* (2008) reported that 0.6% KCl in drinking water reduced the blood pH to 7.31 of 42 d broilers. The possible reasons in the changes in blood pH may have been due to the acidogenic effects of Cl ions or transient metabolic adjustments. The above cited studies demonstrated that during the heat stress in broilers the blood Ca was correlated with K, P, pH, and $p\text{CO}_2$ and before and after heat stress blood pH was correlated with plasma K.

Olanrewaju *et al.* (2006) reported that corticosterone treated birds exhibited higher $p\text{CO}_2$, Hct (Hematocrit), HCO_3 and lower partial pressure of O_2 ($p\text{O}_2$), K, Na and Cl.

The reported studies demonstrated that the measured blood biochemical parameters and the resulting changes due to manipulation of dietary electrolyte concentration and the effects of environmental stresses adequately detected the physiological aspects of acid-base regulation in birds.

PHOSPHORUS TERMINOLOGY AND ESTIMATION METHODS

Terminology

To understand requirements of P for animals, one must be able to understand different terms that are used in nutritional studies to describe different forms of P (Applegate and Angel, 2008). Angel (2011) developed a list of P forms with respective definitions and descriptions.

Total phosphorus (tP) was the broadest term and included all forms of P which is determined after digestion of sample by calorimetry, atomic absorption spectrophotometry (AAS) or inductively coupled plasma spectroscopy (ICPS), and availability of P to the animal was not enumerated.

Phytate P (PP or Phytate P) referred to all organic forms of P that was attached to the six-carbon ring phosphorylated cyclic sugar alcohol (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate; IP6) and is known as phytate or phytic acid. It is measured using high performance liquid chromatography (HPLC) method after sample digestion and purification.

Non-phytate P (NPP), has referred to the P that was not bound to the phytate ($NPP = tP - PP$). This is the most commonly used term for expressing the requirements in poultry nutrition (NRC, 1994).

Available P (AvP), also known as relative bioavailable P, used to express the amount of P that originated from feed and was absorbed and available at tissue level for the animal. The term (AvP) is widely used in literature to express the quantity of P in feed ingredients as well as nutritional P requirements for poultry.

Digestible P, also known as absorbed P is defined as portion of dietary P intake that is digested and absorbed at the distal ileal level.

There are several issues with using relative P bioavailability assays and confusion associated in terminologies used to express P availability and lack of precise P evaluation system therefore led Worlds Poultry Science Association (WPSA) to constitute a group of scientists to develop standard protocol based on pre-cecal digestibility using regression method for determining digestible P in broilers (Angel *et al.*, 2002; Rodehutsord, 2009; Adedokun and Adeola, 2013).

Phosphorus estimation

The P in poultry diets are derived from plant-based feed ingredients, animal-based feed ingredients and inorganic mineral sources. Inorganic P is the major source of feed phosphate is non-renewable and expensive. The P availability can be defined as the amount of P in a feed ingredient that is biologically available for absorption and metabolically utilized by the animal

(Mutucumarana, 2014). Phosphorus is the 3rd most expensive component in poultry diets and the feed formulations needs to be closely managed as chickens require P for bone, muscle growth and a number of metabolic processes. The advances in poultry nutrition research has provided basic knowledge in P utilization and its requirements for various poultry species (NRC, 1994). However, research outcome of past 70 years has provided only the relative values of P availability which is of limited use for production nutritionist for designing precise feed formulation close to requirement of the bird. The biological utilization and content of P varies among the sources of P, with P content ranging from 65-75% in animal-based by products and 55-92% in the mineral P sources (Van der Kilis and Versteegh, 1996). The variation in availability of P from different sources has often led to over formulation of P (Waldroup, 1999). The feed formulation excess of dietary P increases the cost of poultry production and the P excretion has been known to cause environmental problems (Maguire *et al.* 2005).

The P availability and its requirements assessed in reported literature were typically measured using quantitative and qualitative assays (Shastak and Rodehutsord, 2013).

The quantitative testing methods were used for P retention or balance and pre-cecal digestibility studies. The retention of P can be measured using complete excreta collection or using indigestible markers in the test diets with spot sampling of excreta which is calculated using the formula: % retention = $[(\text{tP ingested} - \text{tP excreted}) / \text{tP ingested}] \times 100$ (Leske and Coon, 2002; Rodehutsord and Dieckmann, 2005). The actual reported values are used as direct P availability values which could be used by nutritionists for dietary formulations matrices. Hurwitz *et al.* (1978) demonstrated that the P absorption is almost completed in the distal ileum. Thus, prececal P digestibility evaluation at terminal ileum became a widely used method over the retention method. Though the values obtained were often confounded at

higher P intake due to the fact that urine is the major P excretion pathway in chickens. Both methods demonstrated similar results in evaluation of inorganic P sources when determined at a low level of P intake (Shastak *et al.*, 2012).

The apparent ileal digestibility (AID) of P in broilers were found optimally improved with increasing the dietary (from inorganic P) P up to 5.5 g/kg, beyond which no improvements were observed (Wilkinson *et al.*, 2014). It is suggested that the current industry requirement of 0.40% P (from inorganic P) may be twice of the amount that is necessary for proper maintenance in broiler breeder hen production performance and for the growth performance in broilers (Coon *et al.*, 2007; Ekmay *et al.*, 2012)

The qualitative testing methods for P estimation comprises of several response parameters including bone breaking strength, bone ash and growth data, which could be used individually or in combination to determine relative biological value of P (Coon *et al.*, 2007). The bone ash measurement is widely used for the estimation of P and is used because it is easy to sample. The bone ash analysis uses one of the bones most sensitively responsive to P adequacy and often reflected the differences in dietary Ca and P concentrations, and their differing source bioavailability (Hurwitz, 1964a). Due to these sensitivities, tibia bone ash has become the primary determinant of P requirement (Waldroup, 1999; Hall *et al.*, 2003). The qualitative measurement including the parameters tibia bone ash, body weight gain and feed conversion are preferred methods of estimating P availability from different sources of P (Qian *et al.*, 1996; Cowieson *et al.*, 2011b).

PHYTATE-PHOSPHORUS

The plant-base feed ingredients P is stored as phytic acid and its salt phytate, known as myo-inositol hexaphosphoric acid and myo-inositol hexaphosphate respectively (Cheryan, 1980), of which the latter is most abundant. About two-thirds of the P of plant origin is in the

form of phytic acid (Cosgrove, 1966; Cabahug *et al.*, 1999). The phytate, which is found in most plant-based feed ingredients at concentrations from 5 g/kg to 25 g/kg can potentially contribute between 1.4-7 g/kg phytate-P in the poultry diets (Cowieson *et al.*, 2011) but is the least available form to the chickens. Nelson *et al.* (1976) reported limited phytate-P hydrolysis (0 to 13%) in chicks and laying hens. The proportion of P in form of phytic acid present in the plant-based feed ingredients are poorly utilized due to insufficient phytase enzyme activity to hydrolyze plant phytates in gastrointestinal tract of poultry (Maenz and Classen, 1998; Applegate *et al.*, 2003). Endogenous phytase activity was reported in the intestinal brush border membrane and in the erythrocytes of the chicken (Maenz and Classen, 1998). However, the efficacy of the endogenous mucosal phytase or the microbiota-associated phosphatases in the digestive tract of birds have limited importance in degradation of phytate-P for nutritional benefits (Selle and Ravindran, 2007) and most likely the phytate is chelated to Ca and other minerals before it reaches the intestinal tract where the brush border enzymes are active (Selle *et al.*, 2009).

However, recent studies (Zeller *et al.*, 2015; 2016) measured the effect of high dose (12,500 FTU/kg) of microbial phytase on the degradation of inositol-phosphate (IP₆) isomers and its lower ester, IP₅ in broilers and observed that the IP₆ was hydrolyzed up to 92% followed by a strong degradation of the lower ester IP₅ in the duodenum/jejunum and ileum of the broiler chicken. Sommerfeld *et al.* (2018) reported the appearance of myo-inositol, the end product of inositol-phosphate in duodenum, jejunum, ileum and blood. The above studies, data suggested that the animal has the ability to fully degrade phytate-P when phytase was supplemented. Further complete degradation was achieved by endogenous microbial or epithelial phosphatases to release MI.

MICROBIAL PHYTASE EFFICACY

Microbial phytases are capable of hydrolyzing the ester bond between the inositol ring and phosphate group, thereby releasing P for absorption. Phytase activity is expressed in phytase units (FTU) or fytase units (FYT) and defined in terms of inorganic P released from phytate, where 1 phytase unit equals the amount of enzyme needed to release 1 μmol of inorganic P per minute from 5.1 mM sodium-phytate at 37°C and pH 5.5 (Zyla *et al.*, 2000). It is accepted that the exogenous phytase primarily acts in the acidic environment however is not able to release 100% of phytic P in the gastrointestinal tract of chickens (Selle and Ravindran, 2007).

Leske and Coon (1999) in a 5-d bioassay with male broilers assessed seven poultry feed ingredients and reported that 600 FTU phytase/kg and increased the percentage hydrolyzed phytate-P values of corn (30.8 to 59.0), soybean meal (34.9 to 72.4), wheat (30.7 to 46.8), wheat midds (29.1 to 52.2), barley (32.2 to 71.3), defatted rice bran (33.2 to 48.0) and canola (36.7 to 55.8), respectively. The addition of 600 FTU phytase/kg increased the percent total-P retention of corn (34.8 to 40.9), soybean meal (27.0 to 58.0), wheat (16.0 to 33.8), wheat midds (31.9 to 43.4), barley (40.3 to 55.5), defatted rice bran (15.5 to 26.5) and canola (39.4 to 45.7), respectively.

A similar bioassay with laying hens by Leske and Coon (1999) reported that 300 FTU phytase/kg increased the total-P retention of corn (28.6 to 44.7), soybean meal (36.8 to 53.4), and rice bran (35.9 to 43.0), respectively.

Simons *et al.* (1990) in a landmark paper reported that the application of exogenous phytase to hydrolyze phytate-P improves P availability from the plant-based feed ingredients. The supplementation of 1000 units/kg of phytase to the low-P diets improved the P availability (>60%) and reduced P excretion (50%). Further, the study concluded that exogenous microbial

phytase addition restored the growth performance equivalent to diets supplemented with inorganic phosphate.

Viveros *et al.* (2002) reported that 500 units/kg of phytase improved weight gain (6.7%) at 21d of age in broilers. Cabahug *et al.* (1999) reported that 400 units/kg phytase resulted in greater weight gain (592 to 700 g) and improvement in percentage ash content of dry toe (10.34 to 12.86%) vs. no supplemented phytase in 7-25-d boiler study. On the other hand, Rama Rao *et al.* (1999) reported that when 500 units/kg phytase was added to diets inadequate in Ca (0.75%) and P (0.30%) and fed to broilers to 30 d of age, phytase improved weight gain and feed efficiency. The feed intake improvement from phytase resulted in improved performance was attributed to the improvement in nutrient utilization. Studies were conducted to determine the effect of the exogenous phytase on digestibility of P, Ca and amino acids in broiler diets. Adding phytase enzyme at two levels (500 and 750 FTU/kg of phytase) improved P digestibility of low P diets compared to non-supplemented low P diets due to the reduced P uptake in low P diets and the same effect of improving amino acid digestibility (Rutherford *et al.*, 2004).

Cowieson *et al.* (2015) reported the extra-phosphoric effects with high (1000-3000 units/kg) doses of phytase, apart from improved growth performance, Ca, P availability, an additional improvement in plasma inositol levels was demonstrated, that may be beneficial for nutrient transport and protein deposition. The studies suggested that the growth promoting effect of P effected by phytase was partially attributed to phytate dephosphorylation that released trace minerals and increased the starch digestibility. These results and studies conducted till date supported the concept that phytase is effective in improving P availability and that P level in broiler diets can be lowered with supplementation of phytase (Namkung and Leeson, 1999; Cowieson *et al.*, 2017).

In broiler diets the microbial phytase supplementation is a widely use strategy and phytases have been use in broiler diets to target a release of 0.12-0.15% available P. It has helped the industry to decrease the incorporation of mineral phosphates thus reducing feed cost and the environmental impact of poultry production (Selle and Ravindran, 2007). Elsewhere, published literature in poultry are available on the beneficial effects of exogenous phytase supplementation, on reduction in endogenous losses of nutrients (Cowieson *et al.*, 2004), improvement of amino acid digestibility (Rutherford *et al.*, 2002, 2004), and enhanced protein utilization (Selle *et al.*, 2000; Cowieson *et al.*, 2006).

DIETARY CALCIUM INTERACTION WITH PHYTATE-P

Though Ca and P is interrelated in many biological functions, the requirement of these minerals is interdependent (Mello *et al.*, 2012). Phosphorus availability from phytate-P from phytase is dependent on breakdown of IP6 to lower esters IP and, a number of dietary factors can influence the phytate-P hydrolysis and P absorption along the intestinal tract (NRC, 1994; Zeller *et al.*, 2015). It is known that the phytate-P content and phytate interaction with dietary macro minerals can negatively impact efficacy of exogenous phytase enzyme (Bedford and Rousseau, 2017). Phytic acid in its acidic form binds or chelates multivalent cations including zinc (Zn), copper (Cu), iron (Fe), magnesium (Mg), calcium (Ca). Even though Ca has low affinities for phytate, it has greatest ability to form insoluble Ca-phytate, because it is present in highest concentration around 10 g/kg in the broiler diets (Angel *et al.*, 2002). According to the equation proposed by Nelson (1984), further elucidated by Cowieson *et al.* (2007) one phytate (IP6) molecule can chelate up to five Ca ions and resultant insoluble Ca-phytate complexes are resistant to enzymatic hydrolysis by phytases (Selle *et al.*, 2009). In vitro studies have demonstrated that the precipitated phytate with Ca was not degraded by exogenous phytase enzyme (Tamim and Angel, 2003). In vivo studies demonstrated lower dietary Ca

concentrations to around 5 g/kg resulted in increased P availability to >70% from phytate-P at the distal ileum (Tamim *et al.*, 2004). Shirley and Edwards (2002) reported that Ca negatively impacted phytase efficacy even at higher inclusion rate of 6000 FTU/kg due to high dietary Ca concentrations.

A body of published literature on phytic acid and phytase has provided understanding of both dietary phytate levels and its interaction with dietary cations concentration, particularly Ca which is an important dietary factor that needs to be optimized for achieving a better efficacy of phytase in poultry diets (Tamim *et al.*, 2004; Selle *et al.*, 2009; Cowieson *et al.*, 2011; Walk, 2016). The major dietary factors that has the greatest impact on phytase efficacy includes: dietary Ca and P concentrations, Ca to P ratio, the source and the Ca particle size, and together they constitute an area for active research in poultry nutrition (Driver *et al.*, 2005; Amerah *et al.*, 2014; Anwar *et al.*, 2016; Bradbury *et al.*, 2018).

Dietary Ca: P ratio and Phytase Efficacy

The interrelationship between Ca and P has been extensively studied. Several authors reported improved Ca bioavailability with phytase supplementation (Augspurger and Baker, 2004) through the breakdown of Ca-phytate. The positive effect of narrow Ca: P ratio on phytase efficacy using growth performance is well demonstrated. Olukosi and Fru-Niji (2014a) reported that the best response to lower phytase (1000 FYT/kg) supplementation are with diets containing narrow Ca: tP (2.0: 1) ratio. The high inclusion of phytase (2000 FYT/kg) supplemented to the wider Ca: tP (2.5: 1) ratio ameliorates the negative effect of wide Ca: P ratios to some extent.

Qian *et al.* (1997) showed that the maximum retention of P (51.8 to 57.3%) occurred when dietary Ca: tP ratio was formulated to 1.1: 1 vs. 2.0: 1 with 600 Unit/kg phytase, similarly greater retention of Ca (44.3 to 62.0%) was observed at 1.1: 1. Phytase efficacy maximized

(12.2%) at 1.4: 1 Ca: tP ratio. The study concluded that dietary Ca: tP ratios between 1.1: 1 to 1.4: 1 to be critical for efficiency of phytase in a 21-d broiler study.

Plumstead *et al.* (2008) showed that increasing dietary Ca concentration (4.7 to 11.6 g/kg) decreased phytate-P digestibility coefficient by 71 percentage units. Li *et al.* (2016), evaluated the effects of two dietary Ca concentrations and 3 phytase levels (0, 500 and 1000 FTU/kg) and reported decreased ileal IP₆ disappearance at a higher Ca concentration (10 g/kg) vs. a lower Ca level (7 g/kg).

Delezie *et al.* (2012) observed that phytate-P released greater P (0.21 to 0.27% P/kg) at Ca: tP ratio of 1: 20 vs. 1.6: 1 and retention of Ca was improved (33 to 48%) with 2000 FTU/kg of phytase supplementation in a 39-d broiler study. The high dietary Ca concentrations reduced P availability and hydrolysis of phytate-P as a result of insoluble Ca-phytate complexes.

Amerah *et al.* (2014) showed greater phytate-P degradation (75.9 to 88.4%) at Ca: AvP ratio 1.43: 1 vs. 3.57: 1 and, digestibility of ileal P increased (67.6 to 80.4%) with 1000 units/kg of phytase supplementation at 17-d broiler study. The narrower Ca: AvP ratios improved P digestibility.

The reported studies showed that the phytate-P degradation resulted in improved P and Ca digestibility by phytase enzyme and the absolute concentration of Ca and P was less important than their relative proportions. Currently the industry tends to use higher inclusion of phytases in broiler diets.

Interaction of Phytate-P and Phytase on Protein utilization

Like minerals the interaction between inositol-6-phosphate (IP₆) and protein has been well demonstrated (Selle *et al.*, 2012). Knuckles *et al.* (1989) demonstrated that esters of inositol phosphate reduced the pepsin digestion of casein by between 9 and 14 percentage units,

in a vitro study. The availability of dietary amino acids (AAs) were reduced with presence of IP6 in the diets of poultry has been well reported and comprehensively reviewed by Selle *et al.* (2012).

Cowieson *et al.* (2004) reported that the phytate interacts with the endogenous AA and results in the higher intestinal secretion of mucin in presence of IP6 and, these effects were somewhat mitigated with supplementation of phytase in the diets. Cowieson *et al.* (2006) demonstrated that in presence of IP6 the casein nitrogen utilization and its digestibility of amino acids were decreased and the supplementation of phytase improved the AA digestibility. The data suggested that IP6 resulted in greater endogenous secretion, catabolism and excretion of AA and phytase reduces the interaction between IP₆ and the epsilon amino group (α -NH₂) of AA. However, a detrimental effect of 2000 units/kg of phytase on N utilization of casein was shown in the same study, which could be due the increased endogenous secretion and the first pass catabolism leading to N removal due to imbalance. In a later study Cowieson *et al.* (2011) demonstrated similar stimulatory effects of IP6 on endogenous ileal amino acid flow and described the interaction between sodium (Na) and phytase in reducing endogenous AA ileal flow of specific individual AAs (notably aromatic AA) that would reduce the inimical effect of IP₆. The study concluded that Na, IP6 and phytase influence endogenous AA flow in broiler chickens.

Sebastian *et al.* (1997) reported the improvements in apparent ileal digestibility (AID) of methionine and phenylalanine with phytase supplementation in 21-d broilers. Dilger *et al.* (2004) reported that there was an increase in digestibility of tryptophan and valine with supplementation of 500 units/kg of phytase in 14 d old broilers. Cowieson *et al.* (2006) reported that the supplementation of dietary phytase increased the digestibility of valine (Val), threonine (Thr), and iso-leucine (Ile). Chung *et al.* (2013) reported improved digestibility of several AA;

glycine (Gly), tyrosine (Tyr), histidine (His) and lysine (Lys) as a function of phytase dose response (1500 to 3000 units/kg). Similarly, Rutherford *et al.* (2012) reported increases in AA digestibility of up to 14%. In a recent publish review by Cowieson *et al.* (2017) reported that phytase improved digestibility of all essential and non-essential AA by an average of 4.1% over the P inadequate diets.

The protein-phytate complexes that are formed in the acidic region of the gastrointestinal tract are refractory to pepsin hydrolysis. This may be the main reason, whereby phytate bound to amino acid forms complexes which are not available for further hydrolysis. However, phytase enzyme up to a certain extent is capable of hydrolyzing the protein-phytate complexes and release the AA (Selle *et al.*, 2012; Cowieson *et al.*, 2017).

LIMESTONE PARTICLE SIZE EFFECTS IN BROILER DIETS

Limestone is the major inorganic Ca source used in poultry diets. Dietary Ca and P concentrations are critical for the absorption and post absorptive utilization of Ca and P. In practical feed formulation the proportion of limestone in diets are adjusted according to feed intake. Previous studies have reported that the feeding of coarse limestone rather than fine limestone was beneficial for laying hens and breeder hens (Guinotte and Nys 1991; Rao and Roland., 1990; Ekmay and Coon, 2010). It was evident from the literature that optimum limestone particle size, used as the dietary Ca source, varies between broiler and layer diets due to differences in poultry varietal-requirements of dietary Ca and P and growth rates (Bradbury *et al.*, 2018). There are limited numbers of research reports that have evaluated different particle sizes of limestone for broilers during different successive growth phases.

The Ca from limestone must be solubilized in the gastrointestinal tract before absorption. The gastrointestinal (GI) pH is one of the main factors that regulates the solubility of dietary limestone. The particle size of limestone relates to the solubility, usually, an in vitro

measure of dissociation in a dilution of hydrochloric acid over a given time period which is used for estimation of limestone solubility. Zhang and Coon (1997) reported that the larger particles of limestone >0.8 mm, have lower solubility and retained for a longer period of time in the gizzard of the birds. Rao and Roland (1989) reported the inverse relationship between *in vitro* solubility of limestone is highly correlated with the *in vivo* solubility. Anwar (2017), demonstrated that there was an increased ventriculus Ca content when larger limestone particle size (>1 mm) were fed and that was associated with greater ileal Ca digestibility when compared with fine limestone particles (<0.5 mm). The aforementioned studies in broilers indicated that larger limestone particles of limestone had greater *in vivo* solubility, which was positively correlated with Ca digestibility (Anwar, 2016). Van der Klis et al. (1993) demonstrated that the concentration of small osmo-active molecules in jejunum are critical for absorption. However, high concentration of dietary Ca feeding in birds has a negative impact on the bioavailability of mineral and nutrients. Hurwitz and Bar (1968) demonstrated that the pH in intestinal tract of laying hen showed a remarkable adjustment within 10-20 minutes. The pH in the ileum was regulated efficiently to maintain towards a basic pH, compared to the jejunum. Shafey *et al.* (1991) reported that the increase in dietary Ca concentration (1.1 to 2.5%) resulted in increased GI tract pH which affected the mineral complexes size thus decreased the solubility of minerals. The solubilized Ca from limestone at a low gastric pH can form insoluble calcium-phosphates in the intestinal lumen which reduces the P availability resulting in the lack of counter ion P necessary to utilize Ca thus increases the Ca excretion (Morrissey and Wasserman, 1971; Walk *et al.* 2012).

Recently, Merriman and Stein (2016) reported that the limestone particle size in the range from 200 to 1,125 μm can be used in pigs. However, the study found no differences in growth performance or Ca and P retention when different limestone particle sizes were fed.

A body of published literature on laying hens have reported significant effects of limestone particle size on egg shell quality (Rao and Roland, 1989, 1990; Guinotte and Nys 1991; Zhang and Coon, 1997;). Guinotte and Nys (1991) reported a higher feed intake, BW and greater bone breaking strength in laying hens fed diets with large (>1.18) Ca particles however, no differences were observed in ionized blood Ca and Ca retention. Rao and Roland (1989) reported that percentage of Ca solubilized in the digestive tract was increased when fed diets with larger Ca particles size (2 to 5 mm) compared to smaller Ca particles (0.5 to 0.8 mm). The studies suggested that large particles of Ca were retained longer in the gizzard (ventriculus) of the birds which increased the *in vivo* solubility (Zhang and Coon, 1997). Rao and Roland (1990) reported that Ca particle size influences Ca solubilization in the bird's digestive tract and dietary Ca level and particle size interact with each other to influence *in vivo* Ca source solubilization and Ca retention in laying hens.

However, only few studies have investigated the effect of limestone particle size on broilers compared to laying hens. Guinote *et al.* (1991) reported that there were no benefits in broiler performance when dietary coarse particle limestone was fed and reported that the finely ground limestone to less than 150 μm increased tibia length and was optimum for 28-d broilers. Whereas, McNaughton *et al.* (1981) observed greater tibia ash percentage units with coarse limestone particle size (250 to 840 μm) fed from 1 to 21 d of age for broilers. The recent studies by Anwar *et al.* (2016) reported that coarse limestone particle size (1-2 mm) improved Ca digestibility coefficient (0.71 vs. 0.43) when compared to fine limestone particle size (<0.5 mm). Same author (Anwar, 2017) reported that the true ileal Ca digestibility coefficients of limestone samples varied from 0.56 to 0.62 and the dietary lower dietary P increased the average ileal Ca digestibility of limestone from 0.57 to 0.61. The study's data suggested that

there are species differences associated with feeding of different limestone particle size in animal production.

Phytase enzyme is widely used in poultry diets and dietary limestone particle size is known to have an effect on phytase action. The use of medium to coarse particle limestone as Ca source was shown to be beneficial for phytase efficacy (Selle and Ravindran, 2007). Larger limestone particle size due to its relatively lower solubility when compared to fine limestone particles allows a slow release of Ca thus forming potentially lesser Ca-phytate complexes may improve phytase efficacy. Bradbury *et al.* (2016) reported the negative effects of highly soluble Ca sources on broiler performance. Manangi and Coon (2007) reported that the fine limestone particle size (28 μm) with high solubility (>70%) inhibited phytase action on phytate-P hydrolysis due to Ca-phytate complexes. This study suggested that the limestone particle sizes between 137 μm (56.4% solubility) to 388 μm (53% solubility) with supplementation of phytase 500 units/kg of phytase increased bone mineralization and growth performance in 28-d broilers. Bradbury *et al.* (2018) reported 1000 units/kg of phytase supplemented to diets containing coarse limestone particle size (>500 μm) improved foot ash content, Ca and P availability.

There are limited reported studies on limestone particle size interaction with phytase in broiler nutrition and the nutritional findings in laying hens may not be relevant to the broilers. Therefore, further studies on limestone particle size interaction with dietary Ca, P and phytase in broiler nutrition is required.

RATIONALE

In preparation for the egg production, broiler breeder pullets undergo complex changes in mineral metabolism and regardless of diet formulations wet feces is a frequent problem. It is evident that the onset of egg production plays an important role in water balance. The role of dietary minerals is not well understood during the onset of lay period. The change from grow to lay diets nutrient specifications affects a major change in Ca levels, almost 3-fold increase in Ca concentration occurs. There could be an onset of hypercalcemia, it is possible that Ca may influence water balance. Previous research demonstrated the transitory effect of dietary Ca on excreta moisture (Leeson and Summers, 1987). Onset of lay is associated with decreased excretion of Ca and with increased excretion of P. During the onset of lay period all birds in a flock do start laying at same time however, in practice diets are fed with similar Ca and P concentration, thus dietary excesses are obvious. Egg-shell formation also results in transitory acid-base changes, the role of K a dietary electrolyte is known to exert an alkaline effect. Thus, K ions are involved in electrolyte and water homeostasis. The water content of ileum is not different than the voided urine and feces which implies the efficiency of water conservation by the kidney and the cloaca (Skadhauge, 1981). Larsen *et al.* (2006) reported that solute-solvent coupling a major feature of physiological water handling by kidney is governed by Na^+/K^+ pump. The isotonic transport depends on ATP consumption by the Na^+/K^+ pump and the large component of fluid absorption is due to solute-solvent coupling where K^+ is one of the major forces for water reabsorption. The pre-lay period is difficult to define due to a large spread in maturation and onset of egg production among the individual hens in a flock. Therefore, it was thought prudent to develop an understanding of the effects of dietary phosphorus and potassium concentration on individual broiler breeder pullets coming into lay on excreta moisture and egg characteristics.

In the broilers, use of exogenous phytase and limestone as dietary calcium (Ca) source has been well defined. However, scant information is available on limestone particle size and its interaction with potassium on physiological parameters and the nutritional effect of limestone particle size interaction with phytase enzyme. Dietary composition and chemical properties as well as bird physiology related factors determine the nutrient digestibility. With recent improvements in broiler growth rates there is an increased occurrence of broiler breast myopathies. Higher growth rate is also associated with the production of H ions, physiological compensations through dietary manipulations with dietary electrolytes like, K sources have not been explored much. Broiler diets are formulated with a certain DEB value however, DEB cannot solely be used to evaluate effects of the different mineral sources. Hence, dietary manipulation with an weak electrolyte salt is necessary to discern meaningful inferences. The dietary potassium concentration affects the blood physiology have been studied in relation to the heat stress conditions. While the metabolic rates of broilers have increased, the related literature shows that possibly acid-base balance imbalance occurs and that could be detected in physiological measurements of the live broilers. Further, coarse dietary particles are known to stimulate gizzard function and presumably alter the duodenal pH, the improved nutrient digestibility has been attributed to this phenomenon. Information on dietary limestone particle size effect and its interaction with phytase on broiler performance and nutrient digestibility is scant. Dietary coarse limestone particles are known to alter solubility and release of the Ca could be presumably be beneficial for phytase efficacy. Possible dietary strategies intended to improve physiological and nutritional parameters in relation to fast growing broilers would lend itself to such experimental work.

OBJECTIVES

1. Evaluate the fecal moisture, fecal liquid portion and egg characteristics in response to altered levels of dietary grow phosphorus, lay phosphorus with or without addition of potassium in broiler breeder hens during onset of lay.
2. Determine the effect of limestone particle size and potassium in broiler diets that would elicit a live performance response and changes in the blood physiology parameters.
3. Determine the efficacy of limestone particle size and its interaction with dietary levels of available phosphorus and phytase in broilers on live performance, bone mineralization and nutrient digestibility.

REFERENCES

- Adedokun, S. A., and O. Adeola. 2013. Calcium and phosphorus digestibility: Metabolic limits. *Poult. Sci.* 22:600-608.
- Ahmad, T., T. Khalid, T. Mushtaq, M. A. Mirza, A. Nadeem. M. E. Babar, and G. Ahmad. 2008. Effect of potassium chloride supplementation in drinking water on broiler performance under heat stress conditions. *Poult. Sci.* 87:1276-1280.
- Ait-Boulahsen, A., J. D. Garlich, and F. W. Edens. 1989. Effect of fasting and acute heat stress on body temperature, blood acid-base and electrolyte status in chickens. *Comp. Biochem. Physiol.* 94:683-687.
- Ait-Boulahsen, A., J. D. Garlich, and F. W. Edens. 1995. Potassium chloride improves the thermotolerance of chickens exposed to acute heat stress. *Poult. Sci.* 74:75-87.
- Amerah, A. M., P. W. Plumstead, L. P. Barnard, and A. Kumar. 2014. Effect of calcium level and phytase addition on ileal phytate degradation and amino acid digestibility of broilers fed corn-based diets. *Poult. Sci.* 93:906-915.
- Anderson, J. J. B. Nutritional biochemistry of calcium and phosphorus. 1991. *J. Nutr. Biochem.* 2:300-307.
- Angel, R., N. M. Tamim, T. J. Applegate, A. S. Dhandu, and L. E. Ellestad. 2002. Phytic acid chemistry: influence on phytin-phosphorus availability and phytase efficacy. *J. Appl. Poult. Res.* 11:471-480.
- Angel, R., W. W. Saylor, A. S. Dhandu, W. Powers, and T. J. Applegate. 2005. Effects of dietary phosphorus, phytase, and 25-hydroxycholecalciferol on performance of broiler chickens grown in floor pens. *Poult. Sci.* 84:1031-1044.
- Angel, R. 2011. Calcium and phosphorus requirements in broilers. *Proc. Intl. Symp. Nutritional Requirements of Poultry and Swine. Viçosa, Minas Gerais, Brazil.* 3:77-96.

- Anwar, M. N., V. Ravindran, P. C. H. Morel, G. Ravindran, and A. J. Cowieson. 2016. Effect of limestone particle size and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. *Br. Poult. Sci.* 57:707-713.
- Anwar, M. N. 2017. Measurement of true ileal calcium digestibility of feed ingredients for broiler chicks. Dissertation. Massey University, Palmerston north, New Zealand.
- Applegate, T. J., R. Angel, and H. L. Classen. 2003. Effect of dietary calcium, 25-hydroxy-cholecalciferol, or bird strain on small intestinal phytase activity in broiler chickens. *Poult. Sci.* 82:1140-1148.
- Applegate, T., and R. Angel. 2008. Phosphorus requirements for poultry. *Anim. Sci. Purdue Uni. Coop. Ext. Ser.* West Lafayette. IN.
- Augspurger, N. R., and D. H. Baker. 2004. Phytase improves dietary calcium utilization in chicks, and oyster shell, carbonate, citrate, and citrate-malate forms of calcium are equally bioavailable. *Nutr. Res.* 24:293–301.
- Aureli, R., M. U. Faruk, I. Cechova, P. B. Pedersen, S. G. Elvig-Joer, F. Fru, and J. Broz. 2011. The efficacy of a novel microbial 6-phytase expressed in *aspergillus oryzae* on the performance and phosphorus utilization in broiler chickens. *Int. J. Poult. Sci.* 10:160-168.
- Bar, A., U. Eisner, G. Montecuccoli, and S. Hurwitz. 1976. Regulation of intestinal calcium absorption in the laying quail: independent of kidney vitamin D hydroxylation. *J. Nutr.* 106:1336-1342.
- Bar A., and S. Hurwitz. 1979. The interaction between dietary calcium and gonadal hormones in their effect on plasma calcium, bone, 25hydroxycholecalciferol-1-hydroxylase and duodenal calcium binding protein, measured by radioimmunoassay in chicks. *Endocrinol.* 104:1455-1460.

- Bar, A., and S. Hurwitz. 1984. Egg shell quality, medullary bone ash, intestinal calcium and phosphorus absorption, and calcium-binding protein in phosphorus-deficient hens. *Poult. Sci.* 63:1975-1979.
- Bar, A. 2009. Differential regulation of calbindin in the calcium-transporting organs of birds with high calcium requirements. *J. Poult. Sci.* 46:267-285.
- Bedford, M. R., C. L. Walk, and H. V. Masey O'Neill. 2016. Assessing measurements in feed enzyme research: Phytase evaluations in broilers. *J. Appl. Poult. Res.* 25:305-314.
- Bedford, M., and X. Rousseau. 2017. Recent findings regarding calcium and phytase in poultry nutrition. *Anim. Prod. Sci.* 57:2311-2316.
- Belay, T., C. J. Wiernusz, and R. G. Teeter. 1992. Mineral balance and urinary and fecal mineral excretion profile of broilers housed in thermoneutral and heat-distressed environments. *Poult. Sci.* 71:1043-1047.
- Belay, T., C. J. Wiernusz, and R. G. Teeter. 1993. Broiler water balance and thermobalance during thermoneutral and high ambient temperature exposure. *Poult. Sci.* 72:116-124.
- Belay, T., and R. G. Teeter. 1996. Effects of environmental temperature on broiler mineral balance partitioned into urinary and fecal loss. *Br. Poult. Sci.* 37:423-33.
- Berndt, T. J., S. Schiavi, and R. Kumar. 2005. "Phosphatonins" and the regulation of phosphorus homeostasis. *Am. J. Physiol. Renal Physiol.* 289:1170-1182.
- Berndt, T., and R. Kumar. 2007. Phosphatonins and the regulation of phosphate homeostasis. *Annu. Rev. Physiol.* 69:341-359.
- Berndt, T., and R. Kumar. 2009. Novel mechanisms in the regulation of phosphorus homeostasis. *Physiol.* 24:17-25.
- Berner, W., R. Kinne, and H. Murer. 1976. Phosphate transport into brush-border membrane vesicles isolated from rat small intestines. *Biochem. J.* 160:467-474.

- Biber, J., N. Hernando, and I. Forster. 2013. Phosphate transporters and their function. *Annu. Rev. Physiol.* 75:535-550.
- Bloom, M. A., L. V. Domm, A. V. Nalbandov, and W. Bloom. 1958. Medullary bone of laying chickens. *Am. J. Anat.* 102:411-453.
- Borges, S. A., Fischer da Silva, A V, J. Ariki, D. M. Hooge, and K. R. Cummings. 2003. Dietary electrolyte balance for broiler chickens exposed to thermoneutral or heat-stress environments. *Poult. Sci.* 82:428-435.
- Borges, S. A., B., A. V. Fischer da Silva, A. S. A. A. M. T. Moura, and A. Maiorka, and A. Ostrensky. 2004. Electrolyte balance in broiler growing diets. *Int. J. of Poult. Sci.* 3:623-628.
- Borges, S. A., A. V. Fischer da Silva, and A. Maiorka. 2007. Acid-base balance in broilers. *World's Poult. Sci. J.* 63:73-81.
- Bottje, W. G., and P. C. Harrison. 1985. The effect of tap water, carbonated water, sodium bicarbonate, and calcium chloride on blood acid-base balance in cockerels subjected to heat stress. *Poult. Sci.* 64:107-113.
- Bradbury, E. J., S. J. Wilkinson, G. M. Cronin, P. Thomson, C. L. Walk, and A. J. Cowieson. 2016. Evaluation of the effect of a highly soluble calcium source in broiler diets supplemented with phytase on performance, nutrient digestibility, foot ash, mobility and leg weakness. *Anim. Prod. Sci.* 57:2016-2026.
- Bradbury, E. J., S. J. Wilkinson, G. M. Cronin, C. L. Walk, and A. J. Cowieson. 2018. Effects of phytase, calcium source, calcium concentration and particle size on broiler performance, nutrient digestibility and skeletal integrity. *Anim. Prod. Sci.* 58:271.
- Braun, E. J. 2003. Regulation of renal and lower gastrointestinal function: role in fluid and electrolyte balance. *Comp. Biochem. Physiol.* 136:499-505.

- Cabahug, S., V. Ravindran, P. H. Selle, and W. L. Bryden. 1999. Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorus contents. I. Effects on bird performance and toe ash. *Br. Poult. Sci.* 40:660-666.
- Cheryan, M. 1980. Phytic acid interactions in food systems. *C.R.C. Crit. Rev. Food Sci. Nutr.* 13:297-335.
- Chun, S., T. Bamba, T. Suyama, T. Ishijima, E. Fukusaki, K. Abe, and Y. Nakai. 2016. A High Phosphorus Diet Affects Lipid Metabolism in Rat Liver: A DNA Microarray Analysis. *PLOS.* 11:1553-586.
- Choi, J. H., R. D. Miles, and R. H. Harms. 1979. The response of serum inorganic phosphorus level in laying hens fed low levels of dietary phosphorus. *Poult. Sci.* 58:416-418.
- Chung, T. K., S. M. Rutherford, D. V. Thomas, and P. J. Moughan. 2013. Effect of two microbial phytases on mineral availability and retention and bone mineral density in low-phosphorus diets for broilers. *Br. Poult. Sci.* 54:362-12.
- Cohen, I., and S. Hurwitz. 1974. The response of blood ionic constituents and acid-base balance to dietary sodium, potassium and chloride in laying fowls. *Poult. Sci.* 53:378-383.
- Colvero, L., A. Carrijo, R. Garófallo, R. Bernardi, R. Steffen, and C. Stefanello. 2014. Production aspects of broiler breeders submitted to different drinker types. *Braz. J. Poult. Sci.* 16:61-65.
- Coon, C. N., S. SEO, and M. K. Manangi. 2007. The determination of retainable phosphorus, relative biological availability, and relative biological value of phosphorus sources for broilers. *Poult. Sci.* 86:857-868.
- Copp, D. H. 1972. Calcium regulation in birds. *Gen. Comp. Endocrinol.* 3:441-447.

- Cosgrove, D. J. 1966. The chemistry and biochemistry of inositol polyphosphates. *Rev. Pure Appl. Chem.* 16:209-215.
- Costa, F. G. P., L. R. Rodrigues, C. d. C. Goulart, C. F. S. d. Oliveira, V. P. Rodrigues, and J. H. V. d. Silva. 2011. Nutritional potassium requirement for laying Japanese quails. *R. Bras. Zootec.* 40:2754-2759.
- Cowieson, A. J., T. Acamovic, and M. R. Bedford. 2004. The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. *Br. Poult. Sci.* 45:101-108.
- Cowieson, A. J., and O. Adeola. 2005. Carbohydrases, protease, and phytase have an additive beneficial effect in nutritionally marginal diets for broiler chicks. *Poult. Sci.* 84:1860-1867.
- Cowieson, A. J., T. Acamovic, and M. R. Bedford. 2006. Phytic acid and phytase: implications for protein utilization by poultry. *Poult. Sci.* 85:878-885.
- Cowieson, A. J., and V. Ravindran. 2007. Effect of phytic acid and microbial phytase on the flow and amino acid composition of endogenous protein at the terminal ileum of growing broiler chickens. *Br. J. Nutr.* 98:745:752.
- Cowieson, A. J., and M. R. Bedford, P. H. Selle, and V. Ravindran. 2009. Phytate and microbial phytase: Implications for endogenous nitrogen losses and nutrient availability. *World's. Poult. Sci. J.* 65:401-418.
- Cowieson, A. J., M. R. Bedford, V. Ravindran, and P. H. Selle. 2011a. Increased dietary sodium chloride concentrations reduce endogenous amino acid flow and influence the physiological response to the ingestion of phytic acid by broiler chickens. *Br. Poult. Sci.* 52:613-624.

- Cowieson, A. J., P. Wilcock, and M. R. Bedford. 2011b. Super-dosing effects of phytase in poultry and other monogastrics. *World's Poult. Sci. J.* 67:225-236.
- Cowieson, A. J., R. Aureli, P. Guggenbuhl, and F. Fru-Nji. 2015. Possible involvement of myo-inositol in the physiological response of broilers to high doses of microbial phytase. *Anim. Prod. Sci.* 55:710-719.
- Cowieson, A. J., J. P. Ruckebusch, I. Knap, P. Guggenbuhl, and F. Fru-Nji. 2016. Phytate-free nutrition: A new paradigm in monogastric animal production. *Anim. Feed. Sci. Technol.* 222:180-189.
- Cowieson, A. J., J. -. Ruckebusch, J. O. B. Sorbara, J. W. Wilson, P. Guggenbuhl, and F. F. Roos. 2017. A systematic view on the effect of phytase on ileal amino acid digestibility in broilers. *Anim. Feed. Sci. Technol.* 225:182-194.
- Dacke, C. G., S. Arkle, D. J. Cook, I. M. Wormstone, S. Jones, M. Zaidi, and Z. A. Bascal. 1993. Medullary bone and avian calcium regulation. *J. Exp. Biol.* 184:63-88.
- Danisi, G., H. Murer, and R. W. Straub. 1984. Effect of pH on phosphate transport into intestinal brush-border membrane vesicles. *Am. J. Physiol.* 246:180-186.
- Danisi, G., and H. Murer. 1991. Inorganic phosphate absorption in small intestine. In: *Handbook of physiology*. Chap. 12. P 323-336. Am. Physiol. Soc. Bethesda, MD.
- Davison, S., and R. F. Wideman. 1992. Excess sodium bicarbonate in the diet and its effect on Leghorn chickens. *Br. Poult. Sci.* 33:859-870.
- Davis, B. D. 1958. Importance of being ionized. *Arch. Biochem. Biophys.* 78:497-509.
- Diaz, R., S. Hurwitz, N. Chattopadhyay, M. Pines, Y. Yang, O. Kifor, M. S. Einat, R. Butters, S. C. Hebert, and E. M. Brown. 1997. Cloning, expression, and tissue localization of the calcium-sensing receptor in chicken (*Gallus domesticus*). *Am. J. Physiol.* 273:1008-1016.

- Delezie, E., L. Maertens, and G. Huyghebaert. 2012. Consequences of phosphorus interactions with calcium, phytase, and cholecalciferol on zootechnical performance and mineral retention in broiler chickens. *Poult. Sci.* 91:2523-2531.
- Dilger, R. N., E. M. Onyango, J. S. Sands, and O. Adeola. 2004. Evaluation of microbial phytase in broiler diets. *Poult. Sci.* 83:962-970.
- Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Edwards. 2005. Effects of calcium and nonphytate phosphorus concentrations on phytase efficacy in broiler chicks. *Poult. Sci.* 84:1406-1417.
- Dumont, E. R. 2010. Bone density and the lightweight skeletons of birds. *Proc. R. Soc. B.* 277:2193-2198.
- Ekmay, R. D., and C. N. Coon. 2010. An Examination of the P Requirements of Broiler Breeders for Performance, Progeny Quality and P Balance 1. Non-phytate Phosphorus. *Int. J. Poult. Sci.* 9:1043-1049.
- Ekmay, R. D. 2011. Protein Utilization and Requirements in Broiler Breeders. Dissertation. University of Arkansas.
- Ekmay, R. D., C. Salas, J. England, S. Cerrate, and C. N. Coon. 2012. The effects of pullet body weight, dietary nonphytate phosphorus intake, and breeder feeding regimen on production performance, chick quality, and bone remodeling in broiler breeders. *Poult. Sci.* 91:948-964.
- Engelen, A. J., F. C. van der Heeft, P. H. G. Randsdorp, and E. L. C. Smit. 1994. Simple and rapid determination of phytase activity. *J. AOAC. Int.* 77:730-764.
- Farrell, D. J. 1998. Strategies to improve the nutritive value of rice bran in poultry diets. III. The addition of inorganic phosphorus and a phytase to duck diets. *Br. Poult. Sci.* 39:601-611.

- Favus, M. J., D. A. Bushinsky, and F. L. Coe. 1986. Effects of medium pH on duodenal and ileal calcium active transport in the rat. *Am. J. Physiol.* 251:695-700.
- Favus, M. J., and V. Tembe. 1992. The use of pharmacologic agents to study mechanisms of intestinal calcium transport. *J. Nutr.* 122:683-686.
- Fekry, M. I., P. A. Tipton, and K. S. Gates. 2011. Kinetic consequences of replacing the internucleotide phosphorus atoms in DNA with arsenic. *Chem. Biol.* 6:127-130.
- Forster, I. C., N. Hernando, J. Biber, and H. Murer. 2006. Proximal tubular handling of phosphate: A molecular perspective. *Kidney Int.* 70:1548-1559.
- Francesch, M., and P. A. Geraert. 2009. Enzyme complex containing carbohydrases and phytase improves growth performance and bone mineralization of broilers fed reduced nutrient corn-soybean-based diets. *Poult. Sci.* 88:1915-1924.
- Gaal, T., M. S. Bartlett, W. Ross, C. L. Turnbough Jr., and R. L. Gourse. 1997. Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. *SCIENCE.* 278:2092-2097.
- Gezen, S. S., M. Eren, and G. Deniz. 2005. The effect of different dietary electrolyte balances on eggshell quality in laying hens. *Rev. Med. Vet.* 156:491-497.
- Giral, H., Y. Caldas, E. Sutherland, P. Wilson, S. Breusegem, N. Barry, J. Blaine, T. Jiang, X. X. Wang, and M. Levi. 2009. Regulation of rat intestinal Na-dependent phosphate transporters by dietary phosphate. *Am. J. Physiol. Renal. Physiol.* 279:1466-1475.
- Godwin, J. L., J. L. Grimes, V. L. Christensen, and M. J. Wineland. 2005. Effect of dietary phosphorus and phytase levels on the reproductive performance of large white turkey breeder hens. *Poult. Sci.* 84:485-493.

- Goff, J. P. 2018. Invited review: Mineral absorption mechanisms, mineral interactions that affect acid–base and antioxidant status, and diet considerations to improve mineral status. *J. Dairy Sci.* 101:2763-2813.
- Goldstein, D. L. 2006. Regulation of the avian kidney by arginine vasotocin. *Gen. Comp. Endocrinol.* 147:78-84.
- Goldstein, D. L., and E. Skadhauge. 1999. Renal and extra-renal osmoregulation. In: Whittow, G.C. (Ed.), *Sturkie's Avian Physiology*. Academic Press, pp. 265–297.
- Gonzalez-Vega, J. C., and H. H. Stein. 2014. Calcium digestibility and metabolism in pigs. *Asian-Australas. J. Anim. Sci.* 27:1-9.
- Guinotte, F., and Y. Nys. 1991. Effects of particle size and origin of calcium sources on egg shell quality and bone mineralization in egg laying hens. *Poult. Sci.* 70:583-592.
- Guinotte, F., Y. Nys, and F. de Monredon. 1991. The effects of particle size and origin of calcium carbonate on performance and ossification characteristics in broiler chicks. *Poult. Sci.* 70:1908-1920.
- Guinotte, F., J. Gautron, and Y. Nys. 1995. Calcium solubilization and retention in the gastrointestinal tract in chicks (*Gallus domesticus*) as a function of gastric acid secretion inhibition and of calcium carbonate particle size. *Br. J. Nutr.* 73:125-139.
- Guo, X., K. Huang, F. Chen, J. Luo, and C. Pan. 2008. High Dietary Calcium Causes Metabolic Alkalosis in Egg-Type Pullets. *Poult. Sci.* 87:1353-1357.
- Hall, L. E., R. B. Shirley, R. I. Bakalli, S. E. Aggrey, G. M. Pesti, and H. M. Edwards. 2003. Power of two methods for the estimation of bone ash of broilers. *Poult. Sci.* 82:414-418.

- Hamdi, M., S. López-Vergé, E. G. Manzanilla, A. C. Barroeta, and J. F. Pérez. 2015. Effect of different levels of calcium and phosphorus and their interaction on the performance of young broilers. *Poult. Sci.* 94:2144-2151.
- Hamilton, R. M. G., and B. K. Thompson. 1980. Effects of sodium plus potassium to chloride ratio in practical-type diets on blood gas levels in three strains of white leghorn hens and the relationship between acid-base balance and egg shell strength. *Poult. Sci.* 59:1294-1303.
- Hardin, C. C., and J. A. Knopp. 2013. *Biochemistry: essential concepts*. Oxford University Press. Inc. N. Y.
- Hertelendy, F., and T. G. Taylor. 1961. Changes in the blood calcium associated with egg shell calcification in domestic fowl: 2 changes in the diffusible calcium. *Poult. Sci.* 40:115-123.
- Hodges, R. D., and K. Lorcher. 1967. Possible sources of the carbonate fraction of the egg shell calcium carbonate. *Nature.* 216:609-610.
- Hodges, R. D. 1969. pH and mineral ion levels in the blood of the laying hen (*Gallus Domesticus*) in relation to egg shell formation. *Comp. Biochem. Physiol.* 28: 1243-1257.
- Hodges, R. D. 1970. Blood pH and cation levels in relation to egg-shell formation. *Annal. Biol. Anim. Biochem. Biophysic.* 10:199-213.
- Hofer, A. M., and E. M. Brown. 2003. Extracellular calcium sensing and signaling. *Nat. Rev. Mol. Cell Biol.* 4:530–538.
- Hopkinson, W. I., D. Jessop, D. A. Pass, and D. W. Pethick. 1990. Concentrations of plasma potassium and sodium during the life of a broiler breeder flock. *Avian Pathol.* 19:607-611.

- Hooge, D. M. 2003. Dietary sodium bicarbonate and electrolyte balance for broiler and breeder chickens. PAS. Hooge Consulting Service, Inc. 8775 North Cedar Pass Road Eagle Mountain, and Utah 84043 USA danhooge@fiber.net.
- Huber, K., E. Zeller, and M. Rodehutschord. 2015. Modulation of small intestinal phosphate transporter by dietary supplements of mineral phosphorus and phytase in broilers. *Poult. Sci.* 94:1009-1017.
- Hunter, T. 2012. Why nature chose phosphate to modify proteins. *Phil. Trans. R. Soc. B.* 367:2513-2516.
- Hurwitz, S., and P. Griminger. 1961. Partition of calcium and phosphorus excretion in the laying hen. *Nature.* 189:759-760.
- Hurwitz, S. 1964. Estimation of net phosphorus utilization by the slope method. *J. Nutr.* 84:83-91.
- Hurwitz, S. 1964. Bone composition and Ca retention in fowl as influenced by egg formation. *Am. J. Physiol.* 206:198-204.
- Hurwitz, S., and A. Bar. 1965. absorption of calcium and phosphorus along the gastrointestinal tract of the laying fowl as influenced by dietary calcium and egg shell formation. *J. Nutr.* 86:433-438.
- Hurwitz, S., and A. Bar. 1968. Regulation of pH in the intestine of the laying fowl. *Poult. Sci.* 47:1029-1030.
- Hurwitz, S., and A. Bar. 1970. The sites of calcium and phosphorus absorption in the chick. *Poult. Sci.* 49:324-325.
- Hurwitz, S., and A. Bar. 1971. The effect of pre-laying mineral nutrition on the development, performance and mineral metabolism of pullets. *Poult. Sci.* 50:1044-1055.

- Hurwitz, S., D. Dubrov, U. Elsner, G. Risenfeld, and A. Bar. 1978. Phosphate absorption and excretion in the young turkey as influenced by calcium intake. *J. Nutr.* 108:1329-1335
- Hurwitz, S., S. Fishman, and H. Talpaz. 1987. Model of plasma calcium regulation: System oscillations induced by growth. *Am. J. Physiol.* 252:1173-1181.
- Hurwitz, S. 1989. Calcium homeostasis in birds. *Vitam. Horm.* 45:173–221.
- Hu, Y., X. Liao, Q. Wen, L. Lu, L. Zhang, and X. Luo. 2018. Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. *Br. J. Nutr.* 119:1346-1354.
- Jack El, M. H., and P. E. Lake. 1967. The content of the principal inorganic ions and carbon dioxide in uterine fluids of the domestic hen. *J. Reprod. Fertil.* 13:127-132.
- Jonchère, V., A. Brionne, J. Gautron, and Y. Nys. 2012. Identification of uterine ion transporters for mineralization precursors of the avian eggshell. *BMC Physiol.* 12:10.
- Jongbloed, A. W., Z. Mroz, and P. A. Kemme. 1992. The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. *J. Anim. Sci.* 70:1159.
- Kerschnitzki, M., T. Zander, P. Zaslansky, P. Fratzl, R. Shahar, and W. Wagermaier. 2014. Rapid alterations of avian medullary bone material during the daily egg-laying cycle. *Bone.* 69:109-117.
- Kiela, P. R., and F. K. Ghishan. 2009. Recent advances in the renal-skeletal-gut axis that controls phosphate homeostasis. *Lab. Invest.* 89:7–14.
- Knuckles, B. E., D. D. Kuzmicky, M. R. Gumbmann, and A. A. Betschart. 1989. Effect of myo-inositol phosphate esters on in vitro and in vivo digestion of protein. *J. Food Sci.* 54:1348–1350.

- Kumar, R. 1997. Phosphatonin-a new phosphatoretic hormone? (lessons from tumor-induced osteomalacia and X-linked hypophosphataemia). *Nephrol. Dial. Transplant.* 12:11-13.
- Larsen, E.H., N. Mobjerg, and J. N. Sorensen. 2006. Fluid transport and ion fluxes in mammalian kidney proximal tubule: a model analysis of isotonic transport. *Acta Physiol.* 187:177-189.
- Lavery, G., and E. Skadhauge. 1999. Physiological roles and regulation of transport activities in the avian lower intestine. *J. Exp. Zoo.* 283:480-494.
- Leeson, S., and J. D. Summers. 1987. Effect of dietary calcium levels near the time of sexual maturity on water intake and excreta moisture content. *Poult. Sci.* 66:1918-1923.
- Leeson, S., and J. D. Summers. 1991. *Commercial Poultry Nutrition*, Guelph: University Books.
- Leske, K. L., and C N Coon. 1999. A bioassay to determine the effect of phytase on phytate phosphorus hydrolysis and total phosphorus retention of feed ingredients as determined with broilers and laying hens. *Poult. Sci.* 78:1151-1157.
- Leske, K., and C. Coon. 2002. The development of feedstuff retainable phosphorus values for broilers. *Poult. Sci.* 81:1681-1693.
- Létourneau-Montminy, M. P., A. Narcy, P. Lescoat, J. F. Bernier, M. Magnin, C. Pomar, Y. Nys, D. Sauvant, and C. Jondreville. 2010. Meta-analysis of phosphorus utilization by broilers receiving corn-soybean meal diets: influence of dietary calcium and microbial phytase. *Animal.* 4:1844-1853.
- Leytem, A. B., B. P. Willing, and P. A. Thacker. 2008. Phytate utilization and phosphorus excretion by broiler chickens fed diets containing cereal grains varying in phytate and phytase content. *Anim. Feed Sci. Technol.* 146:160-168.

- Lind, J., B. G. Munck, and O. Olsen. 1980. Effects of dietary intake of sodium chloride on sugar and amino acid transport across isolated hen colon. *J. Physiol.* 305:327-336.
- Li, J., J. Yuan, Y. Guo. 2012. The influence of dietary calcium and phosphorus imbalance on intestinal NaPi-iiib and calbindin mRNA expression and tibia parameters of broilers. *Asian-Australas. J. Anim. Sci.* 25:552–558.
- Li, W., R. Angel, S. -W. Kim, E. Jiménez-Moreno, M. Proszkowiec-Weglarz, and P. W. Plumstead. 2015. Impact of response criteria (tibia ash weight vs. percent) on phytase relative non phytate phosphorus equivalence. *Poult. Sci.* 94:2228-2234.
- Li, X., D. Zhang, T. Y. Yang, and W. L. Bryden. 2016. Phosphorus bioavailability: A key aspect for conserving this critical animal feed resource with reference to broiler nutrition. *Agriculture.* 6:1-15.
- Li, X., X. Li, J. Wang, C. Wang, C. Zhang, C. Tang, and X. Wei. 2016. Effect of dietary phosphorus levels on meat quality and lipid metabolism in broiler chickens. *Food Chem.* 205:289-296.
- Liebert, F., J. K. Htoo, and A. Sünder. 2008. Microbial phytase and nutrient utilization in low phosphorus chicken diets. *Jpn. Poult. Sci.* 45:255-264.
- Long, S., and E. Skadhauge. 1983. Renal acid excretion in the domestic fowl. *J. Exp. Biol.* 104:51-58
- Maenz, D. D., and H. L. Classen. 1998. Phytase activity in the small intestinal brush border membrane of the chicken. *Poult. Sci.* 77:557-563.
- Maenz, D. D. 2000. Enzymatic characteristics of phytases as they relate to their use in animal feeds. Pages 61-84 in *Enzymes in Farm Animal Nutrition*. CABI. Wallingford. UK.
- Maguire, R. O., P. W. Plumstead, and J. Brake. 2005. Impact of diet, moisture, location, and storage on soluble phosphorus in broiler breeder manure. *J. Environ. Qual.* 35:858-865.

- Manangi, M. K., and C. N. Coon. 2007. The effect of calcium particle size and solubility on the utilization of phosphorus from phytase for broilers. *Int. J. Poult. Sci.* 6:85-90.
- Manangi, M. K., F. D. Clark, and C. N. Coon. 2007. Improved colostomy technique and excrement (urine) collection device for broilers and broiler breeder hens. *Poult. Sci.* 86:698-704.
- Manangi M. K., and C. N. Coon. 2008. Phytate phosphorus hydrolysis in broilers in response to dietary phytase, calcium, and phosphorus concentrations. *Poult. Sci.* 87:1577-1586.
- Manangi, M. K., P. Maharjan, and C. N. Coon. 2018. Calcium particle size effects on plasma, excreta, and urinary Ca and P changes in broiler breeder hens. *Poult. Sci.* 97:2798-2806.
- Manghat, P., R. Sodi, and R. Swaminathan. 2014. Phosphate homeostasis and disorders. *Annal. Clin. Biochem.* 0:1-26.
- Martin, E. A. 1998. Strategies to improve the nutritive value of rice bran in poultry diets. IV. Effects of addition of fish meal and a microbial phytase to duckling diets on bird performance and amino acid digestibility. *Br. Poult. Sci.* 39:612-621.
- Marks, J., S. K. Srari, J. Biber, H. Murer, R. J. Unwin, and E. S. Debnam. 2006. Intestinal phosphate absorption and the effect of vitamin D: a comparison of rats with mice. *Exp. Physiol.* 91:531-537.
- Marks, J., E. S. Debnam, and R. J. Unwin. 2010. Phosphate homeostasis and renal-gastrointestinal axis. *Am. J. Physiol. Ren. Physiol.* 299:285-296.
- Martindale, L. 1973. Phosphate excretion in the laying hen (*Gallus Domesticus*). *J. Physiol.* 231: 439-453.
- McDowell, L. R. 2003. Minerals in animal and human nutrition. Chapter 2. Pages 33-100. 2 Ed. Elsevier Sci. B.V.

- McNaughton, J. L. 1981. Effect of calcium carbonate particle size on the available phosphorus requirement of broiler chicks. *Poult. Sci.* 60:197-203.
- McWhorter, T. J., E. Caviedes-Vidal, and, W. H. Karasov. 2009. The integration of digestion and osmoregulation in the avian gut. *Biol. Rev.* 84:533-565.
- Mello, H. H. C., P. C. Gomes, H. S. Rostagno, L. F. T. Albino, R. F. M. d. Oliveira, T. C. d. Rocha, and, C. L. N. Ribeiro. 2012. Requirement of available phosphorus by female broiler chickens keeping the calcium:available phosphorus ratio at 2:1. *R. Bras. Zootec.* 41:2329-2335.
- Miyamoto, K., M. Ito, M. Kuwahata, S. Kato, and H. Segawa. 2005. Inhibition of intestinal sodium-dependent inorganic phosphate transport by fibroblast growth factor 23. *Ther. Apher. Dial.* 9:331-335.
- Mongin, P. 1976. Ionic constituents and osmolality of the small intestinal fluids of the laying hen. *Br. Poult. Sci.* 17:383-392.
- Mongin, P., and B. Sauveur. 1977. Interrelationships between mineral nutrition, acid-base balance, growth and cartilage abnormalities. Pages 235–347 in *Growth and Poultry Meat Production*. Boorman, K. N., Wilson, B. J., eds. *Brit. Poult. Sci.*, Edinburgh, UK.
- Mongin, P., and B. Sauveur. 1979. Plasma inorganic phosphorus concentration during egg-shell formation: Effect of the physical form of the dietary calcium. *Br. Poult. Sci.* 20:401-412.
- Mongin, P. 1981. Recent advances in dietary cation-anion balance: applications in poultry. *Proc. Nutr. Soc.* 40:285–294.
- Morrissey, R. L., and R. H. Wasserman. 1971. Calcium absorption and calcium-binding protein in chicks on differing calcium and phosphorus intakes. *Am. J. Physiol.* 220:1509–1515.

- Mutucumarana, R. K., V. Ravindran, G. Ravindran, and A. J. Cowieson. 2014. Measurement of true ileal digestibility and total tract retention of phosphorus in corn and canola meal for broiler chickens 1. *Poult. Sci* 93:412-419.
- Myers, W. D., P. A. Ludden, V. Nayigihugu, and B. W. Hess. 2004. Technical Note: A procedure for the preparation and quantitative analysis of samples for titanium dioxide. *J. Anim. Sci.* 82:179-183.
- Namkung, H., and S. Leeson. 1999. Effect of phytase enzyme on dietary nitrogen-corrected apparent metabolizable energy and the ileal digestibility of nitrogen and amino acids in broiler chicks. *Poult. Sci.* 78:1317-1319.
- Nelson, T. S. 1967. The utilization of phytate phosphorus by poultry. A review. *Poult Sci.* 46:862-871.
- Nelson, T. S. 1976. The hydrolysis of phytate phosphorus by chicks and laying hens. *Poult. Sci.* 55:2262-2264.
- Nelson, T.S. 1984. Available calcium for poultry. *Proc. Florida Nutr. Conf. Feed Manuf.* PP. 1-7. Orlando, Florida.
- Nie, W., Y. Yang, J. Yuan, Z. Wang, and Y. Guo. 2013. Effect of dietary non-phytate phosphorus on laying performance and small intestinal epithelial phosphate transporter expression in Dwarf pink-shell laying hens. *J. Anim. Sci. Biotechnol.* 4:34-40.
- NRC. 1994. *Nutrient Requirements for Poultry*. National Academy Press, Washington, DC.
- Nusairat, B. M. 2015. Effect of soybean meal varieties, phytase enzyme, and particle size of corn on the performance of broilers. Dissertation. NC State University.
- Ogunji, P. A., R. N. Brewer, S. Roland D A, and D. Caldwell. 1983. Effect of dietary sodium chloride, protein, and strain difference upon water consumption and fecal moisture content of broiler breeder males. *Poult. Sci.* 62:2497-2500.

- Olanrewaju, H. A., S. Wongpichet, J. P. Thaxton, 3. Dozier W A, and S. L. Branton. 2006. Stress and acid-base balance in chickens. *Poult. Sci.* 85:1266-1274.
- Olukosi, O. A., A. J. Cowieson, and O. Adeola. 2007. Age-related influence of a cocktail of xylanase, amylase and protease or phytase individually or in combination in broilers. *Poult. Sci.* 86:77–86.
- Olukosi, O. A., A. J. Cowieson, and O. Adeola. 2008. Influence of enzyme supplementation of maize-soybean meal diets on carcass composition, whole-body nutrient accretion and total tract nutrient retention of broilers. *Br. Poult. Sci.* 49:436-445.
- Olukosi, O., S. Adedokun, K. Ajuwon, and O. Adeola. 2011. Early responses of sodium-dependent phosphate transporter type IIb in broiler chicks to dietary phosphorus intervention. *Br. Poult. Abstr.* 7:39.
- Olukosi, O. A., O. A. Bolarinwa, A. J. Cowieson, and O. Adeola. 2012. Marker type but not concentration influenced apparent ileal amino acid digestibility in phytase-supplemented diets for broiler chickens and pigs. *J. Anim. Sci.* 90:4414-4420.
- Olukosi, O. A. and F. Fru. 2014a. The interplay of dietary nutrient level and varying Ca to phosphorus ratios on efficacy of a bacterial phytase: 1. Growth performance and tibia mineralization. *Poultry Science. Poult. Sci.* 93: 3037-3043.
- Olukosi, O. A., and F. Fru-Nji. 2014b. The interplay of dietary nutrient level and varying calcium to phosphorus ratios on efficacy of a bacterial phytase: 2. Ileal and total tract nutrient utilization. *Poult. Sci.* 93:3044-3052.
- Oude Weernink, P. A., L. Han, K. H. Jakobs, and M. Schmidt. 2007. Dynamic phospholipid signaling by G protein-coupled receptors. *BBA - Biomembranes* 1768:888-900.
- Pearlman, S. M., Z. Serber, and J. E. Ferrell. 2011. A mechanism for the evolution of phosphorylation sites. *Cell.* 147:934-946.

- Pirgozliev, V., O. Oduguwa, T. Acamovic, and M. R. Bedford. 2007. Diets containing *Escherichia coli*-derived phytase on young chickens and turkeys: effects on performance, metabolizable energy, endogenous secretions, and intestinal morphology. *Poult. Sci.* 86:705-713.
- Pirgozliev, V., O. Oduguwa, T. Acamovic, and M. R. Bedford. 2008. Effects of dietary phytase on performance and nutrient metabolism in chickens. *Br. Poult. Sci.* 49:144-154.
- Pirgozliev, V., and M. R. Bedford. 2013. Energy utilisation and growth performance of chicken fed diets containing graded levels of supplementary bacterial phytase. *The British Journal of Nutrition* 109:248-253.
- Powers, W., and R. Angel. 2008. A Review of the capacity for nutritional strategies to address environmental challenges in poultry production. *Poult. Sci.* 87:1929-1938.
- Plumstead, P. W., A. B. Leytem, R. O. Maguire, J. W. Spears, P. Kwanyuen, and J. Brake. 2008. Interaction of calcium and phytate in broiler Diets. 1. Effects on apparent prececal digestibility and retention of phosphorus. *Poult. Sci.* 87:449-458.
- Prashad, D. N., and N. A. Edwards. 1973. Phosphate excretion in the laying fowls. *Comp. Biochem. Physiol.* 46:131-137
- Proszkowiec-Weglarz, M., and R. Angel. 2013. Calcium and phosphorus metabolism in broilers: Effect of homeostatic mechanism on calcium and phosphorus digestibility¹. *J. Appl. Poult. Res.* 22:609-627.
- Qian, H., H. P. Veit, E. T. Kornegay, V. Ravindran, and D. M. Denbow. 1996. Effects of supplemental phytase and phosphorus on histological and other tibial bone characteristics and performance of broilers fed semi-purified diets. *Poult. Sci.* 75:618-626.

- Qian, H., E. T. Kornegay, and D. M. Denbow. 1997. Utilization of phytate phosphorus and calcium as influenced by microbial phytase, cholecalciferol, and the calcium: total phosphorus ratio in broiler diets. *Poult. Sci.* 76:37-46.
- Quamme, G. A., and R. J. Shapiro. 1987. Membrane controls of epithelial phosphate transport. *Can. J. Physiol. Pharmacol.* 65:275-286.
- Rama Rao, S. V., V. Ravindra Reddy, and V. Ramasubba Reddy. 1999. *Anim. Feed. Sci. Technol.* 79:211-222.
- Rama Rao, S. V., and D. Nagalakshmi, and V. R. Reddy. 2002. Feeding to minimize heat stress. *Poult. International.* 41:15.
- Rao, K. S., and D. A. Roland. 1989. Influence of dietary calcium level and particle size of calcium source on *in vivo* calcium solubilization by commercial leghorns. *Poult. Sci.* 68:1499-1505.
- Rao, K. S., and D. A. Roland. 1990. *In vivo* limestone solubilization in commercial leghorns: Role of dietary calcium level, limestone particle size, *in vitro* limestone solubility rate, and the calcium status of the hen. *Poult. Sci.* 69:2170-2176.
- Ravindran, V., 1995. Phytases in poultry nutrition. An overview. *Proc. Aust. Poult. Sci. Sym.* 7:135-139.
- Ravindran, V., S. Cabahug, G. Ravindran, P. H. Selle, and W. L. Bryden. 2000. Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorous levels. II. Effects on apparent metabolizable energy, nutrient digestibility and nutrient retention. *Br. Poult. Sci.* 41:193-200.
- Ravindran, V., P. H. Selle, G. Ravindran, P. C. H. Morel, A. K. Kies, and W. L. Bryden. 2001. Microbial phytase improves performance, apparent metabolizable energy, and ileal amino acid digestibility of broilers fed a lysine-deficient diet. *Poult. Sci.* 80:338-344.

- Ravindran, V., P. C. H. Morel, G. G. Partridge, M. Hruby, and J. S. Sands. 2006. Influence of an *Escherichia coli*-derived phytase on nutrient utilization in broiler starters fed diets containing varying concentrations of phytic acid. *Poult. Sci.* 85:82-89.
- Ravindran, V., A. J. Cowieson, and P. H. Selle. 2008. Influence of dietary electrolyte balance and microbial phytase on growth performance, nutrient utilization, and excreta quality of broiler chickens. *Poult. Sci.* 87:677-688.
- Raup, T. J., and W. G. Bottje. 1990. Effect of carbonated water on arterial pH, pCO₂ and plasma lactate in heat stressed broilers. *Br. Poult. Sci.* 31:377-384.
- Rodehutsord, M., and A. Dieckmann. 2005. Comparative studies with three-week-old chickens, turkeys, ducks, and quails on the response in phosphorus utilization to a supplementation of monobasic calcium phosphate. *Poult. Sci.* 84:1252-1260.
- Rodehutsord, M. 2009. Approaches and challenges for evaluating phosphorus sources for poultry. *Proc. Abstr. 17th European. Symp. Poult. Nutr.* Edinburgh. U.K. pp:2-6.
- Roland, D. A., and D. Caldwell. 1985. Relationship of calcium to wet droppings in laying hens. *Poult. Sci.* 64:1809-1812.
- Romanoff, A. L., and A. J. Romanoff. 1949. *The avian egg.* John Willey & Sons, Inc., New York.
- Rondon, E. O. O., A. E. Murakami, A. C. Furlan, I. Moreira, and M. Macari. 2001. Sodium and chloride requirements of growing broiler chickens fed corn-soybean diets (One to Twenty-One days of age). *Poult. Sci.* 80:592-598.
- Ross, J. 2006. Energy transfer from adenosine triphosphate. *J. Phys. Chem.* 110:6987-6990.
- Rutherford, S. M., T. K. Chung and P. J. Moughan. 2002. The effect of microbial phytase on ileal phosphorus and amino acid digestibility in the broiler chicken. *Br. Poult. Sci.* 44:59-606.

- Rutherford, S. M., T. K. Chung, P. C. H. Morel, and P. J. Moughan. 2004. Effect of microbial phytase on ileal digestibility of phytate phosphorus, total phosphorus, and amino acids in a low-phosphorus diet for broilers. *Poult. Sci.* 83:61-68.
- Rutherford, S. M., T. K. Chung, D. V. Thomas, M. L. Zou, and P. J. Moughan. 2012. Effect of a novel phytase on growth performance, apparent metabolizable energy, and the availability of minerals and amino acids in a low-phosphorus corn-soybean meal diet for broilers. *Poult. Sci.* 91:1118-1127.
- Samiullah, S., J. R. Roberts, and K. Chousalkar. 2015. Eggshell color in brown-egg laying hens a review. *Poult. Sci.* 94:2566-2575.
- Schaal, T. P., J. Arango, A. Wolc, J. V. Brady, J. E. Fulton, I. Rubinoff, I. J. Ehr, M. E. Persia, and N. P. O'Sullivan. 2016. Commercial Hy-Line W-36 pullet and laying hen venous blood gas and chemistry profiles utilizing the portable i-STAT 1 analyzer. *Poult. Sci.* 95:466-471.
- Scheideler, S. E. 1986. utilization of phosphorus in poultry as influenced by dietary calcium and phosphorus source. Dissertation. IOWA State Uni.
- Scott, M., M. C. Nesheim, and R. J. Young. 1982. Nutrition of the Chicken. M. L. Scott and Associates, Publishers, Ithaca, NY.
- Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Lague. 1996. The effects of supplemental microbial phytase on the performance and utilization of dietary calcium, phosphorus, copper, and zinc in broiler chickens fed corn–soybean diets. *Poult. Sci.* 75:729-736.
- Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Lague. 1997. Apparent digestibility of protein and amino acids in broiler chickens fed a corn–soybean diet supplemented with microbial phytase. *Poult. Sci.* 76:1760-1769.

- Selle, P. H., V. Ravindran, A. Caldwell, and W. L. Bryden. 2000. Phytate and phytase: consequences for protein utilization. *Nutr. Res. Rev.* 13:255-278.
- Selle, P. H., and V. Ravindran. 2007. Microbial phytase in poultry nutrition. *Anim. Feed Sci. Technol.* 135:1-41.
- Selle, P. H., A. J. Cowieson, and V. Ravindran. 2009. Consequences of calcium interactions with phytate and phytase for poultry and pigs. *Livestock Sci.* 124:126-141.
- Selle, P. H., A. J. Cowieson, N. P. Cowieson, and V. Ravindran. 2012. Protein–phytate interactions in pig and poultry nutrition: a reappraisal. *Nutr. Res. Rev.* 25:1-17.
- Segawa, H., I. Kaneko, S. Yamanaka, M. Ito, M. Kuwahata, Y. Inoue, S. Kato, and K. Miyamoto. 2004. Intestinal Na-Pi cotransporter adaptation to dietary Pi content in vitamin D receptor null mice. *Am. J. Physiol. Renal Physiol.* 287:39-47.
- Shafey, T. M., M. W. McDonald, and J. G. Dingle. 1991. Effects of dietary calcium and available phosphorus concentration on digesta pH and on the availability of calcium, iron, magnesium, and zinc from the intestinal contents of meat chickens. *Br. Poult. Sci.* 32:185-194.
- Shastak, Y., M. Witzig, K. Hartung, and M. Rodehutsord. 2012. Comparison of retention and prececal digestibility measurements in evaluating mineral phosphorus sources in broilers. *Poult. Sci.* 91:2201-2209.
- Shastak, Y., and M. Rodehutsord. 2013. Determination and estimation of phosphorus availability in growing poultry and their historical development. *World's Poult. Sci. J.* 69:569-586.
- Shirley, R. B., and H. M. Edwards. 2002. Dietary calcium affects phytase activity when phytase is supplemented in excess of industry standards. *Poult. Sci.* 81 (Suppl. 1), 11 (Abstr.).

- Simkiss, K., and T. G. Taylor. 1971. Shell formation. Bell DJ and Freeman BM, editors. In: Physiology and biochemistry of the domestic fowl. 1st ed. New York: Academic Press, Inc. 1331 p.
- Simons, P. C. M., H. A. J. Versteegh, A. W. Jongbloed, P. A. Kemme, P. Slump, K. D. Bos, M. G. E. Wolters, R. F. Beudeker, and G. J. Verschoor. 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. *Br. J. Nutr.* 64:525-540.
- Skadhauge, E. 1981. Osmoregulation in Birds (WS Hoar, B Hoelldobler, K Johansen, H Langer, and G Somero, Eds.). Springer-Verlag, New York.
- Skadhauge, E. 1983. Formation and composition of urine. Pages 108–135 in *Physiology and Biochemistry of the Domestic Fowl*. Freeman, B.M., ed. Academic Press, London.
- Smith, A. H., C. M. Winget, and J. R. Blackard. 1954. The transfer of phosphorus to the hen's egg, under controlled environment, as traced with radiophosphorus (P^{32}). *Poult. Sci.* 33:908-919.
- Smith, A., S. P. Rose, R. G. Wells, and V. Pirgozliev. 2000. The effect of changing the excreta moisture of caged laying hens on the excreta and microbial contamination of their egg shells. *Br. Poult. Sci.* 41:168-173.
- Smith, M.O., and R. G. Teeter. 1987. Effect of ammonium chloride and potassium chloride on survival of broiler chickens. *Nutr. Res.* 7:677-681.
- Sommerfeld, V., M. Schollenberger, I. Kühn, and M. Rodehutscord. 2018. Interactive effects of phosphorus, calcium, and phytase supplements on products of phytate degradation in the digestive tract of broiler chickens. *Poult. Sci.* 97:1177-1188.
- Sturkie, P. D. 1976. *Avian physiology*. Springer, Berlin Heidelberg New York.

- Suhail, M. 2010. Na⁺, K⁺-ATPase: Ubiquitous multifunctional transmembrane protein and its relevance to various pathophysiological conditions. *J. Clin. Med. Res.* 1:1-17.
- Takeda, E., Y. Taketani, N. Sawada, T. Sato, and H. Yamamoto. 2004. The regulation and function of phosphate in the human body. *Biofactors.* 21:345-355.
- Tamim, N. M., and R. Angel. 2003. Phytate phosphorus hydrolysis as influenced by dietary calcium and micro-mineral source in broiler diets. *J. Agric. Food Chem.* 51:4687–4693
- Tamim, N. M., R. Angel, and M. Christman. 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. *Poult. Sci.* 83:1358-1367.
- Talmage, R. V., C. J. VanderWeil, and J. L. Matthews. 1982. Calcitonin and phosphate. *Mol. Cell. Endocrinol.* 24:235-251.
- Taylor, T. G., and J. Kirkley. 1967. The absorption and excretion of minerals by laying hens in relation to egg shell formation. *Br. Poult. Sci.* 8:289-295.
- Talyor, T. 1970. The role of the skeleton in egg-shell formation. *Annal. Biol. Anim. Biochem. Biophy.* 10:83-91.
- Teeter, R. G., M. O. Smith, F. N. Owens, S. C. Arp, S. Sangiah, and J. E. Breazile. 1985. Chronic heat stress and respiratory alkalosis: Occurrence and treatment in broiler chicks. *Poult. Sci.* 64:1060-1064.
- Teeter, R. G., C. J. Wiernusz, and T. Belay. 1996. Animal nutrition in the 21st century a poultry perspective. *Anim. Feed. Sci. Technol.* 58:37-47.
- Temperton, H., and J. Cassidy. 1964. Phosphorus requirements of poultry. *Br. Poult. Sci.* 5:81-86.
- Van der Klis, J. D., A. Van Voorst, and C. Van Cruyningen. 1993. Effect of a soluble polysaccharide (carboxy methyl cellulose) on the physico-chemical conditions in gastrointestinal tract of broilers. *Br. Poult. Sci.* 34:971-983.

- Van der Klis, J. D., and H. A. J. Versteegh. 1996. Phosphorus nutrition in broilers. In *Recent Advances in Animal Nutrition*; Garnsworthy, P.C., Wiseman, J., Haresign, W., Eds.; Nottingham University press: Nottingham, UK, 1996; pp. 71–83.
- Viveros, A., A. Brenes, I. Arija, and C. Centeno. 2002. Effects of microbial phytase supplementation on mineral utilization and serum enzyme activities in broiler chicks fed different levels of phosphorus. *Poultry science* 81:1172-1183.
- Waldroup, P. W. 1999. Nutritional approaches to reducing phosphorus excretion by poultry. *Poult. Sci.* 78:683-691.
- Walk, C. L., E. K. Addo-Chidie, M. R. Bedford, and O. Adeola. 2012. Evaluation of a highly soluble calcium source and phytase in the diets of broiler chickens. *Poultry Science* 91:2255-2263.
- Walk, C. L. 2016. The influence if calcium on phytase efficacy in non-ruminant animals. *Anim. Prod. Sci.* 56:1345-1349.
- Walsh, E. N. 1992. *Phosphorus chemistry*. Am. Chem. Soc. Washington.
- Ward., G. M. 1966. Potassium metabolism of domestic ruminants: a review. *J. Dairy. Sci.* 49:268-276.
- Westheimer, F. H. 1987. Why nature chose phosphates. *Science* 235:1173-1178.
- Whitehead, C. C. 2004. Overview of bone biology in the egg-laying hen. *Poultry science* 83:193-199.
- Wideman, R. F. 1987. Renal regulation of avian calcium and phosphorus metabolism. *J. Nutr.* 117:806-815.
- Wilkinson, S. J., E. J. Bradbury, P. C. Thomson, M. R. Bedford, and A. J. Cowieson. 2014. Nutritional geometry of calcium and phosphorus nutrition in broiler chicks. The effect

- of different dietary calcium and phosphorus concentrations and ratios on nutrient digestibility. *Anim.* 8:1080-1088.
- Xu, H., L. Bai, J. F. Collins, and F. K. Ghishan. 2002. Age-dependent regulation of rat intestinal type IIb sodium-phosphate cotransporter by 1,25-(OH)₂ vitamin D₃. *Am. J. Physiol. Cell Physiol.* 282:487-493.
- Xu, Y., C. R. Stark, P. R. Ferket, C. M. Williams, W. J. Pacheco, and J. Brake. 2015. Effect of dietary coarsely ground corn on broiler live performance, gastrointestinal tract development, apparent ileal digestibility of energy and nitrogen, and digesta particle size distribution and retention time. *Poultry science* 94:53-60.
- Yan, F., R. Angel, and C. M. Ashwell. 2007. Characterization of the chicken small intestine type IIb sodium phosphate cotransporter. *Poultry science* 86:67-76.
- Yan, F., J. H. Kersey, and P. W. Waldroup. 2001. Phosphorus requirements of broiler chicks three to six weeks of age as influenced by phytase supplementation. *Poultry science* 80:455-459.
- Yan, F., J. H. Kersey, C. A. Fritts, P. W. Waldroup. 2006. Effect of phytase supplementation on the calcium requirement of broiler chicks. *Int. J. Poult. Sci.* 5:112-120.
- Yixin, H., L. Xiudong, W. Qian, L. Lu, L. Zhang, and L. Xugang. 2018. Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. *The British Journal of Nutrition* 119:1346-1354.
- Yu, S., A. Cowieson, C. Gilbert, P. Plumstead, and S. Dalsgaard. 2012. Interactions of phytate and myo-inositol phosphate esters (IP1-5) including IP5 isomers with dietary protein and iron and inhibition of pepsin1. *Journal of Animal Science* 90:1824-1832.
- Zeller, E., M. Schollenberger, M. Witzig, Y. Shastak, I. Kuhn, L. E. Hoelzle, and M. Rodehutschord. 2015. Interactions between supplemented mineral phosphorus and

- phytase on phytate hydrolysis and inositol phosphates in the small intestine of broilers. *Poult. Sci.* 94:1018-1029.
- Zeller, E., M. Schollenberger, I. Kühn, and M. Rodehutscord. 2016. Dietary effects on inositol phosphate breakdown in the crop of broilers. *Arch. Anim. Nutr.* 70:57-71.
- Zhang, B., and C. N. Coon. 1997. Improved in vitro methods for determining limestone and oyster shell solubility. *J. Appl. Poult. Res.* 6:94-99.
- Zyla, K., J. Koreleski, S. Swiatkiewicz, A. Wikiera, M. Kujawski, J. Piironen, and D. R. Ledoux, 2000. Effects of phosphorolytic and cell wall-degrading enzymes on the performance of growing broilers fed wheat-based diets containing different calcium levels. *Poultry Sci.* 79:66-76.

MANUSCRIPT I. Effect of Dietary Available Phosphorus and Potassium on Fecal Moisture, Fecal Liquid Portion and Egg Characteristics of Broiler Breeders during the Onset of Lay.

ABSTRACT

Ross 708 broiler breeder pullets were reared on grower diets containing 0.3% or 0.5% available phosphorus (AvP). No adverse effects of treatments were observed during rearing. At 22 wk of age, 64 breeder pullets reared on diets containing either 0.3% or 0.5% available (AvP) were moved to individual cages. Half of the pullets on each rearing diet remained on their corresponding grower AvP levels (0.3% or 0.5%) while the other half were changed to the other AvP level (0.5% and 0.3%), resulting in 4 AvP dietary treatments in a 2 x 2 arrangement. At 23 wk of age, lay diets were also amended with either 0% or 0.2% potassium (K) as potassium carbonate to complete the 2 x 2 x 2 arrangement. Fecal liquid portion (LP), fecal moisture (FM) and characteristics of the second and tenth egg laid were assessed. The LP and FM of each breeder hen were measured at 22 wk of age to establish a basal point and thereafter at 23, 24, 25, 26, 27, 28, and 30 wk of age.

Addition of 0.2% K to the 0.3% AvP layer diet reduced ($P \leq 0.05$) LP at 23, 25, 26 and 28 wk and FM at 23, 24, 25, 26, 27 and 28 wk as compared to the 0.5% AvP layer diet amended with 0.2% K. The non-amended 0.3% and 0.5% AvP layer diets were intermediate. Further, the LP and FM were generally reduced as the breeders achieved peak egg production. The 0.3% AvP grower diet increased ($P \leq 0.05$) the weights of the second egg and its albumen.

It was concluded that while supplementing 0.2% K to the 0.5% AvP layer diet increased LP and FM the opposite was true with the 0.3% AvP layer diet. These findings could be beneficial in controlling excess litter moisture during onset of lay in broiler breeders.

Key words: Broiler breeders, fecal moisture, fecal liquid portion, potassium, phosphorus

INTRODUCTION

Pullets coming into lay are known to exhibit increased fecal moisture content and wet litter is a problem in the broiler breeder industry (Ogunji *et al.* 1983), which results in increased percentage of dirty eggs and management problems associated with increased litter moisture management, such as issues with hygiene and odor. There is a need to understand and develop strategies to minimize the fecal moisture content in order to achieve improved hygiene and economic benefits. Nutritional and non-nutritional factors have been investigated, which include dietary calcium (Ca) and electrolyte balance, phosphate excretion, water restriction (Roberts and Balnave, 1992; Roland and Caldwell, 1985; Leeson and Summers, 1987; Martindale, 1972), mineral requirements (Wideman, 1987), sodium chloride, protein and strain differences (Ogunji *et al.*, 1983). Dietary modification of P with phytase in broiler breeders (Maguire *et al.* 2005) and of dietary minerals (Na, K, Ca and P) in laying hens (Smith *et al.*, 2000) have been reported to alter the fecal moisture.

Acid-Base Homeostasis during Onset of Lay

The acid-base balance of serum and uterine fluid is altered during egg shell calcification. A homeostatic cascade of events to adjust body functions to a new level is triggered when Ca is removed from blood flowing to the shell gland which changes intestinal absorption, kidney function, bone metabolism, and increased P excretion (Taylor and Kirkley, 1967). The formation of calcium carbonate ions in the uterus results in metabolic acidosis which is facilitated by means of hyperventilation that increases H ions, decreases CO₂ and decreases HCO₃⁻ and which leads to acidification of the urine (Mongin and Lacassagne, 1964). During formation of calcium carbonate for the shell calcification H ions increase and P in the blood must be excreted, which results in increased water content of the feces. Further, if the

birds have received a diet with acidogenic potential, decreased plasma bicarbonate will result in poor egg shell quality.

Potassium

Potassium (K), the primary intracellular cation, has an overall dietary alkalogenic effect and is thought to affect litter moisture content. Feed restriction in broiler breeders will limit the K intake, but there is evidence in the literature suggesting that depression in plasma K concentrations will lead to arrhythmia, muscular weakness between 24 to 26 wk of age and may result in sudden death syndrome (Hopkinson *et al.*, 1990). The wet feces observed during onset of lay suggested that there could be an acid-base imbalance associated with DEB which may be related to metabolic acidosis or alkalosis. Potassium, due to its alkalinizing effect when given as a dietary supplement as potassium salt of weak acid may influence the acid-base balance. Halley *et al.* (1995) demonstrated that DEB of 230 mEq/kg corresponding to K, Na and Cl levels in diets containing potassium carbonate supported better performance in broiler breeders. It is apparent that the relationships among various minerals and ratios among K, Cl and Na are important in maintaining homeostasis of water balance in poultry.

Pre-lay Period

The pre-laying period is complex and is a critical phase in reproductive maturation prior to onset of lay. Depending upon management, each flock can follow slightly different pathways, as can the individuals within the flocks (Hurwitz and Bar, 1970). Laying hens mobilize Ca from the medullary bone to synthesize the calcium carbonate of the egg shell and use the Ca they absorb from their diet to replenish the medullary bone. The Ca concentration is controlled within a narrow physiological range through feedback mechanisms among parathyroid hormone (PTH), Vitamin D₃ and calcitonin (Nys and Mongin, 1982; Hurwitz, 1989). Renal 1 α -hydroxylase activation depends on Ca concentration and additionally is

regulated by PTH and calcitonin. Increased Ca can cause CaPO_4 precipitation in soft tissues and increase P and Ca excretion. Thus, excess dietary Ca will interact with P to reduce phosphate buffering capacity which may be causally related to wet litter.

This study was designed to determine whether dietary P and K would interact and have an effect on fecal moisture (FM), fecal liquid portion (LP) and egg characteristics in individual broiler breeders during the onset of lay.

MATERIALS AND METHODS

Animal Welfare

The experimental protocol used in this study was approved by the North Carolina State University Institutional Animal Care and Use Committee. This experiment was designed and conducted in compliance with the Guide and Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Broiler Breeder Rearing Period

A total of 1280 1-d-old Ross 708SF (slow feather) females were raised in 16 floor pens (14.3 m² area; 80 females per pen) on 3 inches of new pine wood shavings in an enclosed fan-ventilated house. All females were identified with a neck tag at placement. Each female rearing pen was initially equipped with 5 tube feeders (DH-4; Kuhl, Flemington, New Jersey). Each feeder pan had a circumference of 132 cm. During the first week of brooding, 6 feeder lids, 4 tube feeders, and 2 font drinkers per female pen were used. From placement to 14 d of age all female pens had 4 tube feeders followed by 3 tube feeders to 10 wk of age. From 11-15 wk, 4 tube feeders were again used, and from 16 wk until birds were moved to the laying house 5 tube feeders were used. Two bell-type Plasson drinkers were used per female pen during rearing. Birds were on a full-feeding allowance for the first 2 wk of age. From 3 wk to 12 wk of age a 4/3 feeding program with 4 feed days and 3 without feed days was followed where the

total feed (g/bird) allowance of 7 days was fed during the 4-day period. Thereafter, a 5/2 feeding program was followed until movement to the laying house. The litter temperature was 35°C (95° F) for the first 2 d and the ambient temperature was 29.4~32.2 °C (85~90° F) through 7 d of brooding. Light was supplied with 100W incandescent lamps for 23 h through 7 d and for 12 h from 8 d to 21 d. From 22 d up to 21 wk, 8 h of light was supplied via 40W incandescent lamps. The house was ventilated to maintain ammonia levels below 25 ppm.

Starter and Grower Diets

Starter feed formulas are shown in Table 1. Day-old chicks were fed a common mash starter diet (17.5% crude protein (CP); 2.9 kcal/g diet) for the first 6 wk of age containing corn ground to 800 µm using a roller mill (Model C128829, RMS, Tea, SD). This was followed by a mash grower diet (15% CP; 2.9 kcal/g diet), with corn ground to 1200 µm with a roller mill, to 21 wk of age. The 2 grower dietary treatments are shown in Table 2. The grower diets had 0.9% calcium (Ca), and either 0.3% or 0.5% available phosphorus (AvP).

Broiler Breeder Cage Placement and Management

For the cage experiment, a total of 64 breeder pullets at 22 wk of age (154 d) were selected from 0.3% or 0.5% AvP treatments, using selection criteria based upon similar body weight (BW) of 2700 ± 21 g/bird, and were randomly assigned to eight dietary treatments. The individual, neck tagged pullets were placed into 0.46 m x 0.33 m x 0.41 m (l x w x h) cages. The birds were acclimatized for 3 d to learn to drink from nipple drinkers and were manually fed daily. Birds were photo-stimulated with an abrupt increase of day length from 8 h to 14 h at 21 wk of age (Ross parent stock handbook, 2018). The day length was subsequently increased to 15 h at 23.5 wk, then to 15.5 h at 5% production (25 wk) and finally to 16 h at 50% production, on a flock average basis, which thereafter was maintained for the duration of the experiment. The house temperature was adjusted weekly to remain between 60° F and 80°

F using curtains and/or heaters and circulating fans. At 22 wk of age, the hens were fed manually on an individual basis with two mash grower diets (15% CP, 2.9 kcal/kg, 1200 microns) and with either 0.3% or 0.5% AvP.

Breeder Dietary Treatments

At 23 wk of age half of the breeder hens each rearing diet remained on their respective grower AvP levels of either 0.3% or 0.5% and half had the AvP levels reversed; Ca was increased to 2.7% for all the diets. Layer diets were also amended with either 0% or 0.2% K as potassium carbonate (Table 3). Thus, the 64 breeder hens were assigned to 8 dietary treatments. These were prepared from a common basal mix that was amended with the required amounts of di-calcium phosphate, limestone, filler, and potassium carbonate as appropriate for each dietary treatment. The common basal was batched, dry ingredients were blended for 180 sec in a twin shaft counterpoise mixer (Model TRDB126-0604, Hayes and Stolz, Fort Worth, TX) followed by addition of fat and mixing for an additional 90 sec. A commercially available potassium carbonate (Arm and Hammer Animal Health, Princeton, NJ) was added during mixing at an inclusion rate of 4 kg/MT to produce the K treatments.

Broiler Breeder Cage Feeding Program

Every morning at 8 AM, each bird was fed individually with a measured quantity of feed throughout the experimental period. All feeding program changes were made weekly except for the period between 5% lay to peak, (from 24 to 26 wk) when amounts were increased daily. The feeding program is shown in Table 4.

Broiler Breeder Fecal Moisture and Fecal Liquid Portion Sampling

On the day of sample collection cages identified with individual tag numbers were fitted with individual aluminum pans covered with oil paper to collect feces and were fitted with a 250 mL beaker to collect the fecal liquid portion that drained from the feces (Figure 1).

The sample collection pan and LP beakers were removed after 24 h collection of feces and LP and were thereafter washed, dried and kept ready for the next collection. Aluminum fecal collection pans were located with enough distance from the drinker and feeder of each pen to avoid water and feed contamination of the feces. There was also an empty cage between birds to further guard against cross-contamination. Samples were collected weekly, and the LP volume that was drained in the LP beaker was measured with a graduated cylinder. The feces and LP were then homogenized in an identified individual plastic bag. This total fecal matter was then placed into a collection pan and dried in an oven for 24 h at 95°C for determination of dry matter (Method 934.01, AOAC, 2006) and fecal moisture (FM). Fecal LP and FM were determined at 22 wk of age (154 d), again at 159 d of age, and thereafter at 23, 24, 25, 26, 27, 28, and 30 wk of age.



Figure 1. Fecal matter and liquid portion collection pan set up.

Broiler Breeder Egg Production and Egg Sampling

Each hen's egg production was recorded daily at 3 PM and expressed as % weekly egg production. Egg weight, eggshell weight, and eggshell thickness, as well as yolk weight and albumen weight and percentage (%) shell were determined for the 2nd and the 10th egg laid by the individual hens. Individual eggs were weighed whole, cracked and the albumen was separated from the yolk. Shells were rinsed and set aside for drying. After complete drying, shells were weighed, and shell thickness was determined at sharp and blunt ends and equator using an Ames Thickness Gauge (56 Series, Framingham, MA). Albumen weight was determined by deducting shell weight and yolk weight from the whole egg weight. These data were used to calculate percentage yolk, percentage albumen, percentage shell, and yolk to albumen ratio.

Statistical Analysis

All data collected in this study were analyzed as fixed factorial effects for Grow AvP, Lay AvP and Lay K in a 2 x 2 x 2 arrangement. Since the cage was the experimental unit it was included using random effects, nested within the combination of Grow AvP, Lay AvP and Lay K using the following statistical model where $Y_{ijkl} = \mu + A_i + B_j + AB_{ij} + C_k + AC_{ik} + BC_{jk} + D_{l(ijk)} + \varepsilon$. Here, i, j, k, l are indexes for, respectively, Grow AvP, Lay AvP, Lay K and cage. The data was analyzed using mixed model of FIT Model platform in JMP 13.2 Pro software. The 3-way interactions between the 2 Grower AvP levels (0.3% and 0.5%), the 2 Layer AvP levels (0.3% and 0.5%) and the 2 Layer K levels (0% and 0.2%) on FM and LP were investigated first using the full factorial arrangement; there were no significant 3-way interactions found. The data was re-analyzed to second term interactions using Factorial to a degree analysis and the main effects and 2-way interactions are shown in the data tables. LSMEANS were considered statistically significant at $P \leq 0.05$, although probability values

up to $P < 0.10$ are shown in the text if the data suggested a numerical trend. When a significant P value was detected, LSMEANS were separated using Student's T-test. The linear regression analysis was performed with Fit Y by X platform of JMP 13.2 software. The week-wise simple linear relationships between the fecal LP and FM at 22 wk, 26 wk and 30 wk were selected for brevity.

RESULTS

The results of fecal moisture (FM) of broiler breeder hens from 22 to 30 wk of age are shown in Table 5. No effects of grow dietary AvP levels were observed on FM of the breeder hens from 22 to 30 wk of age. Fecal moisture of the breeder hens that received 0.2% K lay diets was dependent on the 0.3% and 0.5% AvP layer diets whereas there was no effect of supplementation of 0.2% K on FM of the hens that received the 0.5% AvP lay diets except from 23 to 30 wk of age, which resulted in an interaction ($P \leq 0.05$) between AvP and K lay diets.

The results of fecal liquid portion (LP) of breeder hens from 22 to 30 wk of age are shown in Table 6. No effect of grow dietary AvP level was observed on LP from 22 to 30 wk of age. Fecal liquid portion of the breeder hens that received 0.2% K lay diets was dependent on the 0.3% and 0.5% AvP lay diets at 23, 25, 26 and 28 wk of age, which resulted in an interaction ($P \leq 0.05$) between AvP and K lay diets.

The fecal moisture was associated with fecal liquid portion at 22 wk, 26 wk and 30 wk as shown in Figure 2.

No effect of dietary treatments was observed on the average weekly hen-day egg production from 25 to 29 weeks of age (Table 7).

The 0.3% AvP grower diet increased ($P \leq 0.05$) the weights of the second egg and its albumen (Table 8). However, yolk weights and yolk to albumen ratios were not affected. The

external egg characters, shell weights and thickness were also not affected. In the tenth egg laid there were no effects of dietary treatments observed on the egg characteristics (Table 9).

DISCUSSION

The objective of the present study was to assess the effect of short-term supplementation of two levels of K to two levels of AvP in broiler breeder diets during onset of lay to peak egg production on percentage FM, reproductive performance, and egg quality variables. The study measured the cloacal output of LP and feces over a 24 h period once a week from 22 to 30 wk of age, which took into account the discontinuous nature of feces discharge by chickens to ensure complete collection (Skadhauge and Dawson, 1980). Skadhauge (1981) reported that the water content of the urine-feces mass was in the range between 78 to 88% in domestic fowls. In the present study the FM that was measured weekly in the broiler breeders was found to be within a range between 82% to 90%, which was somewhat consistent with the literature (Skadhauge and Dawson, 1980; Skadhauge, 1981). Earlier information (North, 1978) has indicated that FM can be variable and, that FM can vary significantly with age, where feces from older birds have higher water content than feces from younger birds. Older birds' FM ranged between 75-80% (North, 1978). In the present study, the week-wise linear relationship between LP and FM data showed an increase with age (Figure 2). The variation in FM explained by LP increased as the hens progressed in age and laying cycle as evidenced by the improved R^2 value. Age of birds causing variability in the FM was noted by van der Hoeven-Hangoor *et al.* (2013). Further, Eichner *et al.* (2007) demonstrated increased excreta moisture with increasing age in broiler chickens. In a laying hen study Smith *et al.* (2000) reported that increased intake of sodium, phosphorus, and K was related to increased water consumption and increased FM and the added calcium did not affect the water intake or the FM.

In this study, the lower inclusion rate of AvP (0.3%) caused a trend towards higher FM when supplemented to the grower diet. This finding was unexpected, Ziaei *et al.* (2008) reported that reduced dietary P increased the dry matter of excreta, mainly as a result of increased retention of P. Conversely it was the higher AvP inclusion rate (0.5%) in the layer diet tended to slightly increase FM. The findings were in agreement with Smith *et al.* (2000) who reported increased dietary P resulted in greater excreta moisture in laying hens. Supplemental K at 0.2% reduced FM at 26 wk of age at an early phase of lay. The finding was somewhat similar to that was reported by van der Hoeven-Hangoor *et al.* (2013), greater excreta K level decreased excreta moisture, greater K retention may have been associated with the manipulation of FM levels (Ziaei *et al.*, 2007). However, there were significant Layer AvP x Layer K interactions which indicated that FM was apparently influenced by an additive reaction to 0.5% AvP and 0.2% K while AvP at 0.3% coupled to the response to 0.2% K produced a reduction in FM. These observations suggest that there are physiologically important regulatory events that control body P and K concentrations. These events could be related to endocrine substances such as parathyroid hormone, calcitonin, Vitamin D₃ (Li *et al.*, 2017), and possibly vasotocin (Goldstein, 2006) in the breeder hen.

Skadhauge (1981) reported that water turnover increases at egg laying. In the present experiment it was observed that the LP volume generally increased from 23 wk of age. The LP of the produced feces in the breeder hens was affected by 0.5% AvP inclusion rate to the layer diet in which LP trended higher throughout the early lay period. In the grower diet the inclusion rate of the 0.3% AvP was associated with a higher (not significant) LP than in the hens that received 0.5% AvP. The inclusion of K (0.2%) caused a trend toward lower LP until wk 26 in the early lay period, but thereafter K inclusion at 0.2% was associated with a higher LP trend.

Significant interactions between Layer AvP x Layer K were found indicating that 0.3% AvP in layer diets increased the LP, but the combination of 0.3% AvP with 0.2% K had a significant negative influence on LP. Yet, with an inclusion rate of 0.5% AvP there was no effect on LP, but a combination of 0.5% AvP with K at 0.2% significantly elevated the LP. These observations indicate that there is a significant age/developmental effect that is controlled by excess K even in the presence of excess P.

The physiological events related to the above observed interactions suggested that the intricate regulator of the P and the K would ultimately be the renal system (Long and Skadhauge, 1983). Martindale (1973) reported that P excretion increases during egg shell calcification days and that the homeostasis is maintained via “phosphaturic effect” of PTH leading to greater excretion, which is an additional buffer capable of binding H^+ ions that could lead to excretion of H^+ up to 20% (Long and Skadhauge, 1983). The present study did not measure the P or K load in the FM content; however, it is known that excretion/retention of minerals parallels dietary intake (Satlin, 2009) which influences the excreta dry matter (Ziaei *et al.*, 2008). Studies in rat kidneys by Bomsztyk and Wright (1986) have demonstrated that K ion concentration can influence fluid flux. Additionally, Larsen *et al.* (2006) reported that K, HPO_4^{2-} , and $H_2PO_4^-$ ions cause “solute-solvent coupling” altering water absorption, which may be the possible reason for the 0.5% AvP, 0.2% K interaction which altered the physiological water handling and thus resulted in increased FM and fecal LP by renal handling.

There were no age-related influences of added AvP in grower or layer diets, but overall the effect of added K caused slower onset of maximum egg production in the breeder hens. The combination of added 0.3% AvP along with added 0.2% K in the grower diet was associated with an overall decrease in egg production in the early phase of onset of lay. In the lay diet AvP addition at 0.5% along with added 0.2% K caused a decrease in egg production

through 27 wk, and thereafter there were no differences in egg production. These observations provide further evidence that certain endocrine and other physiological influences in response to added K are having a negative influence on egg production. Ekmay and Coon (2010) observed that dietary non-phytate P at 0.2% did not affect egg production of breeder hens. Costa *et al.* (2011) reported a decrease in egg production in laying quails with an increased concentration of K.

Yet, with the exception of a decrease in egg weight of the second egg caused by 0.5% AvP addition to the grower diet, there were no negative effects of the added P and K on any of the measured egg parameters (egg weight, yolk weight, albumen weight, yolk:albumen ratio, shell weight, or shell thickness).

The dietary Ca concentration of 2.7% fed during onset of lay appeared to be adequate for egg weights from 25-30 wk of lay. Roland (1986b) described that in a population of hens Ca requirement is greatest during peak lay period. In the present study, individually fed breeder egg production was not affected by 2.7% dietary Ca concentration, however, the long-term effect needs further investigation.

In the present study, dietary nutrient requirements were adequate, and hence were not expected to have an effect on external egg characteristics.

CONCLUSIONS

The interaction between lay AvP and lay K on fecal moisture suggested that the effect of lay P seems to be manifested in presence of potassium therefore, it can be concluded that under the conditions of this experiment addition of 0.2% K had a transitory effect on the reduction of the fecal moisture with 0.3% lay AvP lay diets.

Table 1. Composition of breeder starter diets fed from 1-6 wk of age.

Ingredient	Common Starter
	(%)
Corn	64.62
Soybean meal, 48%	23.20
Wheat bran	6.90
Poultry fat	1.31
Dicalcium phosphate, 18.5%	1.99
Limestone	0.84
Salt	0.50
Choline chloride, 60%	0.20
Vitamin premix ¹	0.10
Mineral premix ²	0.20
Selenium premix ³	0.05
Alimet, 88%	0.09
Calculated nutrient content	
Crude protein (%)	17.50
Calcium (%)	0.90
Available phosphorus (%)	0.45
Total Lysine (%)	0.90
Total Methionine (%)	0.40
Total TSAA (%)	0.69
Total Threonine (%)	0.60
Sodium (%)	0.23
Potassium (%)	0.77
Chloride (%)	0.36
Metabolizable energy (kcal/g)	2.90

¹Vitamin premix supplied the following per kg of diet: 6,614 IU vitamin A, 2,000 IU vitamin D₃ (cholecalciferol), 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 1.98 mg menadione (K₃), 1.98 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 3.97 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

²Mineral premix supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt, 1 mg.

³Selenium premix provided 0.2 mg Se (as Na₂SeO₃) per kg of diet.

Table 2. Composition of breeder grower diets fed from 6-22 wk of age.

Ingredient	Dietary Treatments ¹	
	0.3% AvP	0.5% AvP
	(%)	(%)
Corn	68.12	68.12
Soybean meal, 48%	17.51	17.51
Wheat bran	6.43	6.43
Poultry fat	1.43	1.43
Dicalcium phosphate, 18.5%	1.13	2.37
Limestone	1.39	0.67
Salt	0.50	0.50
Choline chloride, 60%	0.20	0.20
L-Lysine	0.04	0.04
Vitamin premix ²	0.10	0.10
Mineral premix ³	0.20	0.20
Selenium premix ⁴	0.05	0.05
Alimet, 88%	0.12	0.12
Vermiculite (inert filler)	2.78	2.26
Calculated nutrients		
Crude protein (%)	15.00	15.00
Calcium (%)	0.90	0.90
Available phosphorus (%)	0.30	0.50
Total Lysine (%)	0.75	0.75
Total Methionine (%)	0.36	0.36
Total TSAA (%)	0.62	0.62
Total Threonine (%)	0.51	0.51
Sodium (%)	0.20	0.20
Potassium (%)	0.65	0.65
Chloride (%)	0.36	0.36
Metabolizable energy (kcal/g)	2.90	2.90

¹Diets contained either 0.3% or 0.5% available phosphorus (AvP).

²Vitamin premix supplied the following per kg of diet: 6,614 IU vitamin A, 2,000 IU vitamin D₃ (cholecalciferol), 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 1.98 mg menadione (K₃), 1.98 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 3.97 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

³Mineral premix supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt, 1 mg.

⁴Selenium premix provided 0.2 mg Se (as Na₂SeO₃) per kg of diet.

Table 3. Composition of breeder lay diets fed from 22-30 wk of age.

Ingredients	Dietary Treatments ¹							
	0.3% Grow AvP				0.5% Grow AvP			
	0.3% Lay AvP		0.5% Lay AvP		0.5% Lay AvP		0.3% Lay AvP	
	% Lay K		% Lay K		% Lay K		% Lay K	
	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2
	(%)							
Corn	68.16	68.16	68.16	68.16	68.16	68.16	68.16	68.16
Soybean meal, 48%	17.25	17.25	17.25	17.25	17.25	17.25	17.25	17.25
Wheat bran	3.49	3.49	3.49	3.49	3.49	3.49	3.49	3.49
Poultry Fat	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Dicalcium phosphate, 18.5%	1.21	1.21	2.45	2.45	2.45	2.45	1.21	1.21
Limestone	6.10	6.10	5.38	5.38	5.38	5.38	6.10	6.10
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride, 60%	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
L-Lysine	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Vitamin premix ²	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mineral premix ³	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Selenium premix ⁴	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Alimet, 88%	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Potassium carbonate	-	0.40	-	0.40	-	0.40	-	0.40
Vermiculite (inert filler)	0.52	0.12	-	-	-	-	0.52	0.12
Calculated nutrients								
Crude protein (%)	14.50	14.50	14.50	14.50	14.50	14.50	14.50	14.50
Calcium (%)	2.70	2.70	2.70	2.70	2.70	2.70	2.70	2.70
Available Phosphorous (%)	0.30	0.30	0.50	0.50	0.50	0.50	0.30	0.30
Potassium (%)	0.70	0.90	0.70	0.90	0.70	0.90	0.70	0.90
Sodium (%)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Chloride (%)								
Total Lysine (%)	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Total Methionine (%)	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37
Total TSAA (%)	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.62
Metabolizable energy (kcal/g)	2.90	2.90	2.90	2.90	2.90	2.90	2.90	2.90

¹Half of the breeders on 0.3% or 0.5% grower available phosphorus (AvP) remained on their respective AvP and, the other half were changed to the other 0.5% or 0.3% AvP, supplemented with or without 0.2% potassium (K).

²Vitamin premix supplied the following per kg of diet: 6,614 IU vitamin A, 2,000 IU vitamin D₃ (cholecalciferol), 33 IU vitamin E, 0.02 mg vitamin B₁₂, 0.13 mg biotin, 1.98 mg menadione (K₃), 1.98 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 3.97 mg vitamin B₆, 55 mg niacin, and 1.1 mg folic acid.

³Mineral premix supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt, 1 mg.

⁴Selenium premix provided 0.2 mg Se (as Na₂SeO₃) per kg of diet.

Table 4. Broiler breeder female feeding to peak program from 5% lay.

Daily Increase	Feed Allocation ¹
(d)	(g/bird/d)
1	110
2	111
3	112
4	113
5	114
6	115
7	116
8	118
9	121
10	124
11	127
12	131
13	135
14	140
15	145
16	151
17	155

¹Females received daily feed amounts as shown above beginning at 5% rate of lay

Table 5. Fecal moisture of female broiler breeders as affected by different levels of percentage available phosphorus (AvP) and percentage potassium (K) in grow and lay diets during onset of lay.

AvP / K	22 wk	23 wk	24 wk	25 wk	26 wk	27 wk	28 wk	30 wk	
	(%)								
Grow AvP									
0.3	84.5	87.5	86.5	88.9	87.5	84.9	83.1	82.1	
0.5	83.7	86.0	84.8	87.2	85.6	83.8	82.6	82.5	
Lay AvP									
0.3	83.7	85.7	84.6 ^x	87.3	86.0	83.3 ^b	81.9 ^x	82.6	
0.5	84.5	87.8	86.8 ^x	88.8	87.2	85.5 ^a	83.8 ^x	82.1	
Lay K									
0.0	-	87.4	85.9	88.5	87.7 ^a	85.0	82.5	81.5	
0.2	-	86.2	85.5	87.5	85.5 ^b	83.8	83.2	83.1	
SEM ¹	0.86	1.01	0.89	0.81	0.78	0.75	0.77	0.86	
Grow AvP x Lay AvP									
0.3 / 0.3	-	86.2	85.3	87.7	86.6	83.6	82.3	83.4	
0.3 / 0.5	-	88.8	87.8	90.1	88.5	86.3	83.9	80.9	
0.5 / 0.3	-	85.3	83.9	86.9	85.5	82.9	81.6	81.8	
0.5 / 0.5	-	86.8	85.7	87.6	85.8	84.7	83.6	83.3	
Grow AvP x Lay K									
0.3 / 0.0	-	87.9	87.1	89.5	88.9	86.0	83.2	80.9	
0.3 / 0.2	-	87.1	86.0	88.2	86.2	83.9	83.0	83.3	
0.5 / 0.0	-	86.8	84.8	87.6	86.6	84.0	81.9	82.1	
0.5 / 0.2	-	85.3	84.9	86.8	84.7	83.7	83.3	83.0	
Lay AvP x Lay K									
0.3 / 0.0	-	88.2 ^{AB}	86.4 ^{AB}	89.4 ^{AB}	88.3 ^a	85.0 ^{ab}	82.8 ^{ab}	83.0 ^{ab}	
0.3 / 0.2	-	83.3 ^B	82.8 ^B	85.1 ^B	83.8 ^b	81.6 ^b	81.1 ^b	82.2 ^{ab}	
0.5 / 0.0	-	86.5 ^{AB}	85.5 ^{AB}	87.7 ^{AB}	87.2 ^{ab}	85.0 ^{ab}	82.2 ^{ab}	80.1 ^b	
0.5 / 0.2	-	89.1 ^A	88.1 ^A	89.9 ^A	87.1 ^{ab}	86.0 ^a	85.3 ^a	84.1 ^a	
SEM ¹	-	1.43	1.26	1.15	1.11	1.06	1.10	1.23	
				(Probability > F)					
Grow AvP	0.51	0.31	0.18	0.16	0.09	0.29	0.63	0.75	
Lay AvP	0.49	0.15	0.09	0.18	0.32	0.04	0.10	0.67	
Lay K	-	0.40	0.72	0.37	0.04	0.26	0.54	0.19	
Grow AvP x Lay AvP	-	0.69	0.76	0.47	0.47	0.66	0.88	0.11	
Grow AvP x Lay K	-	0.80	0.62	0.81	0.72	0.40	0.46	0.53	
Lay AvP x Lay K	-	0.01	0.01	<0.01	0.04	0.04	0.03	0.05	

^{x,y}Means within a column lacking a common superscript differ ($P < 0.10$).

^{a,b}Means within a column lacking a common superscript differ significantly ($P < 0.05$).

¹SEM = Standard error of mean.

Table 6. Fecal liquid portion of female broiler breeders as affected by different levels of percentage available phosphorus (AvP) and percentage potassium (K) in grow and lay diets during onset of lay.

AvP / K	22 wk	23 wk	24 wk	25 wk	26 wk	27 wk	28 wk	30 wk	
	(%)								
Grow AvP									
0.3	40.6	97.8	74.7	114.1	114.3	68.5	61.5	51.0	
0.5	47.7	84.8	68.9	97.4	88.7	66.7	69.7	58.5	
Lay AvP									
0.3	46.7	77.3 ^y	56.7 ^y	88.4 ^b	84.1 ^y	54.5 ^y	53.2 ^y	48.9	
0.5	41.7	105.3 ^x	86.9 ^x	123.0 ^a	118.9 ^x	80.7 ^x	78.0 ^x	60.6	
Lay K									
0.0	-	99.7	79.9	110.3	113.1	62.0	62.6	48.9	
0.2	-	82.9	63.7	101.1	89.9	73.2	68.6	60.6	
SEM ¹	0.86	11.37	13.34	12.72	12.89	10.76	13.96	10.06	
Grow AvP x Lay AvP									
0.3 / 0.3	44.4	80.1	51.5	93.1	94.5	56.8	50.5	54.1	
0.3 / 0.5	36.9	115.5	97.8	135.0	134.2	80.2	72.6	47.8	
0.5 / 0.3	49.0	74.6	61.9	83.7	73.8	52.2	56.0	43.7	
0.5 / 0.5	46.5	95.1	76.0	111.0	103.6	81.1	83.5	73.3	
Grow AvP x Lay K									
0.3 / 0.0	41.1	107.7	73.5	117.2	125.2	61.4	58.4	42.8	
0.3 / 0.2	40.1	87.9	75.8	111.0	103.5	75.6	64.7	59.1	
0.5 / 0.0	56.4	91.7	86.3	103.5	101.0	62.5	66.9	55.0	
0.5 / 0.2	39.1	78.0	51.6	91.3	76.3	70.8	72.6	62.0	
Lay AvP x Lay K									
0.3 / 0.0		102.1 ^{ab}	71.5	114.0 ^{ab}	114.6 ^{ab}	60.6	64.3 ^{ab}	55.1	
0.3 / 0.2		52.6 ^b	41.9	62.9 ^b	53.6 ^b	48.4	42.1 ^b	42.8	
0.5 / 0.0		97.3 ^{ab}	88.3	106.6 ^{ab}	111.6 ^{ab}	63.3	61.0 ^{ab}	42.8	
0.5 / 0.2		113.3 ^a	85.4	139.4 ^a	126.1 ^a	98.1	95.1 ^a	78.4	
SEM ¹		16.08	18.87	17.99	18.23	15.22	13.96	14.22	
				(Probability > F)					
Grow AvP	0.59	0.42	0.76	0.35	0.16	0.90	0.55	0.59	
Lay AvP	0.70	0.08	0.11	0.05	0.06	0.09	0.08	0.41	
Lay K	0.48	0.30	0.39	0.61	0.20	0.46	0.66	0.41	
Grow AvP x Lay AvP	0.84	0.64	0.39	0.68	0.78	0.85	0.85	0.21	
Grow AvP x Lay K	0.53	0.85	0.33	0.87	0.93	0.84	0.98	0.74	
Lay AvP x Lay K	0.98	0.04	0.48	0.02	0.04	0.12	0.04	0.09	

^{x,y}Means within a column lacking a common superscript differ (P < 0.10).

^{a,b}Means within a column lacking a common superscript differ significantly (P < 0.05).

¹SEM = Standard error of mean.

Table 7. Broiler breeder egg production as affected by different levels of percentage available phosphorus (AvP) and percentage potassium (K) in grow and lay diets during onset of lay.

AvP / K	25 wk	26 wk	27 wk	28 wk	29 wk
	(%)				
Grow AvP					
0.3	52.7	62.4	72.8	86.7	88.0
0.5	46.9	69.6	77.2	84.4	85.3
Lay AvP					
0.3	48.2	65.5	74.1	87.1	86.2
0.5	51.3	66.5	75.9	84.0	87.2
Lay K					
0.0	46.4	71.4	80.9	85.8	88.0
0.2	53.1	60.6	69.1	85.3	85.3
SEM ¹	6.88	6.18	5.15	3.36	2.62
Grow AvP x Lay AvP					
0.3 / 0.3	52.7	61.5	72.4	90.2	86.6
0.3 / 0.5	52.6	63.3	73.3	83.1	89.5
0.5 / 0.3	43.7	69.6	75.9	84.0	85.7
0.5 / 0.5	50.0	69.6	78.6	84.8	84.9
Grow AvP x Lay K					
0.3 / 0.0	50.9	61.5	76.0	84.0	92.1
0.3 / 0.2	54.5	63.3	69.6	89.3	84.0
0.5 / 0.0	42.0	81.2	85.8	87.5	84.0
0.5 / 0.2	51.8	58.0	68.6	81.3	86.6
Lay AvP x Lay K					
0.3 / 0.0	43.8	69.6	79.6	88.5	89.3
0.3 / 0.2	52.6	61.5	68.7	85.7	83.0
0.5 / 0.0	49.1	73.1	82.3	83.0	86.7
0.5 / 0.2	53.6	59.8	69.6	85.0	87.6
SEM ¹	9.73	8.74	7.29	4.76	3.70
			(Probability > F)		
Grow AvP	0.55	0.41	0.54	0.63	0.46
Lay AvP	0.74	0.91	0.80	0.51	0.78
Lay K	0.49	0.22	0.11	0.92	0.47
Grow AvP x Lay AvP	0.74	0.92	0.90	0.41	0.62
Grow AvP x Lay K	0.74	0.15	0.46	0.23	0.15
Lay AvP x Lay K	0.82	0.76	0.90	0.62	0.33

¹SEM = Standard error of mean.

Table 8. Second egg characteristics of broiler breeders as affected by different levels of percentage available phosphorus (AvP) and percentage potassium (K) in grow and lay diets during onset of lay.

AvP / K	Egg Wt.	Yolk Wt.	Albumen wt.	Yolk:Alb Ratio	Shell Wt.	Shell Thickness
	(g)			(g:g)	(g)	(mm)
Grow AvP						
0.3	48.42 ^a	12.44	31.64 ^a	0.395	4.33	0.355
0.5	46.30 ^b	12.17	29.91 ^b	0.408	4.16	0.349
Lay AvP						
0.3	47.96	12.43	31.24	0.399	4.27	0.353
0.5	46.77	12.19	30.30	0.404	4.22	0.351
Lay K						
0.0	47.69	12.39	30.95	0.403	4.28	0.352
0.2	47.04	12.22	30.59	0.400	4.21	0.351
SEM ¹	0.69	0.21	0.50	0.007	0.08	0.004
Grow AvP x Lay AvP						
0.3 / 0.3	48.84	12.62	31.83	0.399	4.38	0.356
0.3 / 0.5	48.00	12.27	31.44	0.391	4.28	0.354
0.5 / 0.3	47.08	12.25	30.66	0.399	4.16	0.350
0.5 / 0.5	45.53	12.10	29.16	0.418	4.15	0.347
Grow AvP x Lay K						
0.3 / 0.0	48.99	12.59	32.03	0.395	4.36	0.357
0.3 / 0.2	47.85	12.30	31.24	0.395	4.30	0.353
0.5 / 0.0	46.38	12.20	29.87	0.411	4.19	0.347
0.5 / 0.2	46.23	12.14	29.95	0.406	4.13	0.350
Lay AvP x Lay K						
0.3 / 0.0	48.76	12.44	31.94	0.391	4.37	0.355
0.3 / 0.2	47.16	12.42	30.55	0.407	4.18	0.351
0.5 / 0.0	46.61	12.35	29.96	0.414	4.19	0.350
0.5 / 0.2	46.92	12.02	30.64	0.394	4.25	0.352
SEM ¹	0.98	0.30	0.71	0.01	0.12	0.006
			(Probability > F)			
Grow AvP	0.03	0.38	0.01	0.19	0.17	0.37
Lay AvP	0.23	0.42	0.19	0.62	0.65	0.73
Lay K	0.51	0.58	0.62	0.78	0.60	0.92
Grow AvP x Lay AvP	0.71	0.75	0.44	0.17	0.70	0.89
Grow AvP x Lay K	0.61	0.71	0.54	0.80	1.00	0.62
Lay AvP x Lay K	0.33	0.61	0.15	0.07	0.32	0.70

^{a,b}Means within a column lacking a common superscript differ significantly ($P < 0.05$).

¹SEM = Standard error of mean.

Table 9. Tenth egg characteristics of broiler breeders as affected by different levels of percentage available phosphorus (AvP) and percentage potassium (K) in grow and lay diets during onset of lay.

AvP / K	Egg Wt.	Yolk Wt. (g)	Albumen wt.	Yolk:Alb Ratio (g:g)	Shell Wt. (g)	Shell Thickness (mm)
Grow AvP						
0.3	53.99	14.68 ^x	34.19	0.430	5.10	0.380
0.5	52.78	14.04 ^y	33.85	0.415	5.00	0.378
Lay AvP						
0.3	53.27	14.33	33.96	0.423	5.07	0.380
0.5	53.50	14.39	34.08	0.422	5.03	0.377
Lay K						
0.0	53.81	14.45	34.28	0.422	5.08	0.378
0.2	52.96	14.27	33.76	0.424	5.02	0.380
SEM ¹	0.66	0.26	0.46	0.008	0.08	0.004
Grow AvP x Lay AvP						
0.3 / 0.3	53.82	14.72	34.01	0.434	5.08	0.379
0.3 / 0.5	54.15	14.64	34.37	0.427	5.13	0.381
0.5 / 0.3	52.72	13.95	33.91	0.413	5.07	0.382
0.5 / 0.5	52.85	14.14	33.79	0.418	4.93	0.373
Grow AvP x Lay K						
0.3 / 0.0	54.88	14.99	34.73	0.433	5.15	0.379
0.3 / 0.2	53.09	14.37	33.66	0.428	5.05	0.381
0.5 / 0.0	52.74	13.91	33.83	0.411	5.01	0.377
0.5 / 0.2	52.83	14.18	33.87	0.420	4.99	0.378
Lay AvP x Lay K						
0.3 / 0.0	54.18	14.47	34.54	0.419	5.16	0.381
0.3 / 0.2	52.36	14.20	33.38	0.428	4.99	0.380
0.5 / 0.0	53.44	14.43	34.02	0.425	5.01	0.375
0.5 / 0.2	53.56	14.35	34.15	0.420	5.06	0.379
SEM ¹	0.93	0.37	0.65	0.01	0.12	0.006
			(Probability > F)			
Grow AvP	0.20	0.09	0.60	0.19	0.40	0.69
Lay AvP	0.80	0.88	0.85	0.92	0.73	0.64
Lay K	0.36	0.63	0.43	0.84	0.61	0.79
Grow AvP x Lay AvP	0.91	0.70	0.71	0.61	0.43	0.40
Grow AvP x Lay K	0.31	0.24	0.40	0.51	0.75	0.90
Lay AvP x Lay K	0.30	0.78	0.33	0.55	0.36	0.71

^{a,b}Means within a column lacking a common superscript differ significantly ($P < 0.05$).

¹SEM = Standard error of mean.

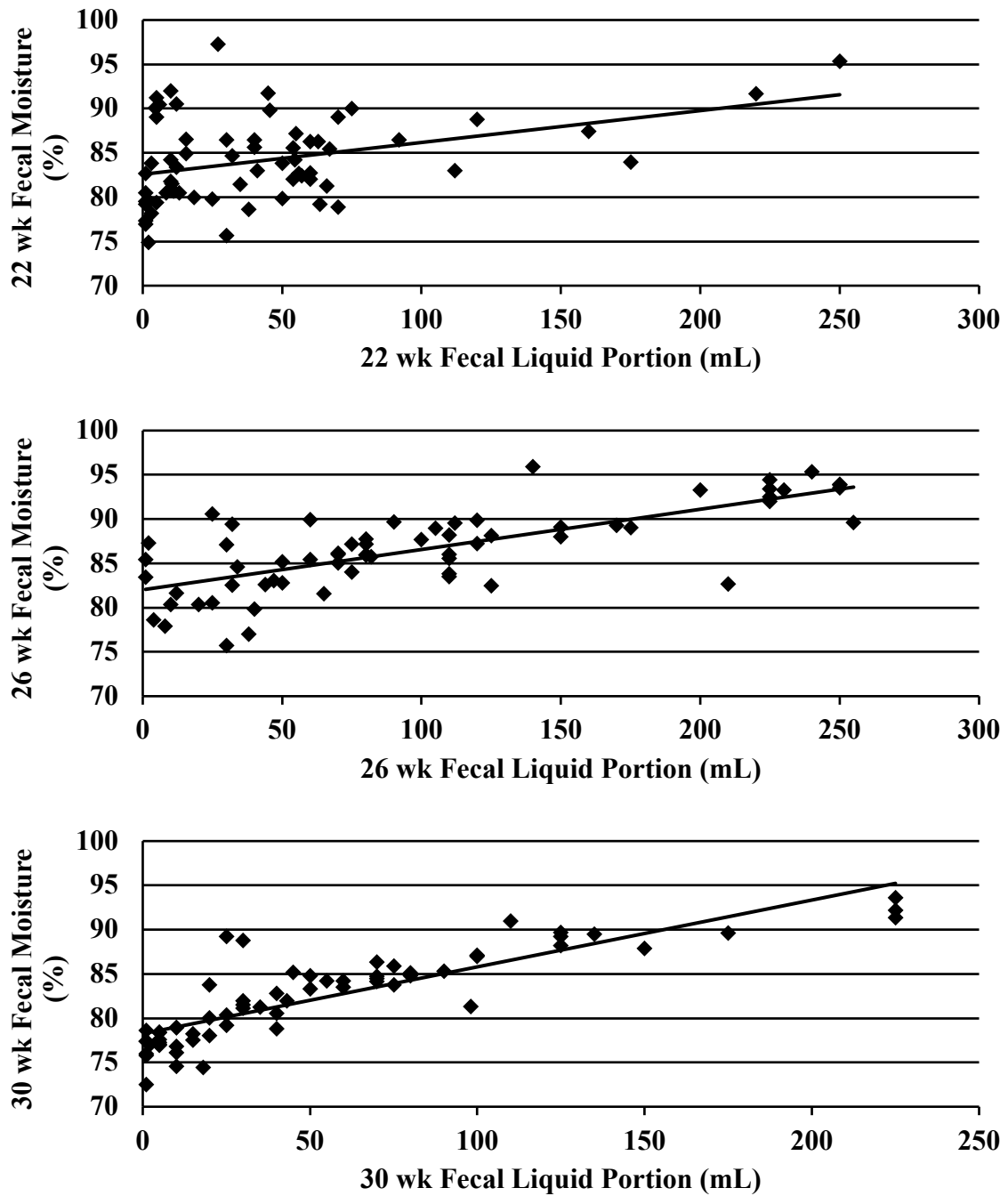


Figure 2. Correlation between fecal liquid portion and fecal moisture at A) 22 wk ($P < 0.01$; $R^2 = 0.14$) B) 26 wk ($P < 0.01$; $R^2 = 0.54$), and C) 30 wk ($P < 0.01$; $R^2 = 0.72$).

REFERENCES

- Adams, A. W., F. E. Cunningham, and L. L. Munger. 1975. Some effects on layers of sodium sulfate and magnesium sulfate in their drinking water. *Poult. Sci.* 54:707-714.
- Ait-Boulahsen, A., J. D. Garlich, and F. W. Edens, F.W. 1989. Effect of fasting and acute heat stress on body temperature, blood acid base balance and electrolytes status in chickens. *Comp. Biochem. and Physiol.* 94:683-687.
- Ait-Boulahsen, A., J. D. Garlich, and F. W. Edens, F.W. 1995. Potassium chloride improves the thermotolerance of chickens exposed to acute heat stress. *Poult. Sci.* 74:75-87.
- Akil, R., and A. H. Zakaria. 2015. Egg laying characteristics, egg weight, embryo development, hatching weight and post-hatch growth in relation to oviposition time of broiler breeders. *Animal Reproduction Science* 156:103-110.
- Arguelles-Ramos, M. 2011. Effects of Phyzyme XP and Avizyme 1502 on the Performance of Broiler Breeders and their Progeny. Page 53. Ph.D. Dissertation, The Graduate School, North Carolina State University, Raleigh, NC.
- Balnave, D., and Muheereza, S.K. 1997. Improving eggshell quality at high temperatures with dietary sodium bicarbonate. *Poult. Sci.* 76:588-593.
- Bar, A. 1976. Regulation of intestinal calcium absorption in the laying Quail: Independent of kidney vitamin D hydroxylation. *J. Nutr.* 106: 1336-1342.
- Belay, T., K. E. Bartels, C. J. Wiernusz, and R. G. Teeter. 1993. A detailed colostomy procedure and its application to quantify water and nitrogen balance and urine contribution to thermobalance in broilers exposed to thermoneutral and heat-distressed environments. *Poult. Sci.* 72:106–115.
- Belay, T., and R. G. Teeter. 1993. Broiler water balance and thermobalance during thermoneutral and high ambient temperature exposure. *Poult. Sci.* 72:116-124.

- Benton, C. E., D. Balnave, J. Brake, L. J. Boyd, and M. A. Brown. 1998. The use of dietary minerals during heat stress in broilers. *Prof. An. Sci.* 14:193-196.
- Bomsztyk, K., and F. D. Wright. 1986. Dependence of ion fluxes on fluid transport by rat proximal tubule. *Am. J. Physiol.-Renal Physiology.* 250:680-689.
- Borges, S. A., A. V. F. Da Silva, and A. Maiorka. 2007. Acid-base balance in broilers. *World's Poult. Sci. J.* 63:73-81.
- Borges, S. A., A. V. Fischer da Silva, A. Majorca, D. M. Hooge, and K. R. Cummings. 2004. Physiological responses of broiler chickens to heat stress and dietary electrolyte balance (sodium plus potassium minus chloride, milliequivalents per kilogram). *Poult. Sci.* 83:1551-1558.
- Braun, E. J. 2003. Regulation of renal and lower gastrointestinal function: role in fluid and electrolyte balance (A review). *Comp. Biochem. and Physiol.* 136:499-505.
- Choi, J. H., R. D. Miles and R. H. Harms. 1979. The response of serum inorganic phosphorus level in laying hens fed low levels of dietary phosphorus. *Poult. Sci.* 58:416-418.
- Cohen, I., and S. Hurwitz. 1974. The response of blood ionic constituents and acid-base balance to dietary sodium, potassium and chloride in laying fowls. *Poult. Sci.* 53:378-382.
- Dantzler, W. H., and E. J. Braun. 1980. Comparative nephron function in reptiles, birds, mammals. *Am. J. Physiol.* 239:197-230.
- Dawson, W. R. 1975. Avian physiology. *Annu. Rev. Physiol.* 37:441-465.
- Deyhim, F., T. Belay, and R. G. Teeter. 1990. The effect of heat distress on blood gas, plasma and urine concentration of Na, K, Cl of broiler chicks. *Poult. Sci.* 69:42 (Abstract).
- Eichner, G., S. L. Vieira, C. A. Torres, J. L. B. Coneglian, D. M. Freitas, and O. A. Oyarzabal. 2007. Litter moisture and footpad dermatitis as affected by diets formulated on an all-

- vegetable basis or having the inclusion of poultry by-product. *J. Appl. Poult. Res.* 16:344-350.
- Enting, H., J. de los Mozos, A. G. del Alamo and P. P. de Ayala. 2009. Influence of minerals on litter moisture. Proceedings of the 17th European Symposium on Poultry Nutrition, August 23-27, Edinburgh, UK. pp: 47-52.
- Ekmay, R. D., and C. N. Coon. 2010. An examination of the P requirements of broiler breeders for performance, progeny quality and P balance 1. Non-phytate phosphorus. *Int. J. Poult. Sci.* 9:1043-1049.
- Ekmay, R. D., C. Salas, J. England, S. Cerrate and C. N. Coon. 2012. The effects of body weight, dietary nonphytate phosphorus intake, and breeder feeding regimen on production performance, chick quality, and bone remodeling in broiler breeder. *Poult. Sci.* 91:948-964.
- Gernat, A. G., and J. Brake. 2007. Effects of phosphorus level and phytase in broiler breeder rearing and laying diets on live performance and phosphorus excretion. *Poult. Sci.* 86:225-231.
- Goldstein, D. L., and E. Skadhauge. 2000. Renal and extrarenal regulation of body fluid composition. In *Sturkie's Avian Physiology* (ed. G. C. Whittow), pp. 265–297. Academic Press, San Diego.
- Goldstein, D. L. 2006. Regulation of the avian kidney by arginine vasotocin. 2006. *Gen. Comp. Endocr.* 147:78-84.
- Guo, X., K. Huang, F. Chen, J. Luo and C. Pan. 2008. High dietary calcium causes metabolic alkalosis in egg-type pullets. *Poult. Sci.* 87:1353-1357.

- Hamilton, R. M. G. 1982. Methods and factors that affect the measurement of egg shell quality. *Poult. Sci.* 61:2002-2039.
- Hamm, L. L., and E. E. Simon. 1987. Roles and mechanisms of urinary buffer excretion. *Am. J. Physiol.* 253:595-605.
- Hamm, L. L., and E. E. Simon. 1987. Roles and mechanisms of urinary buffer excretion. *Am. J. Physiol.* 253:595-605.
- Halley, J. T., T. S. Nelson, L. K. Kirby, and Z. B. Johnson. 1987. Effect of altering dietary mineral balance on growth, leg abnormalities, and blood base excess in broiler chicks. *Poult. Sci.* 66:1684-1692.
- Halley, J. T., S. Cerrate, A. Corzo, and B. Fancher. 1995. Effect of altering dietary electrolyte balance using sodium bicarbonate and potassium carbonate on broiler breeder performance and egg shell parameters. *Poult. Sci.* 95 (E-Suppl. 1): 276.
- Harms, R. H., S. Bootwalla and, H. R. Wilson. 1984. Performance of broiler breeder hens on wire and litter floors. *Poult. Sci.* 63:1003-1007.
- Harms, R. H., K. K. Kuchinski, D. R. Sloan, and G. B. Russell. 1995. Sodium requirement for broiler breeder hens. *Poult. Sci.* 74:1311-1316.
- Hocking, P. M., A. B. Gilbert, M. Walker, and D. Waddington, 1987. Ovarian follicular structure of White Leghorns fed ad libitum and dwarf and normal broiler breeder pullets fed ad libitum or restricted until point of lay. *Br. Poult. Sci.* 28:495-506.
- Hodges, R.D. 1970. Blood pH and cation levels in relation to egg-shell formation. 1970. *Annales de biologie animale, biochimie, biophysique.* 10:199-213.
- Hopkinson, W.I., Griffiths, G.L., Jessop, D. and W. Williams. 1983. Sudden death syndrome in broiler breeders. *Aus. Vet. J.* 60:192-193.

- Hopkinson, W. I., D. Jessop, D. A. Pass and D.W. Pethick. 1990. Concentrations of plasma potassium and sodium during the life of a broiler breeder flock, *Avian Pathol.* 19:4:607-611.
- Hurwitz, S., and A. Bar. 1965. Absorption of calcium and phosphorus along the gastrointestinal tract of the laying fowl as influenced by dietary calcium and egg shell formation. *J. Nutr.* 86:433-438.
- Hurwitz, S., and A. Bar. 1970. Sites of calcium and phosphate absorption in the chick. *Poult. Sci.* 49:324-325.
- Hurwitz, S., and A. Bar. 1971. The effect of pre-laying mineral nutrition on the development, performance and mineral metabolism of pullets. *Poult. Sci.* 50:1044-1055.
- Jonchère, V., A. Brionne, J. Gautron and Y. Nys. 2012. Identification of uterine ion transporters for mineralization precursors of the avian eggshell. *BMC. Physiol.* 12:10.
- Koelkebeck, K.W., P. C. Harrison, and T. J. Madindou. 1993. Effect of carbonated drinkingwater on production performance and bone characteristics of laying hens exposed to high environmental temperatures. *Poult. Sci.* 72:1800-1803.
- Larsen, E. H., N. Mobjerg, and J. N. Sorensen. 2006. Fluid transport and ion fluxes in mammalian kidney proximal tubule: a model analysis of isotonic transport. *Acta Physiol.* 187:177-189.
- Lavery, G., and E. Skadhauge. 1999. Physiological roles and regulation of transport activities in the avian lower intestine. *J. Exp. Zool.* 283:480-494.
- Lavery, G., and E. Skadhauge. 2008. Adaptive strategies for post-renal handling of urine in birds. *Am. J. Physiol. Reg, Integr. Comp. Physiol.* 149:246-254.
- Leech, R.M. 1974. Studies on the potassium requirement of the laying hen. *J. Nutr.* 104:684-686.

- Leeson, S., and J. D. Summers. 1987. Effect of dietary calcium levels near the time of sexual maturity on water intake and excreta moisture content. *Poult. Sci.* 66:1918-1923
- Leytem, A. B., P. W. Plumstead, R. O. Maguire, P. Kwanyuen, and J. Brake. 2007. What aspect of dietary modification in broilers controls litter water-soluble phosphorus: dietary phosphorus, phytase, or calcium? *J. Environ. Qual.* 36:453-63.
- Leytem, A. B., P. W. Plumstead, R. O. Maguire, P. Kwanyuen, J. W. Burton, and J. Brake. 2008. Interaction of calcium and phytate in broiler diets. 2. Effects on total and soluble phosphorus excretion. *Poult. Sci.* 87:459-467.
- Li, X., D. Zhang and W. L. Bryden. 2017. Calcium and phosphorus metabolism and nutrition of poultry: are current diets formulated in excess? *Anim. Prod. Sci.* 57:2304-2310.
- Lilburn, M. S., and D. J. Myers-Miller, 1990. Effect of body weight, feed allowance, and dietary protein intake during the prebreeder period on early reproductive performance of broiler breeder hens. *Poult. Sci.* 69: 1118–1125.
- Long, S., and E. Skadhauge. 1983. Renal acid excretion in the domestic fowl. *J. Exp. Biol.* 104:51-58.
- Maguire, R. O., P. W. Plumstead, and J. Brake. 2005. Impact of diet, moisture, location, and storage on soluble phosphorus in broiler breeder manure. *J. Environ. Qual.* 35:858-865.
- Manangi, M. K., and C. N. Coon. 2006. Effects of varied levels of dietary nonphytate P and Ca on P excretion and relationship between plasma inorganic P and urinary excretion of P in broilers. *Poult. Sci.* 85(E-Suppl. 1):168.
- Manangi, M. K., F. D. Clark, and C. N. Coon. 2007. Improved colostomy technique and excrement (urine) collection device for broilers and broiler breeder hens. *Poult. Sci.* 86:698-704

- Mardsen A., and T. R. Morris. 1987. Quantitative review of the effects of environmental temperature on food intake, egg output, and energy balance in laying pullets. *Br. Poult. Sci.* 28:693-704.
- Mongin, P., and B. Sauveur. 1974. Hourly water consumption and egg formation in the domestic fowl. *Br. Poult. Sci.* 15:361-368.
- Mongin, P., 1980. Electrolytes in nutrition. A review of basic principles and practical applications in poultry and swine. Pages 1-15 in: *Proc. Third Annu. Int. Miner. Conf.* Orlando, FL.
- Mongin, P. 1981. Recent advances in dietary cation-anion balance: applications in poultry. *Proc. Nutr. Soc., Cambridge*, 40:285-294.
- Mizgala, C. L., and G. A. Quamme. 1985. Renal handling of phosphate. *Physiol. Rev.* 65:431-466.
- Naves, L. D. P., P. B. Rodrigues, C. Meneghetti, V. M. P. Bernardino and D. H. de Oliveira. 2016. Efficiency of microbial phytases in diets formulated with different calcium:phosphorus ratios supplied to broilers from 35 to 42 days of age. *J. Appl. Anim. Res.*, 44: 446-453. 13.
- Nesheim, M.C., R. M. Leach, Jr, T. R. Zeigler, and J. A. Serafin. 1964. Interrelationships between dietary levels of sodium, chlorine and potassium. *J. of Nutr.* 84:361-66.
- NRC. 1994. National Research Council. Nutrient requirements of poultry. 8th ed. National Academic Press, Washington, DC.
- Nys, Y., J. Gautron, M. D. McKee, J. M. Garcia-Ruiz and M. Hincke. 2001. Biochemical and functional characterization of eggshell matrix proteins. *World's Poult. Sci. J.* 57:401-403.

- Nys, Y., B. Sauveur, L. Lacassagne, and P. Mongin. 1976. Food, calcium and water intakes by hens lit continuously from hatching. *Br. Poult. Sci.* 17:351-358.
- Odom, T.W., P. C. Harrison, and W. G. Bottje. 1986. Effects of thermal-induced respiratory alkalosis on blood ionized calcium in the domestic hen. *Poult. Sci.* 65:570-573.
- Ogunji, P. A., R. N. Brewer, D. A. Roland, and D. Caldwell. 1983. Effect of dietary sodium chloride, protein, and strain difference upon water consumption and fecal moisture content of broiler breeder males. *Poult. Sci.* 62:2497-2500.
- Oliveira, J. E., L. F. T. Albino, H. S. Rostagno, L. E. Páez, and D. C. O. Carvalho. 2005. Dietary levels of potassium for broiler chickens. *Braz. J. Poult. Sci.* 7:33-37.
- Orloff, J., and D. G. Davidson. 1959. The mechanism of potassium excretion in the chicken. *J. of Clin. Invest.* 38:21-30.
- Palmer, B. F. 2015. Regulation of potassium homeostasis. *Clin. J. Am. Soc. Nephrol.* 10:1050-1060.
- Patterson, P.H., M. L. Sunde, and J. L. Pimentel. 1989. Water consumption and fecal moisture of laying hens fed wheat middlings and corn-soybean-alfalfa meal diets. *Poult. Sci.* 68:830-833.
- Pelicia, K., E. A. Garcia, A. B. G. Faitarone, A. P. Silva, D. A. Berto, A. B. Molino and F. Vercese. 2009. Calcium and available phosphorus levels for laying hens in second production cycle. *Br. J. Poult. Sci.* 11:39-49.
- Plumstead, P. W., H. Romero-Sanchez, R. O. Maguire, A. G. Gernat, and J. Brake. 2007. Effects of phosphorus level and phytase in broiler breeder rearing and laying diets on live performance and phosphorus excretion. *Poult. Sci.* 86:225-231.

- Plumstead, P. W., A. B. Leytem, R. O. Maguire, J. W. Spears, P. Kwanyuen, and J. Brake. 2008. Interaction of Calcium and Phytate in Broiler Diets. 1. Effects on Apparent Prececal Digestibility and Retention of Phosphorus. *Poultry Science*.
- Reece, W.O., J. L. Sell, and D. W. Trampel. 2000. Effects of dietary potassium supplementation for growing turkeys on leg weakness, plasma potassium concentration and selected blood variables. *Poult. Sci.* 79:1120-1126.
- Rice, G. E., and E. Skadhauge. 1982. *J. Comp. Physiol.* 147:65-69.
- Rinehart, K.E., Featherston. W.R. and J. C. Rogler. 1969. Influence of dietary potassium on chick growth, food consumption and blood and tissue composition. *Poult. Sci.* 48:320-325.
- Rinehart, K. E., and J. C. Rogler. 1967. Effects of a dietary potassium deficiency on protein synthesis in the young chick. *J. Nutr.* 95:627–632.
- Robinson, F. E., N. A. Robinson, and R. T. Hardin. 1995. The effects of 20-week body weight and feed allocation during early lay on female broiler breeders. *J. Appl. Poult. Res.* 4:203-210.
- Roland, D. A., R. H. Harms. 1974. Specific gravity of eggs in relation to egg weight and time of oviposition. *Poult. Sci.* 53:1494–1498.
- Roland, D. A., and D. Caldwell. 1985. Relationship of calcium to wet droppings in laying hens. *Poult. Sci.* 64:1809-1812.
- Roland, Sr. D. A., 1986a. Egg shell quality. II. Importance of time of calcium intake with emphasis on broiler breeders. *World Poult. Sci. J.* 40:255-259.
- Roland, D. A., Sr., 1986b. Egg shell quality III: Calcium and phosphorus requirements of commercial leghorns. *World Poult. Sci. J.* 42:154-163.
- Ruiz-Lopez, B., and R. E. Austin. 1993. The effect of selected minerals on the acid-base balance of growing chicks. *Poult. Sci.* 72: 1054-1062.

- Satlin, L. M. 2009. Potassium. Chapter 8:185-204. Springer-Verlag Berlin Heidelberg.
- Sauveur, B., and P. Mongin. 1978. Interrelationships between dietary concentrations of sodium, potassium and chloride in laying hens. *Br. Poult. Sci.* 19:475-485.
- Skadhauge, E. 1981. *Osmoregulation in Birds*. Springer, New York.
- Smith, M.O. and R. G. Teeter. 1987. Potassium balance of the 5 to 8 week-old broiler exposed to constant heat or cycling high temperature stress and the effects of supplemental potassium chloride on body weight gain and feed conversion. *Poult. Sci.* 66:487-492.
- Smith, A. 1996. The effect of dietary composition on the water balance of laying hens. Ph.D. Thesis, Harper Adams Agricultural College.
- Smith, A., S. P. Rose, R. G. Wells and V. Pirgozliev. 2000. Effect of excess dietary sodium, potassium, calcium and phosphorus on excreta moisture of laying hens. *Br. Poult. Sci.* 41:598-607.
- Stallone, J.N., and E. J. Braun. 1986. Contributions of glomerular and tubular mechanisms to antidiuresis in domestic fowl. *Am. J. Physiol.* 249, F842–F850.
- Taylor T.G., and J. Kirkley. 1967. The absorption and excretion of minerals by laying hens in relation to egg shell formation. *Br. Poult. Sci.* 84:289-295.
- van der Hoeven-Hangoor, E., N. D. Paton, I. B. van de Linde, M. W.A. Verstegen, and W. H. Hendriks. 2013. Moisture content in broiler excreta is influenced by excreta nutrient contents. *J. Anim. Sci.* 91:5705-5713.
- Wilson, R.M., M. Wareing, and R. Green. 1997. The role of active transport in potassium reabsorption in the proximal convoluted tubule of the anaesthetized rat. *J. Physiol.* 500.1:155-164.
- Wingo, C. S., and B. D. Cain. 1993. The renal H-K-ATPase: Physiological significance and role in potassium homeostasis. 1993. *Annu. Rev. Physiol.* 55:323-347.

- Zakaria, A. H., P. W. Plumstead, H. Romero-Sanchez, N. Leksrisompong, J. Osborne, and J. Brake. 2005. Oviposition pattern, egg weight, fertility, and hatchability of young and old broiler breeders. *Poult. Sci.* 84:1505–1509.
- Zakaria, A. H., P. W. Plumstead, H. Romero-Sanchez, N. Leksrisompong, and J. Brake. 2009. The effects of oviposition time on egg weight loss during storage and incubation, fertility, and hatchability of broiler hatching eggs. *Poult. Sci.* 88:2712-2717.
- Ziaei, N. J. H. Guy, S. A. Edwards, P. J. Blanchard, J. Ward, and D. Feuerstein. 2007. Effect of gender on factors affecting excreta dry matter content of broiler chickens. *J. Appl. Poult. Res.* 16:226-233.
- Ziaei, N., J. H. Guy, S. A. Edwards, P. J. Blanchard, J. Ward, and D. Feuerstein. 2008. Effect of reducing dietary mineral content on growth performance, water intake, excreta dry matter content and blood parameters of broilers. *Br. Poult. Sci.* 49:195–201.

MANUSCRIPT II. Effect of Dietary Limestone Particle Size and Potassium on Live Performance and Blood Physiology of Male Broiler Chickens.

ABSTRACT

The experiment investigated the effects of limestone particle size and dietary potassium (K) on live performance, blood physiology and muscle myopathies. Limestone particle size was defined as fine (~0.2 mm) or coarse (~0.9 mm) using the US sieve method. A total of 384 Ross male broilers were placed into 24 floor pens and fed four diets in starter (0-16 d of age) and grower (16-33 d of age) phases, containing two limestone particle sizes (fine and coarse) and amended with either 0% basal K (K-) or 0.2% added dietary K (K+) as potassium carbonate to complete the 2 x 2 factorial arrangement. Live performance was measured from 1-33 d of age, blood physiology, hot carcass data, woody breast (WB) and white striping (WS) scores were measured at 35 d of age. The K+ dietary treatment reduced ($P < 0.05$) feed intake, BW and BW gain when compared to the K- during the starter and grower periods. The K+ dietary treatment decreased ($P < 0.05$) blood Na (mmol/L), blood glucose (mg/dL), ionized blood Ca (mg/dL), CO₂ (mmol/L), blood HCO₃ (mmol/L), and base excess in extracellular fluid (mmol/L) when compared to K- of birds of similar BW at 35 d of age. Fine limestone diets reduced ($P = 0.08$) WB scores (2.59 vs. 3.0) when compared to coarse limestone fed diets at 35 d of age.

Key Words: Limestone particle size, potassium, blood physiology, myopathies.

INTRODUCTION

Potassium (K) is the primary cation in the intracellular fluid (ICF), while sodium (Na) and chloride (Cl) are the primary ions in the extracellular fluid (ECF). Each of these ions is involved in the regulation of acid-base balance via extracellular and intracellular buffering (Kaneko *et al.*, 1989). The physiological role of K includes maintaining cell membrane potential, osmotic regulation, glucose uptake and activation of Na-K-ATPase, the sodium/potassium pump for cation transport (Satlin, 2009; Skou, 1965). The NRC (1994) suggested K requirement of broilers is 0.30%; however, Oliveira *et al.* (2005) reported that the optimum BW gain was achieved with K levels of 0.63% from 8-21 d of age, 0.71% from 22-42 d of age, and 0.80% from 43-53 d of age. For maintaining optimum live performance in broilers practical diets are formulated with K levels that are two to three times greater than suggested by the NRC (1994).

The pH in ECF (*i.e.* H ion concentration) is highly regulated. The changes in blood pH are also known to change the relative Na, K and Cl concentration in the blood. Allen *et al.* (2018) described the retention of the Na⁺, and K⁺ and Cl⁻ ions as a compensatory mechanism to offset metabolic or respiratory alkalosis or acidosis depending on blood or tissue pH. Scott *et al.* (2001) described the ratio balance of Na, K and Cl as being more important than the total nutritional value of the individual electrolytes. Mongin (1980) described the electrolyte balance, which is also known as dietary electrolyte balance (DEB), as the formula Na+K-Cl expressed in mEq/kg. The rationale was that the electrolytes Na, K and Cl likely participated in homeostasis of acid-base balance and were easily formulated in the diets. Mongin (1981) suggested the optimal requirement of DEB as 250 mEq/kg.

Edens, (1977; cited by Mujahid *et al.* 2009) reported that blood K levels can be altered by release of stored K from the ICF or leaked from the cells during heat stress. Apart from

thermal stress and hormonal factors, K salts were also shown to influence the blood K status when the manipulations in DEB were achieved by supplementation of K salts such as KCl, KHCO_3 and K_2CO_3 (Teeter and Smith, 1986; Ait-Boulahsen *et al.*, 1995). These studies indicated close relationships between supplemental K, DEB influenced by K and changes in blood chemistry that could be used to assess optimum DEB for acid-base homeostasis during heat stress conditions in broilers. The bird's acid-base status can be assessed by measuring the blood gases and electrolytes (Ait-Boulahsen *et al.*, 1995; Olanrewaju *et al.*, 2006; Schaal *et al.*, 2016; Livingston, 2018).

Recently, Livingston (2018) and Wu (2018) described incidences of muscle myopathies in broilers as Wooden Breast Muscle (WB) that increased the palpable hardness of pectoralis major muscle and White Striping (WS), a myopathy characterized with the presence of white striations parallel to breast muscle fibers (pectoralis major). The WB and WS muscle myopathies have been associated with fast-growing high-yield broiler chickens (Kuttappan *et al.*, 2012; Petracci *et al.*, 2015). Livingston (2018) suggested that the acid-base imbalance may be associated with the muscle myopathies in fast-growing broilers due to altered muscle contraction when the electrical potential of the muscle cell is disrupted due to movement of K from ICF to compensate for altered DEB.

Plumstead *et al.* (2018) described the interaction of P with Ca to form calcium phosphate in the intestinal lumen, which reduced water soluble P and thus reduced the buffering capacity. Anwar (2016) showed that coarse limestone had lower Ca solubility when compared to fine limestone. Hence it could be hypothesized that coarse limestone slows Ca release in the gizzard and would yield less Ca phosphate complexes and aid in phosphate buffering capacity.

This study evaluated the effects of dietary limestone particle size and levels of K on live performance (1-33 d of age), blood physiology and breast muscle WB and WS scores of fast-growing broilers at 35 d of age. The hypothesis tested was altering limestone particle size and addition of K as K_2CO_3 will affect the broiler performance and blood physiology.

MATERIALS AND METHODS

Animal Welfare

The experimental protocol used in this study was approved by the North Carolina State University Institutional Animal Care and Use Committee. This experiment was designed and conducted in compliance with the Guide for Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Bird Management and Data Collection

Ross 708 X YPM (yield PM male) chicks were hatched from eggs collected from the resident broiler breeder flock at the North Carolina State University Chicken Education Unit. After hatching the chicks were feather-sexed, and only male broilers were utilized. A total of 384 chicks were identified with neck tags and placed randomly into 24 1.2 m x 1.8 m floor pens, with 16 chicks/pen for a stocking density of 0.135 m²/bird in the enclosed, fan ventilated house. Pen floors were topped up with 4 cm new pinewood shavings. Each pen had 2 tube feeders and 1 bell drinker; 2 plastic font drinkers were used during the first 7 d after placement, and 3 feeder trays were initially placed in every pen, then reduced to 2 feeders at 5 d, and to 1 at 7 d. The remaining supplemental drinker and feeder were removed at 9 d, and contents of the trays were screened and dumped back into the tube feeders. One tube feeder was used during the starter period and total starter feed was equalized to 0.908 kg per live chick at 7 d. At 16 d, the second tube feeder was added, and a total of 2.72 kg/bird of grower feed was added to each tube feeder in the increment of 0.908 kg/bird until termination of the study at 33 d. For each

dietary phase, the amount of feed supplied to each replicate pen was controlled and adjusted for mortality to assure that all broilers in each replicate pen had similar access to each dietary treatment following complete intake of the starter dietary phase. Feeders were shaken twice daily to maintain uniform and adequate flow of feed from the tubes into the pans until 33 d of age. Shavings were replaced as needed. Floor temperature at chick placement was 96°F, which was reduced to 90°F within 24 h. Air temperature was reduced by approximately 1°F each day until 7 d of age, and was then maintained at approximately 85°F from 8 to 14 d, 80°F from 15 to 21 d, and 75°F from 16 to 33 d. Artificial light was provided by 18-watt fluorescent bulbs. The lighting program during the first 7 d was 23 h of light, which was then reduced to 22 h of light to 14 d, and 20 h of light to 21 d. After 21 d of age, only 15 h of light was used.

Live Performance

Pen body weight (BW), and feed consumption were recorded at placement, 16 d and 33 d for calculation of average BW and average feed intake per bird for each replicate pen. Mortality was weighed and recorded twice daily as necessary. After accounting for the BW of all deceased birds, total BW gain and total feed intake for each pen were used to calculate mortality-adjusted feed conversion ratio (FCR) for each dietary feeding phase.

Blood Physiology Measurements

At 35 d of age average BW per pen was determined and 4 birds near the average BW of each pen were selected and blood was drawn individually from a total of 64 birds. Approximately 2 mL of blood was drawn from the left branchial vein and analyzed using the i-Stat® handheld blood analyzer fitted with a CG8+ cartridge (Abbott Point of Care Inc., Princeton, NJ), which measured packed cell volume (PCV), hemoglobin (Hgb), ionized calcium (iCa), glucose (Glu), Na, K, pH, partial pressure of carbon dioxide (pCO₂), bicarbonate (HCO₃), total carbon dioxide (TCO₂), base excess in the extracellular fluid

(BEecf), partial pressure of oxygen (pO₂), and oxygen saturation of hemoglobin (sO₂) (Martin *et al.*, 2010). A systematic approach to catching broilers, drawing of blood, analysis, and re-introduction to the pen was utilized to assure uniform collection of blood and handling of broilers.

Carcass Measurements

The WB and WS scoring system developed for the Pectoralis major using a one to four-point ordinal scale of measurement described by Livingston *et al.* (2018) was used to determine myopathies. The WB scoring system comprised a hand palpation method where a score of 1 indicated normal or no signs of WB. A score of 2 indicated some firming or hardening of the breast with over 50% of non-affected tissue being pliable. A WB score of 3 indicated that more than 50% of the breast was hard and resisted palpation but with some pliability still present. A WB of 4 indicated no presence of pliability and over 90% of the breast hard to the touch. The WS scoring system was similar with a score of 1 for normal breast tissue or no signs of striping. A WS score of 2 indicated a mild amount of visible striations, a WS score of 3 indicated a moderate amount of striping, and a WS score of 4 indicated severe striations across the ventral portion of the boneless skinless breast fillet. At 35 d of age all birds in the 24 pens with 16 birds/pen were euthanized by cervical dislocation, BW was recorded individually, and hot carcass was scored for WB and WS.

Dietary Treatments and Feed Compounding

A total of 4 corn-soybean meal-based dietary treatments were fed in the starter (0-16 d of age) and grower (16-33 d) feeding phases, with 2 limestone particle sizes of 0.2 mm (**fine**) and 0.9 mm (**coarse**), and 2 levels of K: the control with 0% added K (K-) and 0.2% K added as potassium carbonate (K+). Each treatment had 6 replicate pens and 16 male broilers per pen. The composition of the experimental diets is presented in Table 1. All diets in starter and

grower feeding phases were formulated to be iso-caloric and iso-nitrogenous. The starter diet was calculated to contain 2.99 kcal/g metabolizable energy (ME), and 23.0% crude protein (CP) and grower diets contained 3.00 kcal/g ME, and 20.0% CP. The calculated nutrients in starter and grower diets either met or exceeded the NRC (1994) recommendations for broilers.

Feed Manufacturing

Common basal diets were formulated for both the starter and grower phases and were further manipulated for manufacture of various feed treatments. The common basal starter and grower diets were batched and mixed without poultry fat and potassium carbonate (K_2CO_3). For production of the 4 treatment diets, individual 2000 lbs batches of the basal diets were returned to the mixer for the addition of appropriate K_2CO_3 , and inert filler. The treatment diets were then dry-mixed for 180 sec in a twin shaft counterpoise ribbon mixer (Model TRDB126060, Hayes & Stolz, Fort Worth, TX). This was followed by the addition of poultry fat and mixing for an additional 90 sec wet mix to complete each batch. Each diet was then conditioned at 82 celcius in a single pass conditioner (Model C18LL4/F6, California Pellet Mill, Crawfordsville, IN), pelleted with a 30 HP pellet mill (Model PM1112-2, California Pellet Mill, Crawfordsville, IN), cooled in a counter-flow cooler (Model VK09X09KL, Geelen Counterflow USA, Inc, Orlando, FL), crumbled (starter only; Crumbler, Model 624S, Roskamp Champion, Waterloo, IA), and bagged. Samples of each treatment diet were collected after mixing and post pelleting and were analyzed for particle size determination, proximate nutrient values and K, Na, and, Cl concentration.

The particle size distributions of the two sources of limestone were determined by ASAE S319.3. Limestone particle sizes of ~200 μm and ~900 μm dgw were defined as **fine** and **coarse**, respectively. For starter and grower diets, a ground corn dgw of 800 μm was

targeted and achieved by grinding with a hammer mill equipped with 2 x 2.24 mm (6/64'') screens.

Statistical Analysis

Data collected in this study was analyzed as a completely randomized block design (**CRB**), with 4 treatments observed in each of two complete blocks. Accordingly, the following linear mixed effects model was used for data analysis: $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + B_k + \varepsilon_{ijk}$ where i, j, k are indexes for limestone particle size, K and block, respectively. Here B_k denotes a random block effect. The data was analyzed using the Fit model platform of JMP 13.2 software. Differences were considered statistically significant up to $P < 0.05$, although P values up to ≤ 0.10 are shown in the text if the data suggested a numerical trend. When a significant P value was detected, LSMEANS were separated using Student's T-test. A total of 24 pens were used in this study and each pen was considered an experimental unit. Therefore $n=12$ for each limestone particle size main effect, $n=12$ for each K main effect, and $n=6$ for each interaction.

RESULTS

Formulated and analyzed DEB (Na+K-Cl, mEq/kg) values are shown in Table 1.

The formulated and analyzed DEB with fine or coarse limestone-based diets for K- starter and K- grower were 266, vs. 197, 266 vs. 203, 231 vs. 170 and 231 vs. 180, respectively.

The formulated and analyzed DEB with fine and coarse limestone-based diets for K+ starter and K+ grower were 291 vs. 206, 291 vs. 246, 262 vs. 189 and 262 vs. 178, respectively.

Live Performance

Live performance results from 1-16 d of age are shown in Table 2. Chicks fed K+ diets exhibited decreased feed intake, BW and BW gain when compared to those of chicks fed the K- diets ($P < 0.05$).

Live performance results from 1-33 d of age are shown in Table 3. Chicks fed K⁺ diets exhibited decreased feed intake, BW and BW gain when compared to those of chicks fed the K⁻ diets ($P<0.05$). However, no differences were observed on cumulative FCR to 33 d of age.

Blood Physiology

Blood physiology results at 35 d of age are shown in Table 4. The chicks fed K⁺ diets exhibited decreased blood Na (mmol/L), blood glucose (mg/dL), ionized blood Ca (mg/dL), CO₂ tension (mmol/L), blood HCO₃ (mmol/L), and base excess in extracellular fluid (mmol/L) when compared to chicks of similar BW fed the K⁻ diets ($P<0.05$). In the birds fed K⁻ diets blood pCO₂ was dependent on the limestone particle size where birds fed coarse limestone diets exhibited increased pCO₂ when compared to fine limestone diets, resulted in an interaction ($P<0.10$). The blood chemistry response variables correlation analysis is shown in Figures 1A through 1D. The blood pH was correlated with concentration of blood K (mmol/L) ($P<0.01$; $R^2=0.15$). The pCO₂ (mmHg) was correlated with blood pH ($P<0.01$; $R^2=0.69$). The pCO₂ (mmHg) was correlated with blood K (mmol/L) ($P<0.01$, $R^2=0.13$). The HCO₃ (mmol/L) was correlated with pCO₂ (mmHg) ($P<0.01$, $R^2=0.42$).

Wooden Breast and White Striping

WB and WS score results are shown in Table 5. No differences were found in WB or WS scores among chicks fed K⁺ or K⁻ diets. However, chicks fed diets with fine limestone had numerically decreased WB when compared to those of chicks fed coarse limestone ($P<0.10$).

DISCUSSION

The objective of the present study was to investigate the effect of dietary K supplementation and limestone particle size on live performance, blood physiology and WS and WB myopathies. Analyzed K and DEB values (Table 1) for all diets were less than

calculated, which agreed with Borges *et al.* (2004) where calculated K was greater than analyzed K. The present study calculated values are used as reference in the discussion.

The calculated dietary K in starter and grower was greater than the requirement of K (0.66% to 0.74%) reported by Borges *et al.* (2007) and Oliveira *et al.* (2005) who reported dietary K requirements for optimal BW gain to be 0.63% from 8-21 d of age. However, the reduction in analyzed vs. calculated levels may be the result of decreased K levels in feed ingredients.

The dietary levels of Na and Cl were formulated to be constant in the present study; however, the different dietary K concentrations may be the result of interactions with Na and Cl. Hence, the live performance differences may have reflected the K interrelationship with Na and Cl (Hurwitz *et al.*, 1973), which is also known as DEB (Na+K-Cl, mEq/kg; Mongin 1980). Further, DEB is considered optimized when each electrolyte is neither deficient nor in excess in the diet. In the present study the levels of individual electrolytes Na, Cl and K were similar to those of practical broiler diets (Murakami *et al.*, 2001; Maiorka *et al.*, 2004). The K- starter and grower diets containing a DEB of 280 mEq/kg and 240 mEq/kg, respectively, resulted in significant increases in feed intake and BW gain, which was somewhat in agreement with the values reported by Mongin (1981; 250 mEq/kg). The K+ starter and grower diets contained DEB of 309 and 270 mEq/kg, respectively, which was in the range recommended by Murakami *et al.* (2001) and Oviedo-Rondon *et al.* (2001) who suggested optimal DEB between 246 and 319 mEq/kg for maximum growth of broilers. Hence, the differences in DEB between the K- and K+ diets were found to be in a range that may have resulted in live performance differences. The changes in DEB and acid-base balance are linked to loss of feed consumption similar to the results of Mongin (1981).

However, the live performance responses also could be attributed to the K source (K_2CO_3) rather than only a response to DEB or K *per se*. The K salts commonly used to adjust DEB in broilers are KCl and K_2CO_3 . Previous studies reported benefits of KCl supplementation through drinking water or feed in heat stressed birds. Ait-Boulahsen *et al.* (1995) reported that drinking water with 0.6% and 0.9% KCl were beneficial to heat stress, while a lower concentration (0.3%) provided no response. Borges *et al.* (1997) reported greater BW gain by adding 0.5 and 1.0% of KCl to the diet of growing and finishing broilers in the summer, totaling 0.98 and 1.23% of K, and 172 mEq/kg. The present study demonstrated that 0.2% K_2CO_3 supplemented through feed decreased feed intake and BW gain, which did not agree with Borgatti *et al.* (2004) who reported no growth depression at DEB level of 330 mEq/kg by addition of K_2CO_3 to achieve 1.21% dietary K. Teeter and Smith (1986) also reported reduced BW gain with addition of 0.15% K_2CO_3 and associated the response to reduced water intake.

Potassium is the major intracellular cation of the body and maintains intracellular fluid volume and acid-base balance. Earlier studies by Edens (1997) reported an increase in plasma K levels when the birds were exposed to heat stress which suggested cell damage and leakage of K out of the cell into ECF. Ait-Boulahsen *et al.* (1995) reported that 0.9% dietary KCl supplementation increased plasma K, which was associated with higher K intake. In the present study, the venous blood K was not different between the K- and K+ fed birds, which implied a maintenance mechanism of blood K.

K- diets increased the blood iCa, which was not in agreement with Ait-Boulahsen *et al.* (1995) who reported increased iCa with 0.6% and 0.9% KCl supplementation. However, the exact mechanism is unknown. Differences observed may have been associated with feed intake of the chicks. Similarly, the increased blood Na is indicative of higher ECF volume (Rose,

1984) and increased glucose may have been associated with the increased feed intake of the chicks resulting in greater water intake. This suggested alterations in metabolic activity (Olanrewaju *et al.*, 2007) associated with higher growth and Na role in glucose absorption.

Blood gas parameters are particularly sensitive to changes in acid-base balance. The blood variables involved in the assessment of acid-base status are pH, pCO₂ and HCO₃ (Figures 1A through to 1D). In the present study, the venous blood chemistry values at 35 d of age were within the range reported by Martin *et al.* (2010). There were no differences found in blood pH. However, the blood pH was negatively correlated with the increase in blood K concentration. This was consistent with the findings of Ait-Boulahsen *et al.* (1995) who reported a decrease in blood pH associated with 0.9% dietary KCl. The blood pH values of 7.41 and 7.40, for K⁻ and K⁺ fed birds respectively, were consistent with physiological homeostasis, and the data agreed with Kaya *et al.* (2004) who reported blood pH of 7.4. The K⁺ fed chicks exhibited decreased blood HCO₃, BE_{ecf} and τCO₂ which indicated a lower alkalosis and diminished H⁺ loading capacity (Duke's physiology of domestic animals, 2005). Apparently, no respiratory compensation occurred (partial pressure of CO₂ without significant differences), which was further evidenced by the pCO₂ increase correlated (R²=0.42) with an increase in HCO₃, which could have been a result from the reaction of added carbonate (forming carbonic acid) coming from K₂CO₃ in K⁺ fed diets. These transient reactions leading to blood gas and anion changes, and not otherwise compensated for, may have resulted in reduced live performance. One notable fact was that the HCO₃ concentration followed blood Na in both diets, which agreed with Benton *et al.* (1998).

It has been suggested that genetic background and growth rate play a considerable role in development of muscle myopathies such as WS and WB (Kuttapan *et al.*, 2014; Livingston, 2018). In the present study, though not withstanding statistical significance, the fine limestone

fed chicks exhibited a decreased WB scores ($P=0.08$) at 35 d of age. During the grower period the BW gain in fine limestone fed chicks was numerically lower (2194 g vs. 2208 g) hence, it could be implied that a smaller weight of bird was associated with decreased WB myopathies. Kuttappan *et al.* (2016) reported that the heavier broilers exhibited greater incidences of WB. Livingston *et al.* (2019) observed that reduced BW was associated with lower incidences of muscle myopathies.

CONCLUSIONS

There was no impact of limestone particle size on the live performance and blood physiological parameters assessed. Addition of potassium carbonate as K source decreased the broiler live performance and the venous blood physiology assessed were consistent within the physiological homeostatic range with basal and K⁺ fed diets.

Table 1. Composition of broiler starter and grower diets.

Ingredients	Starter ¹				Grower ²			
	Fine Limestone ³		Coarse Limestone ⁴		Fine Limestone ³		Coarse Limestone ⁴	
	K-	K+	K-	K+	K-	K+	K-	K+
	(%)							
Corn	58.50	58.50	58.50	58.50	65.00	65.00	65.00	65.00
Soybean meal (48% CP)	31.50	31.50	31.50	31.50	26.00	26.00	26.00	26.00
Poultry by-product meal	5.00	5.00	5.00	5.00	4.00	4.00	4.00	4.00
Poultry fat	2.00	2.00	2.00	2.00	2.50	2.50	2.50	2.50
Limestone	0.50	0.50	0.50	0.50	0.36	0.36	0.36	0.36
Defluorinated phosphate	1.05	1.05	1.05	1.05	0.77	0.77	0.77	0.77
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Potassium carbonate ⁵	0.00	0.20	0.00	0.20	0.00	0.20	0.00	0.20
Sand	0.27	0.07	0.27	0.07	0.24	0.04	0.24	0.04
Choline chloride (60%)	0.20	0.20	0.20	0.20	0.10	0.10	0.10	0.10
Vitamin premix ⁶	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Mineral premix ⁷	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Selenium premix ⁸	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
DL-Methionine	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
L-Lysine	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
L-Threonine	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Coccidiostat ⁹	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Total	100	100	100	100	100	100	100	100
<u>Calculated nutrient content</u>								
Metabolizable energy (kcal/g)	2.99	2.99	2.99	2.99	3.09	3.09	3.09	3.09
Crude protein	23.38	23.38	23.38	23.38	20.54	20.54	20.54	20.54
Calcium	0.90	0.90	0.90	0.90	0.71	0.71	0.71	0.71
Available phosphorus	0.45	0.45	0.45	0.45	0.36	0.36	0.36	0.36
Digestible lysine	1.16	1.16	1.16	1.16	1.01	1.01	1.01	1.01
Digestible methionine	0.53	0.53	0.53	0.53	0.50	0.50	0.50	0.50
Digestible methionine + cysteine	0.80	0.80	0.80	0.80	0.75	0.75	0.75	0.75
Digestible threonine	0.79	0.79	0.79	0.79	0.70	0.70	0.70	0.70
Sodium	0.25	0.25	0.25	0.25	0.23	0.23	0.23	0.23
Chloride	0.28	0.28	0.28	0.28	0.26	0.26	0.26	0.26
Potassium	0.92	1.03	0.92	1.03	0.80	0.92	0.80	0.92

Table 1 (Continued). Composition of broiler starter and grower diets.

Ingredients	Starter ¹				Grower ²			
	Fine Limestone ³		Coarse Limestone ⁴		Fine Limestone ³		Coarse Limestone ⁴	
	K-	K+	K-	K+	K-	K+	K-	K+
<u>Analyzed nutrient content</u>	(%)							
Sodium	0.16	0.15	0.17	0.18	0.16	0.13	0.18	0.14
Chloride	0.23	0.29	0.26	0.22	0.25	0.22	0.23	0.22
Potassium	0.75	0.87	0.79	0.90	0.67	0.76	0.65	0.70
Calculated DEB ¹⁰ (mEq/kg)	266	291	266	291	231	262	231	262
Analyzed DEB ¹¹ (mEq/kg)	197	206	203	246	170	189	180	178

¹Starter diet was fed to 16 d of age.

²Grower diet was fed from approximately 16 to 35 d of age.

³Fine limestone particle size; 0.2 mm.

⁴Coarse limestone particle size; 0.9 mm.

⁵Potassium carbonated (as 56.5% K): (K-) basal levels of K; (K+) 0.20% K as potassium carbonate (starter and grower).

⁶Vitamin premix supplied the following per kg of diet: 13,200 IU vitamin A, 4,000 IU vitamin D₃, 33 IU vitamin E, 0.02 mg vitamin B₁₂, 0.13 mg biotin, 2 mg menadione (K₃), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B₆, 55 mg niacin, and 1.1 mg folic acid.

⁷Mineral premix supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt, 1 mg.

⁸Selenium premix provided 0.2 mg Se (as Na₂SeO₃) per kg of diet.

⁹Coccidiostat supplied monensin sodium at 90 mg/kg of food.

¹⁰Dietary electrolyte balance calculated using matrix values of sodium, chloride and potassium.

¹¹Dietary electrolyte balance calculated using analyzed values sodium, chloride and potassium.

Table 2. Effect of limestone particle size and dietary potassium (K) on feed intake, body weight (BW), BW gain, and feed conversion ratio (FCR) from 1-16 d of age.

Limestone ¹	Potassium ²	Feed Intake	BW	BW gain	FCR
		g/bird			(g:g)
Fine		749	606	560	1.33
Coarse		750	596	551	1.36
	K-	763 ^A	612 ^A	567 ^A	1.34
	K+	736 ^B	589 ^B	544 ^B	1.35
	SEM ³	7	5	5	0.01
	Fine / K-	760	616	571	1.33
	Fine / K+	737	595	550	1.34
	Coarse / K-	766	608	563	1.36
	Coarse / K+	736	582	538	1.36
	SEM ⁴	10	8	8	0.01
		(Probability > F)			
	Limestone	0.85	0.21	0.22	0.12
	Potassium	<0.01	<0.01	<0.01	0.64
	Limestone x Potassium	0.67	0.75	0.83	0.91

^{A,B}Means within a column lacking a common superscript differ significantly ($P < 0.01$).

¹Limestone particle size (starter and grower); Fine (0.2 mm) and coarse (0.9 mm).

²Potassium carbonate (starter and grower); (K-) basal levels of K; (K+) 0.20% added K.

³SEM = Standard error of mean for n=12 pens for each main effect.

⁴SEM = Standard error of mean for n=6 pens for each interaction effect.

Table 3. Effect of limestone particle size and dietary potassium (K) on feed intake, body weight (BW), BW gain, and feed conversion ratio (FCR) from 1-33 d of age.

Limestone ¹	Potassium ²	Feed Intake	BW	BW gain	FCR
		g/bird			(g:g)
Fine		3101	2238	2194	1.41
Coarse		3146	2252	2208	1.43
	K-	3172 ^A	2279 ^a	2235 ^a	1.42
	K+	3074 ^B	2212 ^b	2167 ^b	1.42
	SEM ³	26	21	21	0.007
	Fine / K-	3126	2250	2205	1.42
	Fine K+	3074	2227	2182	1.41
	Coarse / K-	3219	2310	2264	1.42
	Coarse / K+	3073	2196	2151	1.43
	SEM ⁴	35	30	30	0.01
		(Probability > F)			
	Limestone	0.20	0.64	0.64	0.28
	Potassium	0.01	0.03	0.03	0.91
	Limestone x Potassium	0.67	0.14	0.15	0.50

^{A,B}Means within a column lacking a common superscript differ significantly ($P < 0.01$).

^{a,b}Means within a column lacking a common superscripts differ significantly ($P < 0.05$).

¹Limestone particle size (starter and grower); Fine (0.2 mm) and coarse (0.9 mm).

²Potassium carbonate (starter and grower); (K-) basal levels of K; (K+) 0.20% added K.

³SEM = Standard error of mean for n=12 pens for each main effect.

⁴SEM = Standard error of mean for n=6 pens for each interaction effect.

Table 4. Effect of limestone particle size (LPS) and dietary potassium (K) on broiler blood pH, sodium (Na), potassium (K), hemoglobin (Hgb), packed cell volume (PCV), glucose (Glu), ionized calcium (iCa), saturated oxygen (sO_2), partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), total carbon dioxide (τCO_2), bicarbonate (HCO_3), base excess in the extra cellular fluid (BEecf) at 35 d of age.

Limestone ^{1,2}	Potassium ^{1,3}	pH	Na —(mmol/L)—	K —(mmol/L)—	Hgb (g/dL)	PCV (%)	Glu —(mg/dL)—	iCa (%)	sO_2 (%)	pO_2 —(mmHg)—	pCO_2 —(mmol/L)—	τCO_2 —(mmol/L)—	HCO_3 —(mmol/L)—	BEecf
Fine		7.41	144.84	5.43	7.41	21.81	229.18	1.37	71.90	37.81	40.41	26.96	25.79	1.31
Coarse		7.40	145.17	5.53	7.40	21.80	228.48	1.36	70.93	37.74	42.29	27.66	26.44	1.75
	K-	7.41	145.73 ^A	5.50	7.38	21.71	235.01 ^A	1.39 ^A	71.27	37.43	41.75	27.87 ^a	26.62 ^a	2.13 ^a
	K+	7.40	142.28 ^B	5.47	7.44	21.90	222.65 ^B	1.34 ^B	71.56	38.12	40.95	26.75 ^b	25.61 ^b	0.93 ^b
	SEM ⁴	0.008	0.35	0.08	0.09	0.28	2.30	0.01	1.31	0.71	1.04	0.38	0.33	0.35
	Fine / K-	7.42 ^x	145.68	5.51	7.33	21.56	236.75	1.39	72.62	37.62	39.63 ^y	27.37	26.17	2.00
	Fine / K+	7.40 ^x	144.00	5.56	7.49	22.06	221.62	1.35	71.18	38.00	41.20 ^{xy}	26.56	25.40	0.62
	Coarse / K-	7.40 ^x	145.79	5.49	7.43	21.86	233.28	1.39	69.93	37.24	43.87 ^x	28.38	27.06	2.26
	Coarse / K+	7.41 ^x	144.56	5.38	7.38	21.75	223.68	1.33	71.93	38.25	40.71 ^{xy}	26.93	25.81	1.25
	SEM ⁵	0.01	0.65	0.12	0.14	0.41	3.36	0.02	1.92	1.03	1.42	0.56	0.51	0.53
(Probability > F)														
	Limestone	0.54	0.52	0.41	0.96	0.98	0.82	0.72	0.59	0.94	0.20	0.19	0.18	0.38
	Potassium	0.54	<0.01	0.77	0.67	0.62	<0.01	<0.01	0.87	0.48	0.58	0.03	0.04	0.02
	Limestone x Potassium	0.06	0.65	0.48	0.46	0.43	0.39	0.72	0.35	0.74	0.10	0.55	0.62	0.72

^{x,y}Means within a column lacking a common superscript differ significantly ($P < 0.10$).

^{a,b}Means within a column lacking a common superscript differ significantly ($P < 0.05$).

^{A,B}Means within a column lacking a common superscript differ significantly ($P < 0.01$).

¹Main effect means calculated using 64 broilers at 35 d of age.

²Limestone particle size; Fine (0.2 mm) and coarse (0.9 mm).

³Potassium carbonate (starter and grower); (K-) basal levels of K; (K+) 0.20% added K.

⁴SEM = Standard error of mean calculated using n=64

⁵SEM = Standard error of mean calculated using n=32

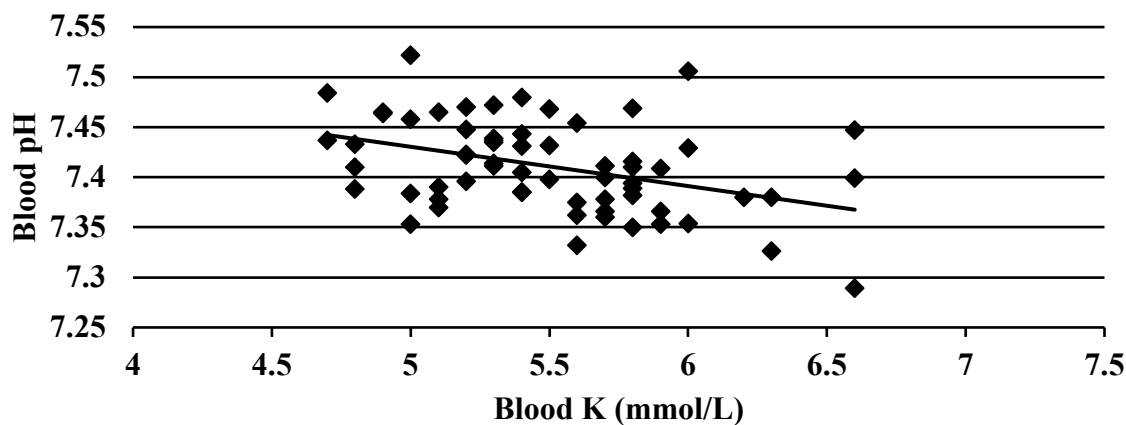


Figure 1A. Correlation between blood K and blood pH at 35 d of age, $P < 0.01$; $R^2 = 0.15$.

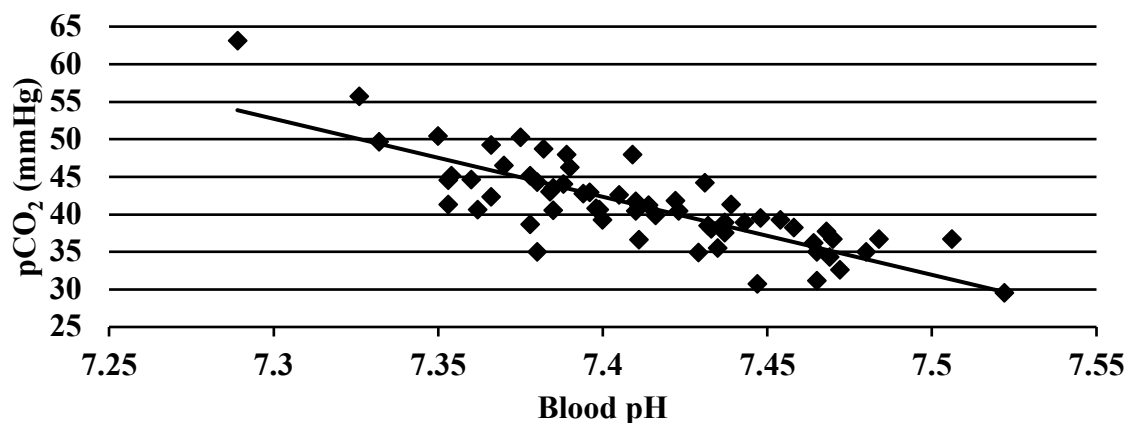


Figure 1B. Correlation between blood pH and pCO₂ at 35 d of age, $P < 0.01$, $R^2 = 0.69$.

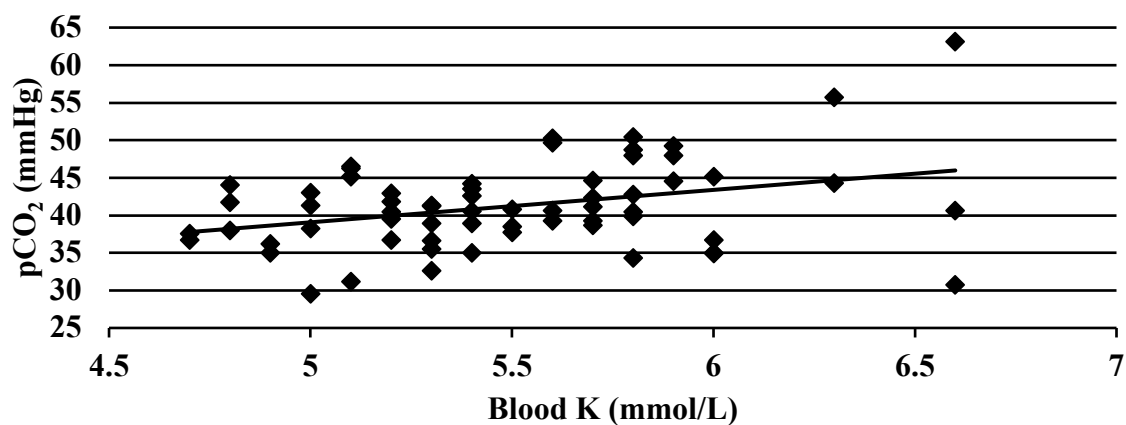


Figure 1C. Correlation between blood K and pCO₂ at 35 d of age, $P < 0.01$; $R^2 = 0.13$.

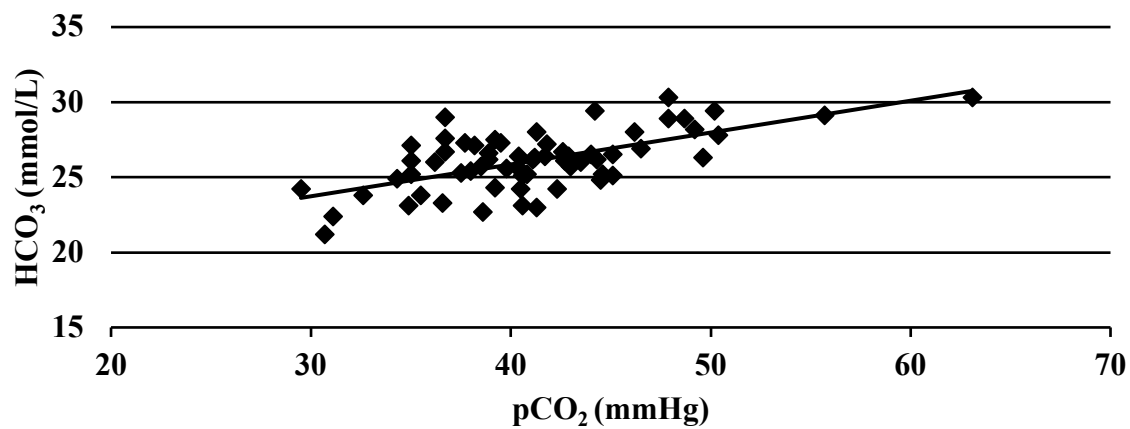


Figure 1D. Correlation between pCO₂ and HCO₃ at 35 d of age, $P < 0.01$; $R^2 = 0.42$.

Table 5. Effect of limestone particle size and dietary potassium (K) on wooden breast (WB) and white striping (WS) scores of broilers at 35 d of age.

Limestone ^{1,2}	Potassium ^{1,3}	Wooden Breast	White Striping
Fine		2.59 ^y	2.09
Coarse		3.00 ^x	2.12
	K-	2.74	2.03
	K+	2.84	2.19
	SEM ⁴	0.16	0.10
	Fine / K-	2.62	1.93
	Fine / K+	2.56	2.25
	Coarse / K-	2.85	2.13
	Coarse / K+	3.12	2.12
	SEM ⁵	0.22	0.14
		(Probability > F)	
	Limestone	0.08	0.81
	Potassium	0.66	0.29
	Limestone x Potassium	0.47	0.27

^{x,y}Means within a column lacking a common superscript differ significantly ($P < 0.10$).

¹Main effect means calculated using 64 broilers at 35 d of age.

²Limestone particle size (starter and grower); Fine (0.2 mm) and coarse (0.9 mm).

³Potassium carbonate (starter and grower); (K-) basal levels of K; (K+) 0.20% added K.

⁴SEM = Standard error of mean using n=64 birds.

⁵SEM = Standard error of mean using n=32 birds.

REFERENCES

- Allen, J. C. 2018. Advanced Nutrition and Metabolism: What is the quest? PP. 87:89. Linus Learning. Ronkonkoma, NY.
- Ahmad, T., T. Khalid, T. Mushtaq, M. A. Mirza, A. Nadeem, M. E. Babar, and G. Ahmad. 2008. Effect of potassium chloride supplementation in drinking water on broiler performance under heat stress conditions. *Poult. Sci.* 87:1276-1280.
- Ahmad, T., and M. Sarwar. 2006. Dietary electrolyte balance: implications in heat stressed broilers. *World's Poult. Sci. J.* 62:638-653.
- Ahmad, T., T. Mushtaq, M. U. Nisa, M. Sarwar, D. M. Hooge, and M. A. Mirzaa. 2006. Effect of different non-chloride sodium sources on the performance of heat stressed broiler chickens. *Br. Poult. Sci.* 47:249-256.
- Ait-Boulahsen, A., J. D. Garlich, and F. W. Edens. 1995. Potassium chloride improves the thermotolerance of chickens exposed to acute heat stress. *Poult. Sci.* 74:75-87.
- Ait-Boulahsen, A., J. D. Garlich, and F. W. Edens. 1989. Effect of fasting and acute heat stress on body temperature, blood acid-base and electrolyte status in chickens. *Comp. Biochem. Physiol.* 94:683-687.
- Benton, C. E., D. Balnave, J. Brake, L. J. Boyd, and M. A. Brown. 1998. The use of dietary minerals during heat stress in broilers. *Prof. An. Sci.* 14:193–196.
- Borgatti, L., R. Albuquerque, N. Meister, L. Souza, F. Lima, and M. Trindade Neto. 2004. Performance of broilers fed diets with different dietary electrolyte balance under summer conditions. *Bra. J. Poult. Sci.* 6:153-157.
- Borges, S. A., Fischer da Silva, A V, A. Maiorka, D. M. Hooge, and K. R. Cummings. 2004. Physiological responses of broiler chickens to heat stress and dietary electrolyte

- balance (sodium plus potassium minus chloride, milliequivalents per kilogram). *Poult. Sci.* 83:1551-1558.
- Borges, S. A., Fischer da Silva, A V, J. Ariki, D. M. Hooge, and K. R. Cummings. 2003. Dietary electrolyte balance for broiler chickens exposed to thermoneutral or heat-stress environments. *Poult. Sci.* 82:428-435.
- Borges, S. A., B., A. V. Fischer da Silva, A. S. A. A. M. T. Moura, and A. Maiorka, and A. Ostrensky. 2004. Electrolyte balance in broiler growing diets. *Int. J. of Poult. Sci.* 3:623-628.
- Borges, S. A., A. V. Fischer da Silva, and A. Maiorka. 2007. Acid-base balance in broilers. *World's Poult. Sci. J.* 63:73-81.
- Cohen, I., and S. Hurwitz. 1974. The response of blood ionic constituents and acid-base balance to dietary sodium, potassium and chloride in laying fowls. *Poult. Sci.* 53:378-383.
- Costa, F. G. P., L. R. Rodrigues, C. d. C. Goulart, Oliveira, C. F. Santos de Oliveira, V. P. Rodrigues, and Silva, J. V. da Silva. 2011. Nutritional potassium requirement for laying Japanese quails. *R. Bras. Zootec.* 40:2754-2759.
- Danny M. Hooge, PAS Hooge Consulting Service, Inc. 8775 North Cedar Pass Road Eagle Mountain, and Utah 84043 USA danhooge@fiber.net. Dietary sodium bicarbonate and electrolyte balance for broiler and breeder chickens.
- Dawson, W. R. 1975. *Annu. Rev. Physiol.* 37:441-465.
- Davison, S., and R. F. Wideman. 1992. Excess sodium bicarbonate in the diet and its effect on leghorn chickens. *Br. Poult. Sci.* 33:859-870.
- Goldstein, D. L. 2006. Regulation of the avian kidney by arginine vasotocin. *Gen. and Comp. Endocrin.* 147:78-84.

- Hamilton, R. M. G., and B. K. Thompson. 1980. Effects of sodium plus potassium to chloride ratio in practical-type diets on blood gas levels in three strains of white leghorn hens and the relationship between acid-base balance and egg shell strength. *Poult. Sci.* 59:1294-1303
- Hayat, J., D. Balnave, and J. Brake. 1999. Sodium bicarbonate and potassium bicarbonate supplements for broilers can cause poor performance at high temperatures. *Br. Poult. Sci.* 40:411-418.
- Hodges, R. 1970. Blood pH and cation levels in relation to egg-shell formation. *Annales de biologie animale, biochimie, biophysique* 10:199-213.
- Hopkinson, W. I., D. Jessop, D. A. Pass, and D. W. Pethick. 1990. Concentrations of plasma potassium and sodium during the life of a broiler breeder flock. *Avi. Pathol.* 19:607-611.
- Hossain, S., and A. G. Bertechini. 1999. Effects of varying levels of magnesium and available phosphorus on performance of layers. *Ani. Feed. Sci. and Tech.* 71:363-368.
- Kaneko, J. J. 1989. *Clinical Biochemistry of Domestic Animals*. 4th Edition. ACADEMIC PRESS, INC. San Diego, CA.
- Kaya, I., B. Karademir, and O. Ucar. 2012. The effects of diet supplemented with sodium bicarbonate upon blood pH, blood gases and eggshell quality in laying geese. *Vet. Med.* 49:201-206.
- Koreleski, J., S. Świątkiewicz, and A. Arczewska-Włosek. 2011. The effect of different dietary potassium and chloride levels on performance and excreta dry matter in broiler chickens. *Czech J. of Anim. Sci.* 56:53-60.
- Kuang, Q., P. Purhonen, and H. Hebert. 2015. Structure of potassium channels. *Cell. Mol. Life Sci.* 72:3677-3693.

- Kuttappan, V. A., Y. S. Lee, G. F. Erf, J.-F. C. Meullenet, S. R. McKee, and C. M. Owens. 2012. Consumer acceptance of visual appearance of broiler breast meat with varying degrees of white striping. *Poult. Sci.* 91:1240–1247.
- Larsen, E. H., N. Møbjerg, and J. N. Sørensen. 2006. Fluid transport and ion fluxes in mammalian kidney proximal tubule: a model analysis of isotonic transport. *Acta Physiol.* 187:177-189.
- Leach, JR. R. M. 1974. Studies on the potassium requirement the laying hen. *J. Nutr.* 104:684-686.
- Leeson, S., and J. D. Summers. 2001. *Scott's Nutrition of the Chicken*. 4th Ed. UNIVERSITY BOOKS. Guelph, Ontario.
- Livingston, M. L. 2018. Physiological and nutritional aspects of broiler breast myopathies. Dissertation. NC State University.
- Martin, M. P., M. Wineland, and H. Barnes. 2010. Selected blood chemistry and gas reference ranges for broiler breeders using the i-STAT handheld clinical analyzer. *Avian Diseases.* 54:1016-1020.
- Mongin, P. 1981. Recent advances in dietary cation-anion balance: applications in poultry. *Proc. Nutr. Soc.* 40:285–294.
- Mongin, P., 1980. Electrolytes in nutrition. A review of basic principles and practical applications in poultry and swine. Pages 1-15 in: *Proc. Third Annu. Int. Miner. Conf.* Orlando, FL.
- Mongin, P., and B. Sauveur. 1977. Interrelationships between mineral nutrition, acid-base balance, growth and cartilage abnormalities. Pages 235–347 in *Growth and Poultry Meat Production*. Boorman, K. N., Wilson, B. J., eds. *Brit. Poult. Sci.*, Edinburgh, UK.

- Mujahid, A., Y. Akiba, and M. Toyomizu. 2009. Progressive changes in the physiological responses of heat-stressed broiler chickens. *J. Poult. Sci.* 46:163-167.
- Murakami, A. E., E. A. Saleh, S. E. Watkins, and P. W. Waldroup. 2000. Sodium source and level in broiler diets with and without high levels of animal protein. *J. Appl. Poult. Res.* 9:53-61.
- Murakami, A. E., E. O. Oviedo-Rondón, E. N. Martins, M. S. Pereira, and C. Scapinello. 2001. Sodium and chloride requirements of growing broiler chickens (twenty-one to forty-two days of age) fed corn-soybean diets. *Poult. Sci.* 80: 289 - 294.
- Mushtaq, M. M. H., and T. N. Pasha. 2013. Electrolytes, dietary electrolyte balance and salts in broilers: an updated review on acid-base balance, blood and carcass characteristics. *World's Poult. Sci. J.* 69:833-852.
- NRC. 1994. National Research Council. Nutrient requirements of poultry. 8th ed. National Academic Press, Washington, DC. NRC. 1994. National Research Council. Nutrient requirements of poultry. 8th ed. National Academic Press, Washington, DC.
- Ogunji, P. A., R. N. Brewer, S. Roland D A, and D. Caldwell. 1983. Effect of dietary sodium chloride, protein, and strain difference upon water consumption and fecal moisture content of broiler breeder males. *Poult. Sci.* 62:2497-2500.
- Olanrewaju, H. A., S. Wongpichet, J. P. Thaxton, 3. Dozier W A, and S. L. Branton. 2006. Stress and acid-base balance in chickens. *Poult. Sci.* 85:1266-1274.
- Oliveira, J. E., L. F. T. Albino, H. S. Rostagno, L. E. Páez, and D. C. O. Carvalho. 2005. Dietary levels of potassium for broiler chickens. *Br. J. Poult. Sci.* 7:33-37.
- Oviedo-Rondón, E. O., A. E. Murakami, A. C. Furlan, I. Moreira, and M. Macari. 2001. Sodium and chloride requirements of young broiler chickens fed corn-soybean diets (one to twenty-one days of age). *Poult. Sci.* 80:592-598.

- Palmer, B. F. 2015. Regulation of potassium homeostasis. *Clin. J. Am. Soc. Nephrol.* 10:1050–1060.
- Petracci, M., S. Mudalal, F. Soglia, and C. Cavani. 2015. Meat quality in fast-growing broiler chickens. *World's Poult. Sci. J.* 71:363–374.
- Rice, G. E., and E. Skadhauge. 1982. Colonic and coprodeal transepithelial transport parameters in NaCl-loaded domestic fowl. *J. Comp. Physiol.* 147:65-69.
- Roland SR. D. A., S. T. McCready, R. H. Stonerock, and R. H. Harms. 1977. Hypercalcemic effect of potassium iodide on serum calcium in domestic fowl. *Poult. Sci.* 56:1310-1314.
- Ruiz-Lopez, B., M. Rangel-Lugo, and R. E. Austic. 1993. Effects of selected minerals on acid-base balance and tibial dyschondroplasia in broiler chickens. *Poult. Sci.* 72:1693-1704.
- Satlin, L. M. 2009. Potassium. Chapter 8:185-204. Springer-Verlag Berlin Heidelberg.
- Schaal, T. P., J. Arango, A. Wolc, J. V. Brady, J. E. Fulton, I. Rubinoff, I. J. Ehr, M. E. Persia, and N. P. O'Sullivan. 2016. Commercial Hy-Line W-36 pullet and laying hen venous blood gas and chemistry profiles utilizing the portable i-STAT1 analyzer. *Poult. Sci.* 95:466-471.
- Scott, M. L., M. C. Nesheim, and R. J. Young. 2001. *Nutrition of the chicken*. 4 Ed. University Books. Guelph, Ontario, Canada.
- Skou, J. C. 1965. Enzymatic basis for active transport of Na⁺ and K⁺ across cell membrane. *Physiol. Rev.* 45:596–617.
- Teeter, R. G., and M. O. Smith. 1986. High chronic ambient temperature stress effects on broiler acid-base balance and their response to supplemental ammonium chloride, potassium chloride, and potassium carbonate. *Poult. Sci.* 65:1777-1781.

- Wilson, R. W., M. Wareing, and R. Green. 1997. The role of active transport in potassium reabsorption in proximal tubule of the anesthetized rat. *J. Physiol.* 274:1109-1112.
- Wingo, C., and B. Cain. 1993. The renal H-K-ATPase: physiological significance and role in potassium homeostasis. *Annu. Rev. Physiol.* 55:323-347.
- Wu, C. 2018. Effects of diet and management practices on turkey and broiler meat quality and muscle myopathies. Dissertation. NC State University.

MANUSCRIPT III. Effect of Dietary Limestone Particle Size, Available Phosphorus, and Phytase on Live Performance, Bone Mineralization, and Apparent Ileal Digestibility of Amino Acids and Minerals of Male Broiler chickens.

ABSTRACT

The experiment that is reported herein investigated the effects of limestone particle size, available phosphorus (AvP) and phytase on live performance, tibia bone ash, and apparent ileal digestibility of calcium (Ca), phosphorus (P), and amino acids (AA) of male broiler chickens in floor pens. Two particle sizes of limestone were defined as fine (~0.2 mm) and coarse (~0.9 mm) by using the US sieve method. The analyzed Ca concentrations of both limestone sources were similar (~395 g/kg). Diets were formulated with 2 limestone particle sizes and with or without Ca, available phosphorus (AvP) matrix values for phytase as the negative control diet (NC) or the positive control diet (PC), respectively, and with the same Ca:AvP ratio (2:1). Phytase was included at either 1000 or 2000 phytase units (FYT)/kg (NC+1000, NC+2000), thus producing a 2 x 4 factorial arrangement. Eight experimental diets were randomly allocated to 6 replicate pens with 16 birds per pen and fed starter (0-16 d) and grower (16-33 d) diets. Apparent ileal digestibility of Ca, P, and amino acids were determined at 33 d using titanium dioxide (5 g/kg) as an indigestible marker. Bone ash was determined at 33 d. Dietary phytase supplementation of the NC diet improved ($P \leq 0.05$) BW gain, feed intake, and FCR at 16 d. Weight gain, feed intake, and bone ash were improved ($P \leq 0.05$) at 33 d by phytase supplementation. Birds that were fed fine limestone-based diets returned lower ($P \leq 0.05$) FCR at 16 d whereas the feed intake was increased with phytase; 1000 FYT/kg in coarse limestone-based diets resulted in limestone particle size x diet interaction ($P \leq 0.05$). The feed intake, digestibility of Ca and AA at 33 d of age were increased in the chicks fed coarse limestone-based diets ($P \leq 0.05$). The P digestibility was improved by phytase 1000 FYT/kg in fine limestone-based diets resulted in limestone x diet interaction ($P \leq 0.05$). Phytase

supplementation to NC diet improved the live performance and bone ash. Fine limestone improved FCR in the starter phase but coarse limestone diets resulted in greater feed intake and improved digestibility of Ca, P and, AA at 33 d of age. These data suggested that the effect of phytase supplementation on P utilization was similar to P adequate diets when the broilers were fed either fine or coarse limestone. Phytase and particle size of dietary limestone should be an important consideration when formulating broiler diets.

Key words: Coarse limestone, digestibility, amino acids, calcium, phosphorus

INTRODUCTION

Limestone is a common source of Ca used in almost all poultry diets. Past research was reported on a range of limestone particle sizes (0.02 mm to 3.35 mm) indicated that dietary limestone particle size of <0.5 mm could promote improved performance in broilers (McNaughton *et al.* 1974; Guinotte *et al.* 1991, Manangi and Coon 2007; Anwar *et al.* 2016). McNaughton *et al.* (1974) observed that the broiler BW gain was increased with fine limestone particle size ~0.15 mm, when compared to a larger particle size >2.36. Guinotte *et al.* (1991) demonstrated that fine limestone particle size >0.15 mm was beneficial for BW gain, feed conversions and tibia ash for 28 d broilers. In broilers, limestone particle size was found to influence gastrointestinal (GI) passage rate of Ca ions and its absorption (Zhang and Coon, 1997).

In the last decade, studies conducted on broilers focused on understanding the interaction between limestone particle size and solubility on Ca-phytate complexes to evaluate phytase efficacy. Manangi and Coon (2007) demonstrated that phytase efficacy was greater with limestone solubility of 53% from ~0.4 mm limestone particle size and resulted in increased BW gain and tibia ash when compared to solubility of >70% from ~0.28 mm limestone particle size. Results indicated that fine limestone particle size with greater *in vivo* solubility led to formation of Ca-phytate complexes which reduced phytate-P solubility, thus negatively impacting phytase efficacy. Walk *et al.* (2012) compared limestone and a highly soluble Ca source (HSC) and documented that when HSC was used at a reduced dietary Ca level (0.45% vs 0.90%), that phytase improved ileal P and Ca digestibility. At an adequate dietary Ca level (0.9%) limestone improved Ca digestibility and phytase efficacy on live performance, tibia ash and nutrient digestibility when compared to HSC fed broilers (Walk *et al.*, 2012; Bradbury *et al.*, 2018). It is apparent that *in vivo* solubility of Ca sources is an

important factor, which influences Ca digestibility and phytase efficacy. However, at an optimal dietary Ca level of 0.9%, limestone is a preferred source of Ca in broilers diets.

A few studies have included phytase and evaluated amino acid (AA) digestibility using different sources of limestone and particle sizes (Bradbury et al., 2018). Additional information is required to further elucidate the relationship between dietary limestone particle size, and phytase on Ca, P and AA digestibility.

This study was designed to investigate the effects of two limestone particle sizes (0.2 mm and 0.9 mm), available phosphorus (AvP) and phytase on live performance, tibia bone ash, and apparent ileal digestibility of Ca, P, and AA of male broiler chickens reared in floor pens.

MATERIALS AND METHODS

Animal Welfare

The experimental protocol used in this study was approved by the North Carolina State University Institutional Animal Care and Use Committee. This experiment was designed and conducted in compliance with the Guide and Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Broiler Management and Data Collection

Ross 708 x YPM (yield PM male) chicks were hatched from eggs laid by the resident broiler breeder flock at the North Carolina State University Chicken Education Unit. After hatching, chicks were feather-sexed, and only male broilers were utilized for this study. A total of 768 Ross 708 x YPM male broilers chicks were identified with neck tags and placed randomly into 48 1.2 m x 1.8 m floor pens, with 16 chicks/pen for a stocking density of 0.135 m²/bird in the enclosed, fan ventilated house. Pen floors were covered with new pinewood shavings. Each pen had two tube feeders and one bell drinker, two plastic font drinkers were

used during the first 7 d after placement. Three feeder trays were initially placed in every pen, reduced to 2 feeders at 5 d, and finally reduced to one at 7 d. The supplemental drinker and feeder were removed at 9 d. Contents of the trays were screened and dumped back into the tube feeders at 9 d. In the starter period, 1 tube feeder was used, and total starter feed was equalized to 0.908 kg per live chick at 7 d. At 16 d, the second tube feeder was added, and a total of 2.72 kg/bird of grower feed was added to each tube feeder in increments of 0.908 kg/bird until termination of the study at 33 d. For each dietary phase, the amount of feed supplied to each replicate pen was controlled and adjusted for mortality to assure that all broilers in each replicate pen had similar access to each dietary phase feed following complete intake of the starter dietary phase. Feeders were shaken twice daily to maintain uniform and adequate flow of feed from the tubes into the pans until 33 d of age. Shavings were replaced as needed. Floor temperature at chick placement was 95°F, which was reduced to 90°F within 24 h after all chicks had been placed. Air temperature was reduced by approximately 1°F each day until 7 d of age, thereafter it was then maintained at approximately 85°F from 8 to 14 d, 80°F from 15 to 21 d, and 75°F from 16 to 33 d. Artificial light was provided by ceiling mounted 18-watt fluorescent bulbs. The lighting program during the first 7 d was 23 h of light, that was reduced to 22 h of light up to 14 d, 20 h of light up to 21 d. After 21 d of age, only 15 h of light was used.

Pen body weight (BW), and feed consumed were determined at placement, 16 d, and 33 d for calculation of average BW and average feed intake per bird for each replicate pen. Mortality was weighed and recorded twice daily as necessary. After accounting for the BW of all deceased birds, total BW gain and total feed intake for each pen were used to calculate mortality-adjusted feed conversion ratio (FCR) for each dietary feeding phase.

At 33 d of age average BW per pen was determined and 2 birds, near the average BW of each pen were euthanized by cervical dislocation. The ventriculus ventricular contents were collected in plastic bags for calcium solubility determination. The distal 13 cm of ileum was removed 3 cm anterior to the ileo-cecal junction, ileal contents were gently expressed, pooled per pen, and stored at -20°C before being freeze-dried for determination of apparent ileal digestibility (AID).

The left tibia was excised, cleaned of any tissues and immersed for 48 h in ethyl alcohol for fat extraction. After tibias were dried for 12 h at 110°C, the bone-ashing was done in ceramic crucibles for 24 h at 600°C in a muffle furnace (Hall et al., 2003). The percentage of ash was determined relative to dried defatted weight of tibia and expressed as grams of ash/bone and as a percentage of the defatted tibia. Bone ash was calculated using the formula: Bone ash% = (tibia ash weight / defatted tibia weight) x 100.

Dietary Treatments and Feed Compounding

A total of 8 corn-soybean meal based dietary treatments were tested in the starter and grower feeding phases, with 2 limestone particle sizes (fine and coarse), and 4 dietary formulations to achieve positive control (PC), negative control (NC), NC+1000, and NC+2000 phytase FYT/kg. The composition of the experimental diets is presented in Table 5. The 4 dietary treatments were formulated to achieve 2 levels of AvP in the starter 0-16 d (PC = 0.45% and NC = 0.30%) and grower 16-33 d (PC = 0.36% and NC = 0.24%) with similar Ca to AvP ratio of 2:1. Each treatment had 6 replicate pens and 16 male broilers per pen. The factors were 2 limestone particle sizes of **fine** limestone (0.2 mm) and **coarse** limestone (0.9 mm), and 4 dietary formulations, for the starter period, positive control (**PC**) was calculated to yield AvP 0.45% and Ca 0.90% and negative control (**NC**) was calculated to yield AvP 0.30% and Ca 0.70%. Similarly, for grower diets the PC was calculated to yield AvP 0.36% and Ca 0.72%

and NC calculated to yield AvP 0.21% and Ca 0.58%. Two dosages of Ronozyme HiPhos (DSM Nutritional Products Inc., Parsippany, NJ) was supplemented at 1000, 2000 FYT/kg to yield **NC+1000** and **NC+2000**, in the starter and grower diets, respectively. The ingredients, poultry by product meal, SBM, corn, di-calcium phosphate and de-flourinated phosphate (DFP) were analyzed for total Ca and P, and digestibility coefficients of P were used (Garcia *et al.*, 2006) to calculate dietary AvP concentration. The total Ca (tCa) and AvP concentrations were achieved with amended DCP and DFP in the PC, NC, and NC+FYT diets. Phytase was given a matrix value of 0.20% Ca and 0.15% AvP. All diets in starter and grower feeding phases were formulated to be iso-caloric and iso-nitrogenous. The starter diet was calculated to contain 2.99 kcal/g metabolizable energy (ME), and 23.0% crude protein (CP) and grower diets contained 3.00 kcal/g ME, and 20.0% CP. The calculated nutrients in starter and grower diets either met or exceeded the NRC (1994) recommendations for broilers, with the exception of Ca and AvP in the NC diets. In the grower diets titanium dioxide (TiO₂) was added at the rate of 5 g/kg as an indigestible marker to allow for the calculation of apparent nutrient digestibility.

Feed Manufacturing

A common basal diet was formulated for the starter and grower phase diets, respectively and were further manipulated for manufacture of various feed treatments. The common basal starter and grower diets that were mixed without poultry fat and phytase enzyme (or sand filler for PC diets), and, stored in 1-ton bins. Afterwards, production of the 8 treatment diets, individual batches of 2000 lbs each were returned to the mixer for the addition of appropriate phytase enzyme, DCP, DFP, limestone, and sand were dry-mixed for 180 sec in a twin shaft counterpoise ribbon mixer (Model TRDB126060, Hayes & Stolz, Fort Worth, TX). This was followed by the addition of poultry fat and mixing for an additional 90 sec wet mix

to complete each mixing. Each diet was then conditioned at 82°C in a single pass conditioner (Model C18LL4/F6, California Pellet Mill, Crawfordsville, IN), pelleted with a 30 HP pellet mill (Model PM1112-2, California Pellet Mill, Crawfordsville, IN), cooled in a counter-flow cooler (Model VK09X09KL, Geelen Counterflow USA, Inc, Orlando, FL), crumbled (starter only; Crumbler, Model 624S, Roskamp Champion, Waterloo, IA), and bagged. Samples of each treatment diets were collected post-pelleting and were analyzed for particle size determination, proximate analysis for nutrients, and phytase units.

Determination of Particle Size

The particle size distribution was determined by ASAE S319.3. The particle size (d_{gw}) analysis of ingredients and compounded feed is shown in Table 1. The DFP was manually sieved using a #40 sieve to achieve a geometric mean diameter (d_{gw}) of ~150 μm . The two limestone particle sizes distributions of ~200 μm and ~900 μm d_{gw} were called fine and coarse, respectively. For starter and grower diets, a d_{gw} of ~875 μm for 50% of the grind corn and soy amount was targeted and achieved by grinding with a hammer mill equipped with a pair of 2.24 mm screens.

Chemical Analyses

Dry matter: The dry matter content of frozen samples of feed and ileal contents were analyzed using a freeze-drying method at -50°C at -0.05 mBar pressure, for 72 h (Labconco-FreeZone® Model 77520, Kansas City, MO.). Samples of feed and dried ileal digesta were ground (< 2 mm) for analysis. Diets and ileal samples were analyzed to calculate AID.

Titanium dioxide: The TiO₂ of diets and ileal samples were determined using the method of Myers et al. (2004).

Calculations: The percent apparent ileal digestibility (AID) of amino acids (AA) and minerals (Min) in the diets were calculated using the titanium ratio in the diets and digesta:-

Percent AID = $100 \times [1 - (\text{Ti Diet}/\text{Ti Ileal}) \times (\text{No}/\text{Nd})]$, where

Ti diet is the titanium concentration in the diet, Ti Ileal is the concentration of titanium in the ileal digesta, No is the concentration of the individual AA or mineral in the ileal digesta, and Nd is the concentration of individual AA or minerals in the diet. All analyzed values were expressed as percent dry matter.

Phytase Units: The recovery of Ronozyme HiPhos in feed was confirmed and one unit of phytase activity (FYT) was defined as the activity that releases 1 μmol inorganic phosphate from 5.0 mM phytate per minute at pH 5.5 and 37°C. (Williams, 2014).

HPLC assay for phytic acid: For the analysis of inositol-6-phosphate (IP_6) in ventriculus ventricular digesta samples, 0.5 g freeze-dried digesta from the ventriculus ventricular were ground to allow passage through a 1 mm screen, which were extracted with 10 ml of 0.5 mol/L HCl for for 1 h at 20°C with a magnetic stirrer. One mL was centrifuged at 2200 X g for 10 min and 400 μL supernatant was filtered using 13 mm syringe filter with a 0.45 μm GHP (general hydrophilic polypro) membrane filter and placed in a Microcon membrane filter device with a cut-off of 30 kDa (Millipore) containing a cation exchanger (H^+) (Dowex, Merck) and centrifuged again at 19000 X g for 30 min at 5°C. Filtrates were analyzed by high-performance ion chromatography using ion chromatography AG7 (guard) and AS7 (analytical) columns (Dionex Corp., Sunnyvale, CA) to measure phytic acid (Phillippy and Johnston, 1988). The eluant, 0.25 M HNO_3 , was sparged continuously with a slow stream of helium to prevent air bubble formation. A 10 μm inlet filter is attached to the forward end of the eluant line. The eluant was pumped at a rate of 1 mL/min. The effluent from the analytical column is directed into a PEEK tee used as a post-column reactor, where it was combined with 0.1% $\text{Fe}(\text{NO}_3)_3$ in 2% HClO_4 (Phillippy and Bland, 1988). A total flow rate of 1.5 mL/min was maintained by pressurizing the post-column reservoir with approximately 30

psi N₂ gas. The mixture entered a Waters UV/visible detector equipped with a flow cell, and absorbance was read at 290 nm. Sample solution injections were bracketed by standard injections of 25 nmol dodecasodium phytate (Sigma) in 25 µl water.

Calculations: The sample solution peak height divided by the average of the bracketing standard solution peak heights was multiplied by 25 to give nmol Inositol-6-phosphates per injection. The nmol IP₆ per injection divided by the sample solution injection volume was multiplied by 1000 µL and divided by 0.5 g to give nmol IP₆ per g digesta. The nmol IP₆ in 0.5 g digesta was multiplied by the total g digesta to give total nanomoles IP₆. The difference in IP₆ between ventriculus with or without phytase was divided by the IP₆ in the ventriculus without phytase and multiplied by 100% to give the disappearance of IP₆.

Limestone solubility: In vitro solubility of fine and coarse limestone and digesta samples from the ventriculus was determined by weight loss modified method (NFIA Laboratory method compendium, 1991). Calcium solubility was determined by solubilizing a weighed 2g sample of limestone with heating 0.1N HCl at 42°C for 10 minutes with agitation and recovering quantitatively the non-solubilized residue. The difference between the weighed sample and its non-solubilized residue was the soluble portion and was expressed as ratio percentage (Insoluble portion / Sample wt.) x 100.

Statistical Analysis

All the data collected in this study were analyzed as a 2 x 4 completely randomized factorial arrangement of treatments with the Standard Least Squares ANOVA within the Fit model platform of JMP 13.2 software. , The linear model is: $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$ with $n=6$ for each i -th and j -th fixed factor levels α (2 levels of phytase), β (4 diets) and their interactions. Differences among means were considered significant at $P < 0.05$, although P values up to ≤ 0.10 are shown in the text if the data suggest a numerical trend. When a

significant P values was detected, LSMEANS were separated using Student's T-test in JMP 13.2. A total of 48 pens were used in this study and each pen was considered an experimental unit. Therefore $n=24$ for each limestone particle size main effect, $n=12$ for each diet main effect, and $n=6$ for each interaction.

To discern apparent ileal digestibility variables that might influence Ca, P and AA digestibility, correlations were estimated using bivariate analysis and was performed with Fit Y by X platform of JMP 13.2 software. The correlation between the response variables, Ca digestibility and P digestibility and the correlation between Na digestibility and 17 AA digestibility assessed Na digestibility between Ca and, P were selected for brevity.

RESULTS

The two limestone particle sizes used in the study were classified as fine (~ 0.2 mm) and coarse (~ 0.9 mm). The limestone solubility results are shown in Table 4. The *in vitro* solubility of fine limestone was greater (86.3% vs 61.7%) than coarse limestone. In the ventriculus contents, the solubility of coarse limestone was greater (81.3% vs 75.3%) than fine limestone.

The particle size distribution of the starter and grower diets is presented in the Figure 1A through 1D. The d_{gw} of the starter and grower diets was determined to be 650 μm . However, the coarse limestone diets contained 5% more particles over 400 μm when compared to fine limestone diets.

The analyzed Ca concentration of fine and coarse limestone particles was similar (~ 395 g/kg) (Table 5). The analyzed Ca and P concentrations in the experimental diets were similar to the formulated concentrations. The phytase recoveries in the starter and grower diets were similar to the formulated levels (Table 2).

The Inositol-6-phosphate (IP₆) percentage disappearance measured by high performance ion exchange chromatography (HPIC) was found greater in coarse limestone fed diets (Table 3).

Live Performance

The live performance results from 1-16 d of age are shown in Table 6. The chicks fed diets based on fine limestone decreased FCR when compared to coarse limestone fed diets ($P < 0.05$). Chicks fed the NC diets with phytase at 1000 FYT/kg or 2000 FYT/kg increased BW, BW gain, and decreased FCR when compared to chicks fed the NC diets and to the same extent as chicks fed the PC diets ($P < 0.01$). There was an interaction between limestone particle size and diet on feed intake ($P < 0.05$). The feed intake of the chicks fed diets based on fine limestone was dependent on phytase dose whereas there was no effect of increasing phytase dose on the feed intake of the chicks fed diets based on coarse limestone.

The live performance results from 1-33 d of age are shown in Table 7. The chicks fed diets with coarse limestone exhibited increased feed intake when compared to chicks fed diets with fine limestone ($P < 0.05$). The chicks fed diets NC+1000 or NC+2000 had increased ($P < 0.01$) feed intake, BW, BW gain and, lower ($P < 0.10$) FCR compared to the chicks fed NC diets and to the same extent as chicks fed the PC diets.

Tibia Bone Minerals

The results for the tibia bone data measured at 33 d of age is shown in Table 8. The addition of phytase to NC diet at dose of 1000 or 2000 FYT/kg increased ($P < 0.01$) tibia weight and percent bone ash when compared to the chicks fed NC diets, and to the same extent of the PC fed diets.

The results for tibia bone ash mineral concentration of Calcium (Ca), phosphorus (P), sodium (Na), chloride (Cl), magnesium (Mg), potassium (K), manganese (Mn), zinc (Zn) and

iron (Fe), measured at 33 d are shown in Table 9. Neither limestone particle size nor the diets affected Ca concentration in tibia bone ash. However, the P concentrations in the tibia bone ash of chicks that were fed NC diets were decreased when compared to the chicks fed the PC diets, and with the chicks that were fed the diets NC+1000 or 2000 FYT/kg being intermediate to both the PC and NC ($P<0.10$).

The Na concentration in the tibia bone ash of the chicks fed NC diets or NC+1000 FYT/kg was found greater than the chicks that were fed the PC diets and NC+2000 FYT/kg ($P<0.01$).

The Cl concentration in the tibia bone ash of the chicks fed NC diets was greater than in the chicks that were fed the PC diets and NC+1000 or 2000 FYT/kg ($P<0.01$).

There was an interaction between limestone particle size and diet on tibia bone ash Mg concentration ($P<0.05$). Phytase supplementation of the NC diets with fine and coarse limestone increased tibia bone ash Mg concentration with 1000 or 2000 FYT/kg.

The K concentration in the tibia bone ash of the chicks fed NC diets and NC+1000 FYT/kg was greater when compared with the chicks that were fed PC diets and NC+2000 FYT/kg ($P<0.01$).

There was an interaction between limestone particle size and diet on tibia bone ash Mn concentration ($P<0.01$). The Mn concentration in the tibia bone ash of the chicks that were fed diets with fine limestone was dependent on the dose of phytase whereas there was no effect of increasing phytase dose on tibia Mn in the chicks that were fed diets with coarse limestone.

The Zn concentration in the tibia bone ash of the chicks fed diets NC+1000 or 2000 FYT/kg was greater when compared to the chicks that were fed the NC and PC diets ($P<0.01$).

There was an interaction between limestone particle size and diet on tibia bone ash Fe concentration ($P<0.01$). The Fe concentration in the tibia bone ash of the chicks fed diets with

fine limestone was dependent on the phytase dose whereas there was no effect of increasing phytase dose on tibia Zn in the chicks that were fed diets with coarse limestone.

Apparent Ileal Digestibility of Minerals

The AID of minerals; Ca, P, K, Na and Cl are shown in Table 10. The chicks that were fed diets with coarse limestone had higher AID of Ca ($P<0.01$) and, AID of P ($P<0.01$), when compared to the fine limestone fed diets. However, there was an interaction between limestone particle size and diet on the AID of both, Ca ($P\leq 0.05$) and, P ($P<0.01$). For chicks that were fed diets with fine limestone the digestibility of Ca and, P was dependent on the phytase dose whereas there was no effect of increasing phytase dose on the digestibility of Ca and, P when fed diets with coarse limestone. To discern the digestibility variables that might influence the Ca and P digestibility, correlations were estimated among all response variables. The correlation analysis is shown in Figure 2A through 2C. The AID of Ca was correlated with AID of P ($P<0.01$; $R^2=0.56$). The AID of Na was correlated with AID of Ca ($P<0.01$; $R^2=0.15$), similarly, AID of Na was correlated with the AID of P ($P<0.01$; $R^2=0.14$).

There was an interaction between limestone particle size and diet on the AID of K ($P<0.10$). The AID of K of the chicks with fine limestone fed diets NC+2000 FYT/kg was decreased compared to the chicks fed diets PC and NC which was dependent on the dose of phytase whereas there was no effect of increasing the dose of phytase with coarse limestone fed diets.

The AID of Na were negative values and this is explained later in the discussion. The chicks fed diets with coarse limestone increased the AID of Na when compared to the diets fed with fine limestone ($P\leq 0.05$). The AID of Na of the chicks fed with PC diets was increased when compared to the chicks that were fed NC+1000 FYT/kg, with the chicks that were fed

both NC diets and the NC+2000 FYT/kg being intermediate to both PC diets and NC+1000 FYT/kg ($P<0.05$).

Apparent Ileal Digestibility of Amino Acids

The AID of amino acids; lysine (Lys), methionine (Met), cysteine (Cys), threonine (Thr), valine (Val), leucine (Leu), isoleucine (Ile), glycine (Gly), serine (Ser), arginine (Arg), glutamic acid (Glu), histidine (His), alanine (Ala), aspartic acid (Asp), tyrosine (Tyr), phenylalanine (Phe) and proline (Pro) measured at 33 d of age are shown in Table 11. The chicks fed the coarse limestone diets had an increased AID for all the amino acids when compared to the chicks that were fed fine limestone diets ($P<0.05$), but there was an exception ($P\leq 0.10$) for the AID for Met.

To discern the digestibility variables that might influence the AA digestibility, correlations were estimated among all response variables. The correlation analysis is shown in Figure 3A through 3Q. The AID of Na was significantly correlated with the AID of 17 AA assessed.

DISCUSSION

The objective of the present study was to investigate the interactivity of limestone particle size, dietary AvP and Ca concentration and phytase supplementation effects on live performance, tibia mineralization and AID of amino acids and minerals. Different particle sizes of limestone as a dietary source of Ca have been evaluated in broilers with varying results (McNaughton *et al.*, 1974; Guinotte *et al.*, 1991). Diets containing larger limestone particle size (1.18 to 4.75 mm) were associated with decreased live performance of the chicks, whereas the chicks fed diets with smaller limestone particle size (0.25 to 1 mm) showed no differences in live performance (Guinotte *et al.*, 1991; Anderson *et al.*, 1984). Only a few studies have

investigated the effects of limestone particle size and phytase supplementation on broiler chicks.

In this experiment, during 1 to 16 d of age, the chicks fed diets with fine limestone particle size had lower FCR, which is consistent with the findings of Guinotte *et al* (1991). A similar trend was observed where chicks that were fed diet based on fine limestone particle size (<0.15 mm) improved 28 d broiler performance. During 1 to 33 d of age, the chicks fed diets with coarse limestone had higher feed intake, although this did not affect the FCR. The results agreed with Manangi and Coon (2007) who observed feed intake differences with no effect on FCR of the chicks fed limestone particle sizes between 28 μm to 1306 μm at 28 d of age. These results are not in agreement with the findings of Anwar (2017) who observed that limestone particle size (<0.5 mm or >1.0 mm) did not affect feed intake at 24 d of age. Phytase supplementation with 1000 and 2000 FYT units/kg improved BW gain and FCR of the NC diets-fed chicks, similar to that of PC fed-diets in both growth phases. These effects were consistent with several other investigations that reported increased growth performance when phytase was supplemented to P-inadequate diets (Simons *et al.*, 1990; Cabahug *et al.*, 1999; Cowieson *et al.*, 2006; Olukosi *et al.*, 2013).

In this experiment, during 1 to 16 d of age, the feed intake for the birds that received diets based on fine limestone was dependent on phytase dose. This was evidenced by feed intake being increased in fine limestone-based diets with phytase at a dose of 2000 FYT units/kg compared to coarse limestone-based diets, which returned similar feed intake with phytase at a dose of 1000 FYT units/kg. The data suggest that greater solubility of fine limestone may have resulted in insoluble Ca-phytate complexes impeding phytase efficacy (Lei *et al.*, 1994). It is known that the effects of dietary enzymes are more obvious in young

birds (Acamovic, 2000). Thus, the higher than standard phytase inclusion rates could be beneficial in fine limestone containing diets, particularly in younger aged birds.

However, the general lack of interaction between limestone particle size and phytase supplemented diets was unexpected. The overall growth data agreed with Bradbury *et al.* (2018) who reported no effect of limestone particle size on BW gain or FCR but phytase supplementation improved the live performance.

At 33 d of age, the dietary limestone particle size did not affect weight of either the tibia, weight of the ash, or percent bone ash. The results agreed with Manangi and Coon (2007) who observed no differences in tibia bone ash in chicks fed diets with limestone particle sizes between 28 μm to 1306 μm . This is also consistent with the findings of Bradbury *et al.* (2018), observed no effect of limestone particle sizes <0.5 mm or >0.5 mm on foot ash percentage.

Phytase supplementation at 1000 or 2000 FYT units/kg to NC diets increased tibia weight and percent bone ash of the chicks. Thus, phytase was effective in maintaining the P status of the chicks. The increase in bone mineralization was related to increased weight gain. Williams *et al.* (2004) indicated that the growth rate of fast-growing birds was responsible for rapid bone deposition. The finding of this study was in agreement with several studies (Qian *et al.*, 1996; Onyango *et al.*, 2004; Olukosi *et al.*, 2013; Olukosi and Fru-Nji, 2014a) that reported phytase supplementation improved weight gain and bone mineralization. This investigation also demonstrated that chicks are sensitive to low Ca/AvP diets and the phytase supplementation at 1000 or 2000 FYT units/kg, equally improved, live performance and tibia bone ash percent.

Phytase supplementation did not change the concentration of Ca and P in tibia bone ash and similar results have been reported by Broz *et al.* (1994). The tibia bone ash concentration was related to phytase activity, 2000 FYT units/kg in NC diets, which reduced the

concentration of tibia Na, Cl and K, similar to the responses of chicks given the PC diets. In contrast, Sebastian *et al.* (1996) reported that phytase did not affect the concentration of minerals in whole tibia ash. The tibia bone ash concentration of Mg, Mn and Zn, was increased with supplementation of phytase at a dose of 1000 or 2000 FYT units/kg in both fine and coarse limestone diets. The increase in these minerals in tibia bone ash has been demonstrated previously in chickens (Mohanna and Nys, 1999; Qian *et al.*, 1996; Viveros *et al.*, 2002).

The *in vitro* solubility of fine limestone was found to be greater (~24%) than coarse limestone, whereas the ventriculus contents the solubility of coarse limestone was found greater (~7%). The findings were in agreement with Zhang and Coon (1997) who reported that in laying hens the *in vivo* solubility of limestone was negatively correlated with *in vitro* solubility this phenomenon was explained by the longer retention time of larger particle size limestone (>0.8 mm) in the ventriculus, which increased the *in vivo* solubility of coarse limestone particles. The linear correlation between AID of Ca and AID of P in the chicks was positive ($R^2=0.56$) which suggested that these minerals are intricately related in their absorption mechanisms (Hurwitz and Bar, 1971). Coarse limestone impacted the AID of Ca and, P positively, due to the lower solubility.

Several studies reported that dietary Ca was associated with lower phytase efficiency (Selle and Ravindran, 2007) due to formation of insoluble Ca-phytate complexes (Tamim *et al.* 2004). The significant interaction between limestone and diets on Ca and, P digestibility investigation indicated that coarse limestone-fed diets increased the overall Ca and P digestibility and was unaffected by phytase supplementation at activities of either 1000 or 2000 FYT units/kg. Those results were similar to those obtained by Zhang and Coon (1997), which indicated that feeding larger particle size limestone increased the overall Ca retention for laying hens. In contrast, Bradbury *et al.* (2018) showed that addition of phytase improved Ca

digestibility in diets that contained limestone particle size of >0.5 mm. In the present study, coarse limestone NC diets produced similar Ca and P digestibility as the PC diets, which made the phytase effects on Ca digestibility redundant. It is also known that when marginal Ca levels are fed, homeostatic mechanisms will upregulate Ca absorption. The fine limestone-fed diets made P digestibility of NC diets more dependent on the phytase, and as was expected, phytase supplementation at 1000 FYT units/kg increased P digestibility to a level comparable to that reported by Dilger *et al.* (2004) and Ravindran *et al.* (2006). An additional dose of 1000 FYT units/kg did not affect P digestibility of the NC diets but was similar to that of PC diets, which raises the possibility that the rate of phytate-P release may not be related to phytase activity (Plumstead *et al.*, 2008). It is more likely that the phytate molecule was inaccessible due to unidentified phytate complexes of which Ca-phytate is one potential factor (Tamim *et al.*, 2004). Although the HPLC data did indicate similar phytic acid degradation by both levels of phytase activity in coarse and fine limestone ventriculus content, overall, the observation on P utilization indicated that phytase was more beneficial in fine limestone-fed diets.

The Na digestibility values were found negative in this study, which is somewhat similar to the Na digestibility values reported in the literature (Ravindran *et al.*, 2006, 2008; Truong *et al.*, 2014, 2015; Bradbury *et al.*, 2018). In this study the Na digestibility was improved with coarse limestone fed diets and the magnitude of this negativity was significantly reduced in the coarse limestone fed diets to -38.9% from -54.1% found in the fine limestone fed diets. The findings were in agreement with Bradbury *et al.* (2018) who reported improvement in Na digestibility when limestone particle sized was increased to >0.5 mm from <0.2 mm. The data indicated that coarse limestone particle size augmented the Na homeostasis whereas in the fine limestone-based diets the increased appearance of Na may have been due to the NaHCO₃ secretion associated with the pancreatic juices and largely an interaction with

phytate which can result in endogenous secretion of Na to maintain homeostasis (Cowieson *et al.*, 2006). The effect of dietary Ca and P levels on the negative Na digestibility values was described by Bradbury *et al.* (2018), the present study analyzed dietary Ca and P were in a similar range of 0.82% and 0.65%, respectively, may have been associated with the negative values for Na digestibility.

Currently, there is little information available on broiler AID of AA with respect to dietary limestone particle size. It is well established that AA are transported in jejunum as small peptides rather than free amino acids (Silk *et al.*, 1985). Separate systems exist for transport of free AA (neutral system, basic systems, acidic systems, iminoglycine system and β -amino acid system) that enters the same port used by glucose and it is also known that AA uptake is most rapid in the ileum. Coarse limestone based-diets increased the ileal digestibility of 16 of the 17 amino acids that were assessed herein. The coarse limestone *vs.* fine limestone diets increased the digestibility of Ca (58.8% *vs.* 39.9%), P (74.6% *vs.* 52.5%), and Na (-38.9% *vs.* -54.1%) thus, shared a significant trend with AID of AA. One possible reason for these observations would appear to be due to formation of lesser binary or ternary protein-phytate complexes in the small intestine (Cowieson and Cowieson, 2011; Sommerfeld *et al.*, 2018). Further, it may be implied that the presence of coarse limestone (less soluble) particle having a lesser effect on gastric pH due to lower acid binding capacity thus leading to optimal protein hydrolysis affected by pepsin activation, which is pH dependent (Cowieson *et al.*, 2017). The PC diets, based on coarse limestone, returned significantly greater feed intake. Selle *et al.* (2012) suggested that the differences in the feed intake may have an influence on the digestibility of AA. However, no correlation could be discerned between the response variables; feed intake and AID of AA. However, there was a significant correlation detected between the Na digestibility and, all the 17 AA digestibility assessed (Figure 3A through 3Q).

Skou (1965) described that the activation of ATP-hydrolyzing enzyme system (later known as sodium pump) in addition to Mg requires Na and, K, and when K is substituted with NH₄ led to considerable increase in the ATPase enzyme system. Sodium is known to play a crucial role in the intestinal uptake of peptides and AA because of its role in the Na dependent Na⁺, K⁺, ATPase enzyme transport mechanism (Sklan and Noy, 2000; Broer, 2008; Ravindran *et al.*, 2008). It could be implied that at the intestinal level this mechanism may have resulted in increased uptake of AA. Truong *et al.* (2014) assessed the effect of phytase on AA digestibility and reported that the Na digestibility was correlated with increased AA retention. In the present study the variation in AA digestibility was explained by the AID of Na to an extent however, it could be implied that the Na-dependent AA transporters may have caused this common relationship, hence further investigation is required.

Similar to the findings of the present study, some broiler trials reported no effect of phytase on AA digestibility (Sebastian *et al.* 1997; Peter and Baker, 2001). In the present study, despite a lack of significant limestone x diet interaction, phytase at a dose of 2000 FYT/kg to NC diets appeared to return a similar AA digestibility to those of the PC-fed diets.

CONCLUSIONS

In the broiler diets supplementation of phytase enzyme at dose of 1000 FYT/kg and 2000 FYT/kg allowed the reduction up to 0.15% dietary AvP. The broilers fed diets based on coarse limestone diets returned an increased nutrient digestibility of calcium, phosphorus, sodium and amino acids.

Table 1. Particle size (d_{gw}) analysis of ingredients and compounded feed.

Item	Geometric mean diameter (d_{gw}) ¹	Standard deviation (S_{gw}) ²
De-flourinated phosphate	166 μm	1.58
Fine limestone	208 μm	1.85
Coarse limestone	925 μm	1.99
Starter diet with Fine limestone	668 μm	2.16
Starter diet with coarse limestone	662 μm	2.16
Grower diet with fine limestone	615 μm	1.90
Grower diet with coarse limestone	605 μm	1.90

¹ d_{gw} : Geometric mean diameter by mass.

² S_{gw} : Geometric standard deviation of particle size variation by mass.

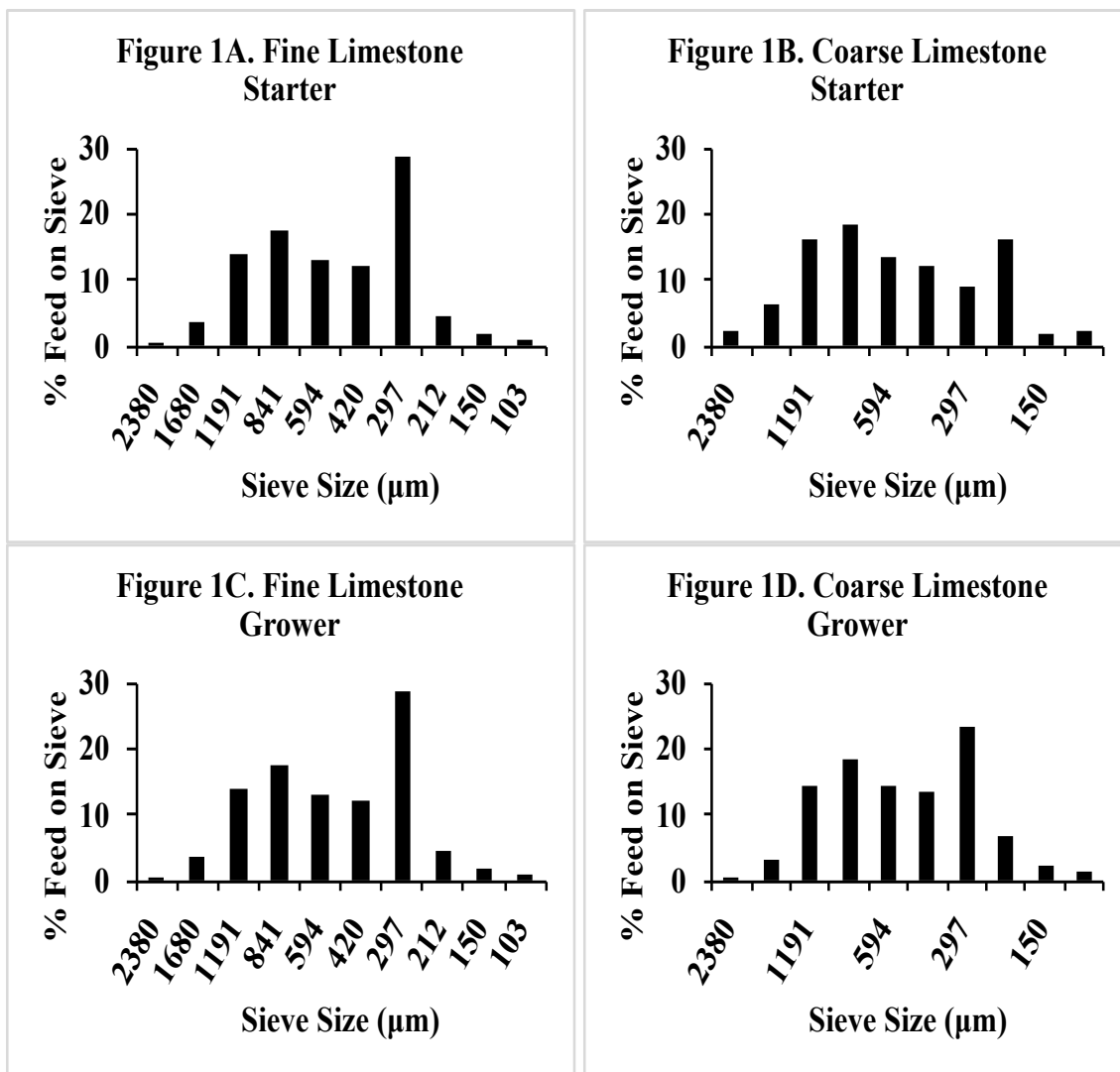


Figure 1 (A-D). Particle size distribution of diets.

Table 2. Phytase recovery (FYT units/kg) from the compounded feeds.

Limestone ¹	Diet	Phytase (FYT units/kg)
Fine	C + 1000 FYT units/kg	952
Fine	C + 2000 FYT units/kg	2168
Coarse	G + 1000 FYT units/kg	1110
Coarse	H + 2000 FYT units/kg	2521

²Limestone: Fine; (~200 µm) and Coarse; (~900 µm).

Table 3. Inositol-6-phosphate disappearance measure by HPIC¹ in ventriculus digesta.

Limestone ²	Phytase (FYT units/kg)	% Disappearance of IP ₆	n
Fine	1000	56.7	4
Fine	2000	69.1	3
Coarse	1000	67.0	3
Coarse	2000	72.9	2

¹HPIC: High performance ion exchange chromatography.

²Limestone: Fine; (~200 µm) and Coarse; (~900 µm)

Table 4. Limestone solubility percentage in ventriculus digesta.

Limestone ¹	In-vitro	Solubility (%)	Ventriculus
Fine	86.30		75.30
Coarse	61.70		85.50

¹Limestone: Fine; (~200 μm) and Coarse; (~900 μm).

Table 5. Composition of experimental diets.

Ingredients	Dietary treatment								
	Starter ¹				Dietary treatment (%)	Grower ²			
	PC	NC	NC+	NC+		PC	NC	NC+	NC+
Corn	58.50	58.50	58.50	58.50	64.50	64.50	64.50	64.50	
Soybean meal 48% CP	31.50	31.50	31.50	31.40	26.00	26.00	26.00	26.00	
Poultry by product meal	5.00	5.00	5.00	5.00	4.00	4.00	4.00	4.00	
Poultry fat	2.00	2.00	2.00	2.00	2.50	2.50	2.50	2.50	
Limestone ³	0.50	0.55	0.55	0.55	0.36	0.57	0.57	0.57	
Defluorinated phosphate ⁴	1.05	0.30	0.30	0.30	0.77	0.07	0.07	0.07	
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	
Vitamin premix ⁵	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
Mineral premix ⁶	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	
Selenium premix ⁷	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
DL-Methionine	0.20	0.20	0.20	0.20	0.11	0.11	0.11	0.20	
L-Lysine	0.11	0.11	0.11	0.11	0.10	0.10	0.10	0.11	
L-Threonine	0.07	0.07	0.07	0.07	0.06	0.06	0.06	0.07	
Choline chloride 60%	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	
Coccidiostat ⁸	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
Titanium dioxide	0.00	0.00	0.00	0.00	0.50	0.50	0.50	0.50	
Phytase	0.00	0.00	0.04	0.08	0.00	0.00	0.04	0.08	
Sand (inert filler)	0.22	0.92	0.88	0.84	0.23	0.72	0.68	0.64	
Calculated nutrients									
ME (kcal/g)	2.99	2.99	2.99	2.99	3.07	3.07	3.07	3.07	
Crude protein	23.38	23.38	23.38	23.38	20.44	20.44	20.44	20.44	
Crude fat	4.84	4.84	4.84	4.84	5.36	5.36	5.36	5.36	
Calcium	0.90	0.70	0.70	0.70	0.71	0.58	0.58	0.58	
Available phosphorus	0.45	0.30	0.30	0.30	0.36	0.24	0.24	0.24	
Digestible lysine	1.16	1.16	1.16	1.16	1.00	1.00	1.00	1.00	
Digestible methionine	0.53	0.53	0.53	0.53	0.41	0.41	0.41	0.41	

Table 5 (Continued). Composition of experimental diets.

Ingredients	Dietary treatment							
	Starter ¹				NC+ 2000 (%)	Grower ²		
	PC	NC	NC+ 1000	NC+ 2000		PC	NC	NC+ 1000
Digestible TSAA	0.80	0.80	0.80	0.80	0.65	0.65	0.65	0.65
Digestible threonine	0.79	0.79	0.79	0.79	0.70	0.70	0.70	0.70
Sodium	0.25	0.21	0.21	0.21	0.23	0.19	0.19	0.19
Chloride	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Potassium	0.92	0.92	0.92	0.92	0.80	0.80	0.80	0.80
<u>Analyzed nutrients</u>								
Crude Fat	4.36	3.88	4.16	4.28	5.38	5.09	5.67	5.49
Calcium	0.82	0.69	0.70	0.64	0.71	0.57	0.59	0.57
Phosphorus	0.61	0.55	0.58	0.50	0.55	0.47	0.47	0.47

¹Starter was fed to 16 d of age.

²Grower was fed to 33 d of age.

³Limestone particle size: 0.2 mm and 0.9 mm.

⁴Deflourinated phosphate analyzed phosphorus concentration 179 g/kg.

⁵Vitamin premix supplied the following per kg of diet: 6,614 IU vitamin A, 1,984 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 1.98 mg menadione (K3), 1.98 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 3.97 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

⁶Mineral premix supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; cobalt, 1 mg.

⁷Selenium premix provided 0.3 mg Se (as Na₂SeO₃) per kg of diet.

⁸Coccidiostat supplied monensin sodium at 90 mg/kg of feed.

Table 6. Effect of limestone particle size and diet formulation on feed intake, body weight (BW), BW gain, and feed conversion ratio (FCR) of male broilers from 1-16 d of age.

Limestone ¹	Diet ²	Feed	BW	BW gain	FCR
		Intake	g/bird		(g:g)
Fine		749	601	556	1.35 ^b
Coarse		754	596	550	1.37 ^a
	SEM ³	5	3	3	0.007
	PC	763 ^A	612 ^A	567 ^A	1.36 ^A
	NC	721 ^B	560 ^B	515 ^B	1.40 ^A
	NC+1000	758 ^A	605 ^A	560 ^A	1.35 ^B
	NC+2000	763 ^A	616 ^A	571 ^A	1.34 ^B
	SEM ⁴	7	4	4	0.010
	Fine / PC	760 ^{ab}	616	571	1.33
	Fine / NC	731 ^{cd}	566	521	1.40
	Fine / NC+1000	739 ^{bc}	600	555	1.33
	Fine / NC+2000	765 ^{ab}	621	576	1.33
	Coarse / PC	766 ^{ab}	608	563	1.36
	Coarse / NC	712 ^d	555	509	1.40
	Coarse / NC+1000	778 ^a	610	564	1.38
	Coarse / NC+2000	761 ^{ab}	611	565	1.35
	SEM ⁵	9	6	6	0.014
		(Probability > F)			
	Limestone	0.43	0.26	0.26	0.04
	Diet	<0.01	<0.01	<0.01	<0.01
	Limestone x Diet	0.02	0.30	0.32	0.28

^{A,B}Means within a column lacking a common superscript differ significantly ($P \leq 0.01$).

^{a,b}Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).

¹Limestone particle size (starter); Fine (0.2 mm) and Coarse (0.9 mm).

²Diets; PC: positive control (AvP = 0.45%), NC: negative control (AvP = 0.30%), NC+1000: NC+Phytase 1000 FYT/kg, NC+2000: NC+Phytase 2000 FYT/kg.

³SEM = Standard error of mean for n=24 pens for each main effect of limestone particle size.

⁴SEM = Standard error of mean for n=12 pens for each main effect of diet.

⁵SEM = Standard error of mean for n=6 pens for each interaction effect of limestone particle size and diet.

Table 7. Effect of limestone particle size and diet formulation on feed intake, body weight (BW), BW gain, and feed conversion ratio (FCR) of male broilers from 1-33 d of age.

Limestone ¹	Diet ²	Feed	BW	BW gain	FCR
		Intake	g/bird		(g:g)
Fine		3067 ^b	2219	2174	1.41
Coarse		3136 ^a	2248	2203	1.42
	SEM ³	23	15	3	0.006
	PC	3172 ^A	2279 ^A	2234 ^A	1.42 ^{xy}
	NC	2964 ^B	2109 ^B	2063 ^B	1.44 ^y
	NC+1000	3128 ^A	2263 ^A	2218 ^A	1.41 ^x
	NC+2000	3143 ^A	2284 ^A	2239 ^A	1.40 ^x
	SEM ⁴	32	21	21	0.008
	Fine / PC	3126	2249	2205	1.42
	Fine / NC	2951	2103	2057	1.43
	Fine / NC+1000	3047	2235	2190	1.39
	Fine / NC+2000	3144	2291	2245	1.40
	Coarse / PC	3218	2309	2264	1.42
	Coarse / NC	2977	2114	2069	1.44
	Coarse / NC+1000	3210	2292	2246	1.43
	Coarse / NC+2000	3141	2277	2232	1.40
	SEM ⁵	46	30	30	0.012
		(Probability > F)			
	Limestone	0.03	0.19	0.18	0.15
	Diet	<0.01	<0.01	<0.01	0.06
	Limestone x Diet	0.28	0.56	0.57	0.47

^{A,B}Means within a column lacking a common superscript differ significantly ($P \leq 0.01$).

^{a,b}Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).

^{x,y}Means within a column lacking a common superscript differ significantly ($P \leq 0.10$).

¹Limestone particle size (grower); Fine (0.2 mm) and Coarse (0.9 mm).

²Diets; PC: positive control (AvP = 0.45%), NC: negative control (AvP = 0.30%), NC+1000: NC+Phytase 1000 FYT/kg, NC+2000: NC+Phytase 2000 FYT/kg.

³SEM = Standard error of mean for n=24 pens for each main effect of limestone particle size.

⁴SEM = Standard error of mean for n=12 pens for each main effect of diet.

⁵SEM = Standard error of mean for n=6 pens for each interaction effect of limestone particle size and diet.

Table 8. Effect of limestone particle size and diet formulation on weight of tibia, weight of ash and tibia bone ash percentage of male broilers at 33 d of age.

Limestone ¹	Diet ²	Weight of	Weight of	Bone Ash
		de-fatted Tibia	Ash	
		g/bird		(%)
Fine		9.2	4.0	44.0
Coarse		9.1	4.1	44.8
	SEM ³	0.21	0.07	0.50
	PC	9.3	4.3 ^a	45.9 ^a
	NC	8.9	3.7 ^b	41.9 ^b
	NC+1000	9.3	4.1 ^a	44.1 ^a
	NC+2000	9.1	4.1 ^a	45.6 ^a
	SEM ⁴	0.29	0.10	0.70
	Fine / PC	9.1	4.2	45.9
	Fine / NC	8.7	3.5	41.4
	Fine / NC+1000	9.6	4.1	43.1
	Fine / NC+2000	9.4	4.3	45.6
	Coarse / PC	9.5	4.3	46.0
	Coarse / NC	9.2	3.8	42.3
	Coarse / NC+1000	9.0	4.0	45.0
	Coarse / NC+2000	8.9	4.0	45.6
	SEM ⁵	0.41	0.14	0.99
		(Probability > F)		
	Limestone	0.81	0.67	0.30
	Diet	0.80	<0.01	<0.01
	Limestone x Diet	0.45	0.28	0.75

^{A,B}Means within a column lacking a common superscript differ significantly ($P \leq 0.01$).

^{a,b}Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).

¹Limestone particle size (starter and grower); Fine (0.2 mm) and Coarse (0.9 mm).

²Diets; PC: positive control (AvP = 0.45%), NC: negative control (AvP = 0.30%), NC+1000: NC+Phytase 1000 FYT/kg, NC+2000: NC+Phytase 2000 FYT/kg.

³SEM = Standard error of mean for n=24 pens using 2 birds/pen for each main effect of limestone particle size.

⁴SEM = Standard error of mean for n=12 pens using 2 birds/pen for each main effect of diet.

⁵SEM = Standard error of mean for n=6 pens using 2 birds/pen for each interaction effect of limestone particle size and diet.

Table 9. Effect of limestone particle size and diet formulation on tibia mineral concentration of male broilers at 33 d of age.

Limestone ¹	Diet ²	Ca	P	Na	Cl	Mg	K	Mn	Zn	Fe
				(%)				(ppm)		
Fine		37.0	17.9	1.45	0.38	0.86 ^x	6299	15.3	374	538 ^a
Coarse		37.0	17.8	1.40	0.36	0.85 ^x	6103	14.3	375	474 ^b
	SEM ³	0.16	0.07	0.03	0.02	0.005	221	0.45	3.41	21.6
	PC	37.1	18.0 ^x	1.31 ^B	0.32 ^{BC}	0.85 ^B	5670 ^{BC}	12.7 ^C	355 ^B	539
	NC	36.7	17.6 ^y	1.52 ^A	0.50 ^A	0.80 ^C	7169 ^A	12.4 ^C	356 ^B	470
	NC+1000	37.2	17.8 ^{xy}	1.50 ^A	0.38 ^B	0.89 ^A	6512 ^{AB}	16.2 ^B	391 ^A	484
	NC+2000	37.0	17.9 ^{xy}	1.35 ^B	0.28 ^C	0.89 ^A	5454 ^C	18.0 ^A	395 ^A	532
	SEM ⁴	0.22	0.10	0.04	0.03	0.007	316	0.64	4.87	30.5
	Fine / PC	37.1	17.9	1.34	0.36	0.85 ^c	6098	10.5 ^E	357	425 ^D
	Fine / NC	36.8	17.6	1.56	0.55	0.80 ^d	7433	12.1 ^{DE}	353	489 ^{CD}
	Fine / NC+1000	37.0	17.9	1.52	0.38	0.89 ^{ab}	6212	16.8 ^B	384	552 ^{BC}
	Fine / NC+2000	37.2	18.0	1.36	0.23	0.91 ^a	5455	21.7 ^A	401	685 ^A
	Coarse / PC	37.0	18.0	1.28	0.27	0.85 ^c	5242	14.9 ^{BC}	352	653 ^{AB}
	Coarse / NC	36.6	17.6	1.48	0.45	0.80 ^d	6905	12.6 ^{CDE}	360	450 ^D
	Coarse / NC+1000	37.4	17.8	1.49	0.38	0.88 ^b	6812	15.5 ^B	397	417 ^D
	Coarse / NC+2000	36.7	17.7	1.35	0.33	0.86 ^{bc}	5454	14.3 ^{BCD}	390	378 ^D
	SEM ⁵	0.32	0.14	0.06	0.05	0.01	442	0.90	6.81	43.2
		(Probability > F)								
	Limestone	0.81	0.30	0.30	0.48	0.08	0.53	0.14	0.86	0.04
	Diet	0.48	0.09	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.30
	Limestone x Diet	0.53	0.60	0.96	0.17	0.02	0.39	<0.01	0.28	<0.01

^{A,B}Means within a column lacking a common superscript differ significantly ($P \leq 0.01$).

^{a,b}Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).

^{x,y}Means within a column lacking a common superscript differ significantly ($P \leq 0.10$).

¹Limestone particle size (starter and grower); Fine (0.2 mm) and Coarse (0.9 mm).

²Diets; PC: positive control (AvP = 0.45%), NC: negative control (AvP = 0.30%), NC+1000: NC+Phytase 1000 FYT/kg, NC+2000: NC+Phytase 2000 FYT/kg.

³SEM = Standard error of mean for n=24 pens using 2 birds/pen for each main effect of limestone particle size.

⁴SEM = Standard error of mean for n=12 pens using 2 birds/pen for each main effect of diet.

⁵SEM = Standard error of mean for n=6 pens using 2 birds/pen for each interaction effect of limestone particle size and diet.

Table 10. Effect of limestone particle size and diet formulation on apparent ileal digestibility of calcium (Ca), phosphorus (P), potassium (K), sodium (Na), and chloride (Cl) of male broilers at 33 d of age.

Limestone ¹	Diet	Ca	P	K	Na	Cl
		(%)				
Fine		39.9 ^B	52.5 ^B	83.3	-54.1 ^b	15.5 ^x
Coarse		60.0 ^A	74.8 ^A	84.0	-38.9 ^a	25.2 ^x
	SEM ³	2.23	2.67	0.85	5.35	3.91
	PC	49.6 ^{xy}	57.0 ^y	84.1	-35.0 ^a	18.8
	NC	56.6 ^x	62.3 ^{xy}	85.5	-46.8 ^{ab}	18.1
	NC+1000	49.0 ^{xy}	70.8 ^x	83.0	-65.2 ^b	16.9
	NC+2000	44.6 ^y	64.4 ^{xy}	82.0	-39.0 ^{ab}	27.6
	SEM ⁴	3.16	3.78	1.21	7.57	5.54
	Fine / PC	33.6 ^{de}	40.7 ^B	86.2 ^x	-39.6	10.4
	Fine / NC	49.6 ^{bc}	45.6 ^B	85.2 ^{xy}	-56.0	17.6
	Fine / NC+1000	44.7 ^{cd}	71.6 ^A	81.3 ^{yz}	-72.0	8.5
	Fine / NC+2000	31.7 ^e	52.0 ^B	80.3 ^z	-48.8	25.6
	Coarse / PC	65.5 ^a	73.3 ^A	82.0 ^{xyz}	-30.5	27.3
	Coarse / NC	63.7 ^a	79.1 ^A	85.8 ^{xy}	-37.6	18.7
	Coarse / NC+1000	53.3 ^{abc}	70.0 ^A	84.6 ^{xyz}	-58.4	25.3
	Coarse / NC+2000	57.6 ^{ab}	76.7 ^A	83.8 ^{xyz}	-29.2	29.6
	SEM ⁵	4.47	5.35	1.71	10.7	7.83
		(Probability > F)				
	Limestone	<0.01	<0.01	0.52	0.05	0.08
	Diet	0.07	0.09	0.22	0.03	0.51
	Limestone x Diet	0.05	<0.01	0.09	0.95	0.63

^{A,B}Means within a column lacking a common superscript differ significantly ($P \leq 0.01$).

^{a,b}Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).

^{x,y}Means within a column lacking a common superscript differ significantly ($P \leq 0.10$).

¹Limestone particle size (starter and grower); Fine (0.2 mm) and Coarse (0.9 mm).

²Diets; PC: positive control (AvP = 0.45%), NC: negative control (AvP = 0.30%), NC+1000: NC+Phytase 1000 FYT/kg, NC+2000: NC+Phytase 2000 FYT/kg.

³SEM = Standard error of mean for n=24 pens using 2 birds/pen for each main effect of limestone particle size.

⁴SEM = Standard error of mean for n=12 pens using 2 birds/pen for each main effect of diet.

⁵SEM = Standard error of mean for n=6 pens using 2 birds/pen for each interaction effect of limestone particle size and diet.

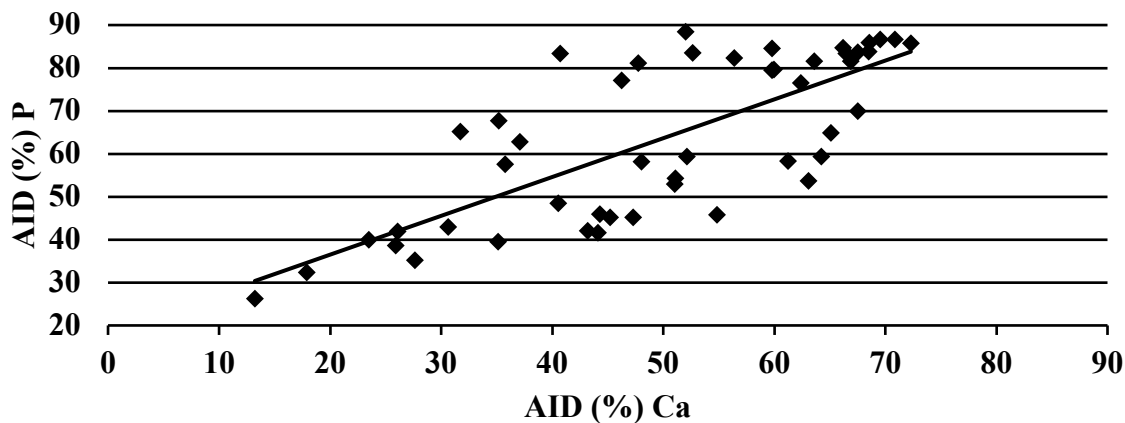


Figure 2A. Correlation between AID of Ca and AID of P at 33 d of age, $P < 0.01$; $R^2 = 0.56$.

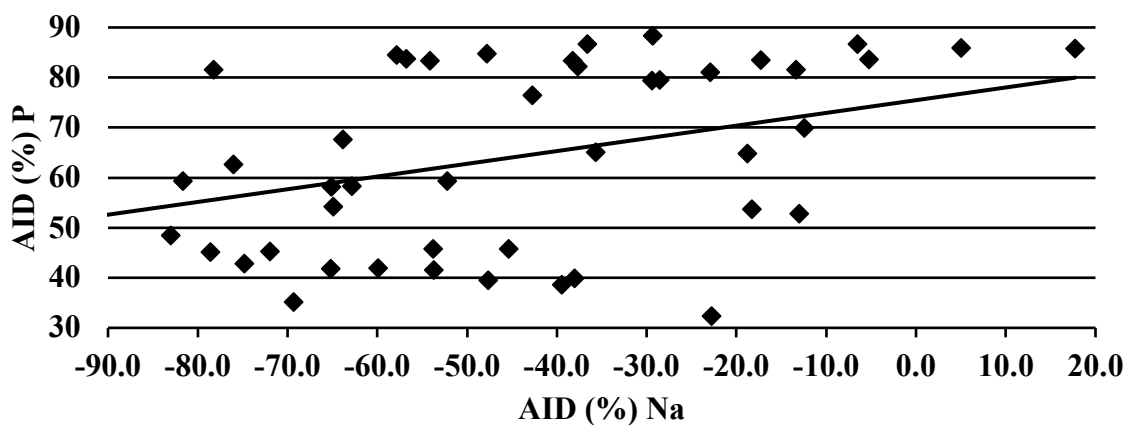


Figure 2B. Correlation between AID of Na and AID of P at 33 d of age, $P < 0.01$; $R^2 = 0.15$.

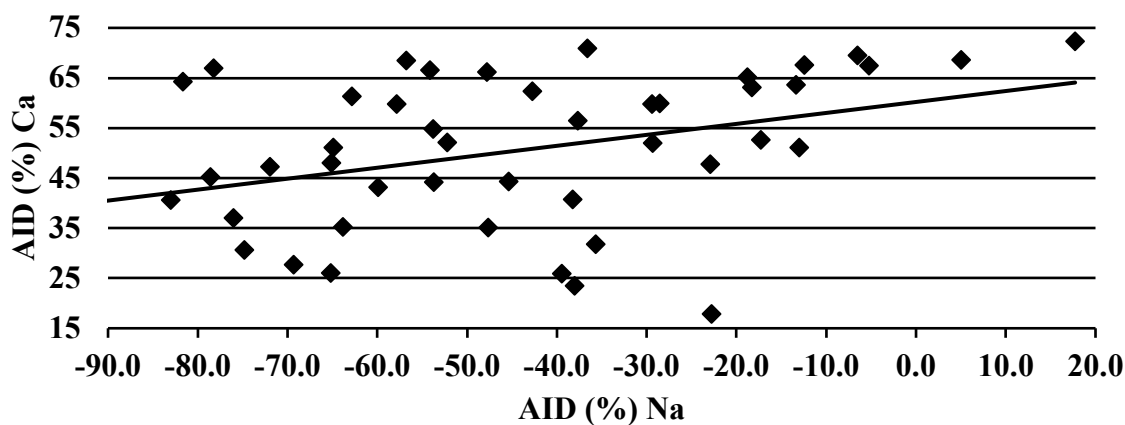


Figure 2C. Correlation between AID of Na and AID of Ca at 33 d of age, $P < 0.01$; $R^2 = 0.14$.

Table 11. Effect of limestone particle size and diet formulation on apparent ileal digestibility percentage of amino acids of male broilers at 33 d of age.

Limestone ¹	Diet ²	Lys	Met	Cys	Thr	Val	Leu	Ile	Gly	Ser
						(%)				
Fine		82.1 ^B	86.9 ^y	60.3 ^B	70.3 ^B	72.8 ^B	78.9 ^B	76.9 ^B	75.9 ^B	75.1 ^B
Coarse		84.4 ^A	88.3 ^x	65.4 ^A	74.7 ^A	76.7 ^A	81.8 ^A	79.9 ^A	79.3 ^A	79.0 ^A
	SEM ³	0.63	0.60	1.30	0.91	0.89	0.80	0.83	0.80	0.88
	PC	83.1	87.1	60.1	72.0	74.1	80.0	77.9	77.3	76.2
	NC	83.5	88.2	61.7	72.4	75.0	80.4	78.4	77.3	76.5
	NC+1000	82.3	86.6	63.3	71.2	73.5	79.5	77.6	76.9	76.2
	NC+2000	84.0	88.3	66.2	74.4	76.3	81.6	79.7	78.8	79.3
	SEM ⁴	0.89	0.84	1.84	1.83	1.26	1.13	1.17	1.14	1.24
	Fine / PC	81.9	86.2	55.7	69.3	71.5	78.4	76.0	75.3	73.9
	Fine / NC	83.1	88.4	61.4	71.7	74.0	79.5	77.6	76.5	75.3
	Fine / NC+1000	80.2	84.6	59.9	67.6	70.4	76.7	74.6	74.2	73.1
	Fine / NC+2000	83.2	88.2	64.1	72.7	75.2	81.1	79.3	77.6	78.2
	Coarse / PC	84.4	88.0	64.6	74.8	76.8	81.7	79.8	79.3	78.4
	Coarse / NC	83.9	88.1	62.1	73.1	76.0	81.3	79.1	78.2	77.7
	Coarse / NC+1000	84.4	88.6	66.7	74.8	76.7	82.3	80.6	79.6	79.3
	Coarse / NC+2000	84.7	88.4	68.3	76.1	77.4	82.1	80.2	79.9	80.4
	SEM ⁵	1.26	1.20	2.60	1.20	1.78	1.61	1.66	1.61	1.76
		(Probability > F)								
	Limestone	0.01	0.10	<0.01	<0.01	<0.01	0.01	0.01	<0.01	<0.01
	Diet	0.62	0.42	0.13	0.42	0.45	0.61	0.59	0.66	0.23
	Limestone x Diet	0.58	0.28	0.44	0.28	0.53	0.51	0.43	0.65	0.62

Table 11 (Continued). Effect of limestone particle size and dietary formulation on apparent ileal digestibility percentage of amino acids of male broilers at 33 d of age.

Limestone ¹	Diet ²	Arg	Glu	His	Ala	Asp	Tyr	Phe	Pro
		(%)							
Fine		84.8 ^B	82.8 ^b	79.4 ^b	77.2 ^B	74.2 ^B	79.2 ^B	79.1 ^B	75.9 ^B
Coarse		86.9 ^A	85.1 ^a	81.8 ^a	80.4 ^A	77.9 ^A	82.2 ^A	82.1 ^A	79.3 ^A
	SEM ³	0.59	0.67	0.76	0.81	0.83	0.75	0.78	0.80
	PC	85.6	83.5	80.3	78.9	75.2	79.8	80.0	77.3
	NC	86.0	84.0	80.6	79.0	76.0	80.2	80.5	77.3
	NC+1000	85.3	83.3	80.1	77.5	75.2	80.8	80.0	76.9
	NC+2000	86.7	85.0	81.4	79.8	77.7	82.0	82.0	78.8
	SEM ⁴	0.84	0.94	1.07	1.15	1.18	1.07	1.10	1.14
	Fine / PC	84.4	82.1	78.5	77.0	72.7	78.7	78.4	75.3
	Fine / NC	85.5	83.4	80.3	78.1	75.1	78.9	79.4	76.5
	Fine / NC+1000	83.3	81.2	77.9	74.8	72.1	77.5	77.1	74.2
	Fine / NC+2000	86.3	84.6	80.9	78.9	76.7	81.6	81.6	77.6
	Coarse / PC	86.8	84.9	82.0	80.8	77.8	80.8	81.7	79.3
	Coarse / NC	86.5	84.5	80.9	79.9	76.9	81.5	81.5	78.2
	Coarse / NC+1000	87.3	85.5	82.3	80.3	78.3	84.1	83.0	79.6
	Coarse / NC+2000	87.1	85.4	82.0	80.6	78.8	82.4	82.5	79.9
	SEM ⁵	1.19	1.34	1.52	1.62	1.67	1.51	1.56	1.61
		(Probability > F)							
	Limestone	0.01	0.02	0.03	<0.01	<0.01	<0.01	<0.01	<0.01
	Diet	0.65	0.61	0.82	0.58	0.40	0.47	0.53	0.66
	Limestone x Diet	0.53	0.53	0.54	0.60	0.49	0.27	0.43	0.65

^{A,B}Means within a column lacking a common superscript differ significantly (P≤0.01).

^{a,b}Means within a column lacking a common superscript differ significantly (P≤0.05).

¹Limestone particle size (starter and grower); Fine (0.2 mm) and Coarse (0.9 mm).

²Diets; PC: positive control (AvP = 0.45%), NC: negative control (AvP = 0.30%), NC+1000: NC+Phytase 1000 FYT/kg, NC+2000: NC+Phytase 2000 FYT/kg.

³SEM = Standard error of mean for n=24 pens using 2 birds/pen for each main effect of limestone particle size.

⁴SEM = Standard error of mean for n=12 pens using 2 birds/pen for each main effect of diet.

⁵SEM = Standard error of mean for n=6 pens using 2 birds/pen for each interaction effect of limestone particle size and diet.

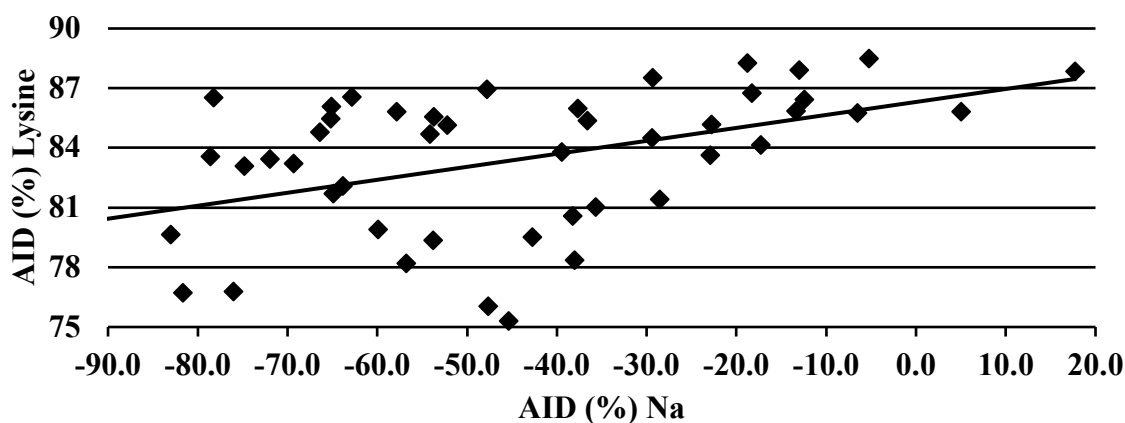


Figure 3A. Correlation between AID of Na and AID of lysine at 33 d of age, $P < 0.01$; $R^2 = 0.21$.

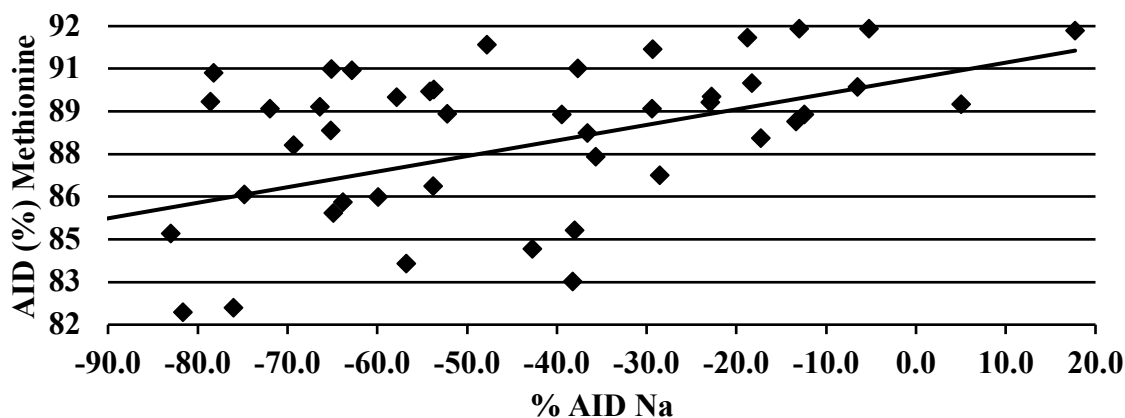


Figure 3B. Correlation between AID of Na and AID of methionine at 33 d of age, $P < 0.01$; $R^2 = 0.18$.

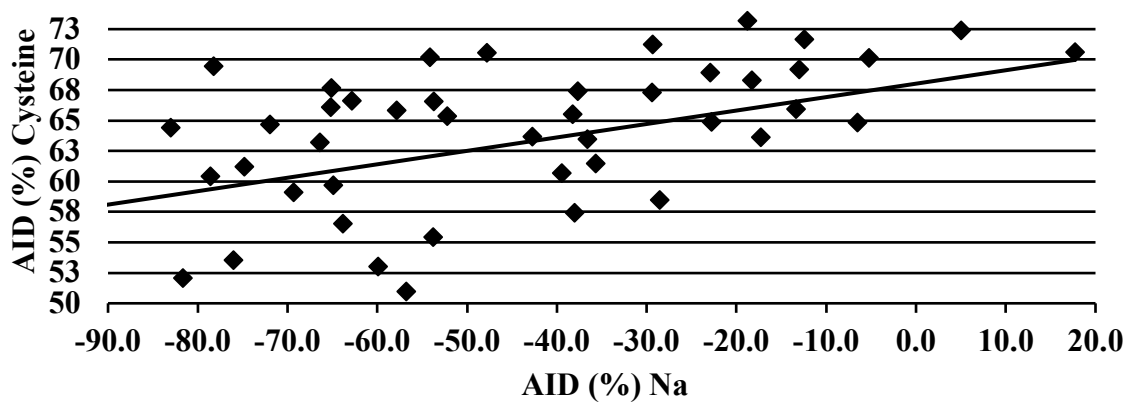


Figure 3C. Correlation between AID of Na and AID of cysteine at 33 d of age, $P < 0.01$; $R^2 = 0.16$.

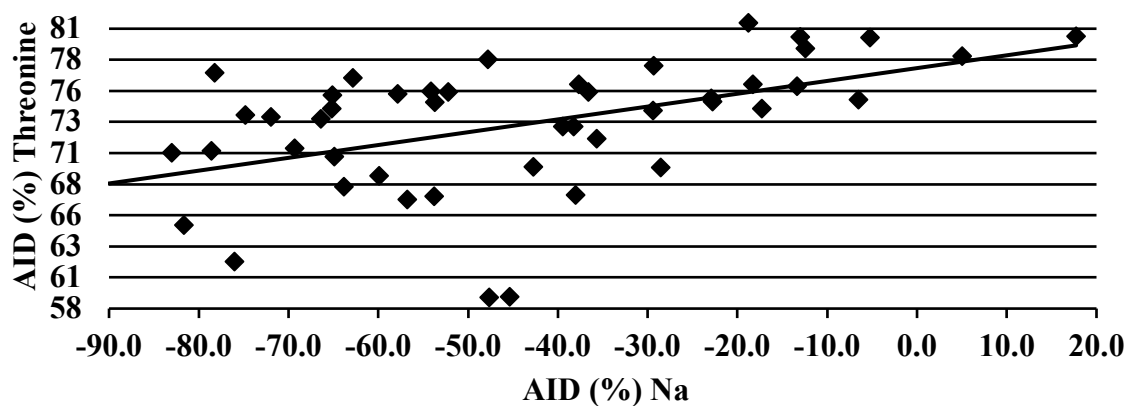


Figure 3D. Correlation between AID of Na and AID of threonine at 33 d of age, $P < 0.01$; $R^2 = 0.26$.

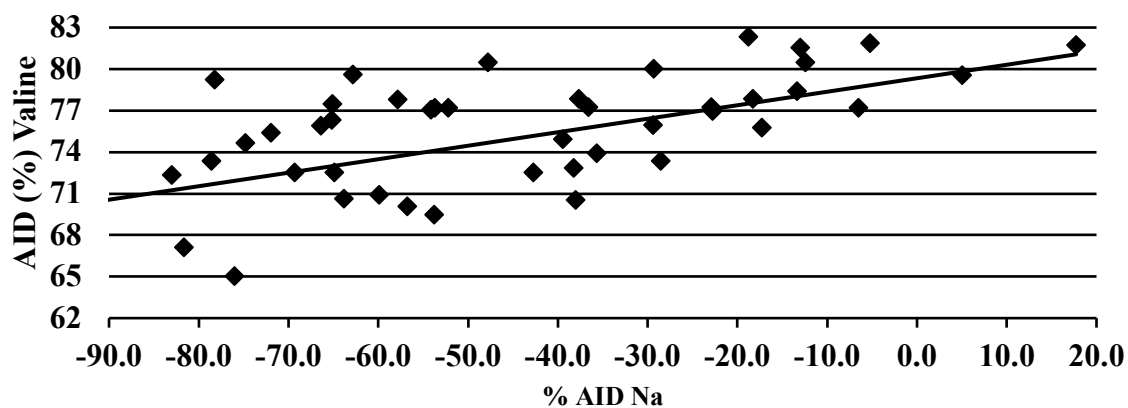


Figure 3E. Correlation between AID of Na and AID of valine at 33 d of age, $P < 0.01$; $R^2 = 0.25$.

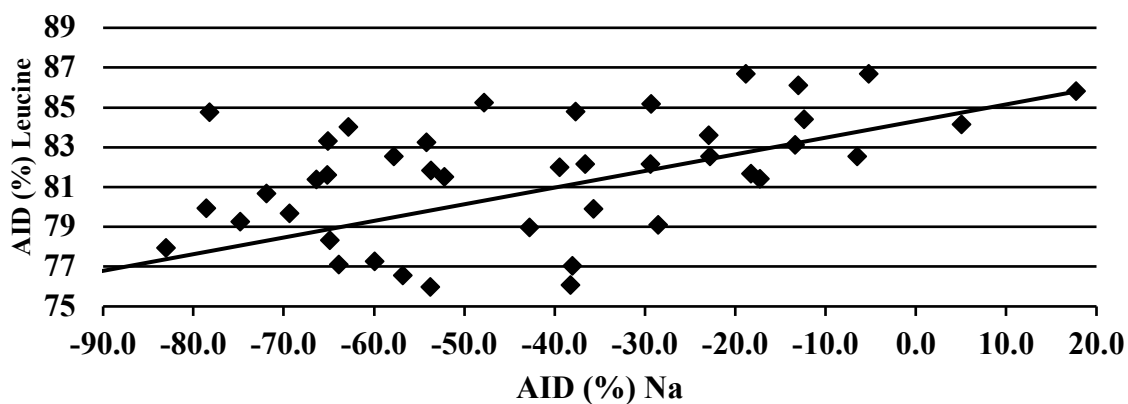


Figure 3F. Correlation between AID of Na and AID of leucine at 33 d of age, $P < 0.01$; $R^2 = 0.25$.

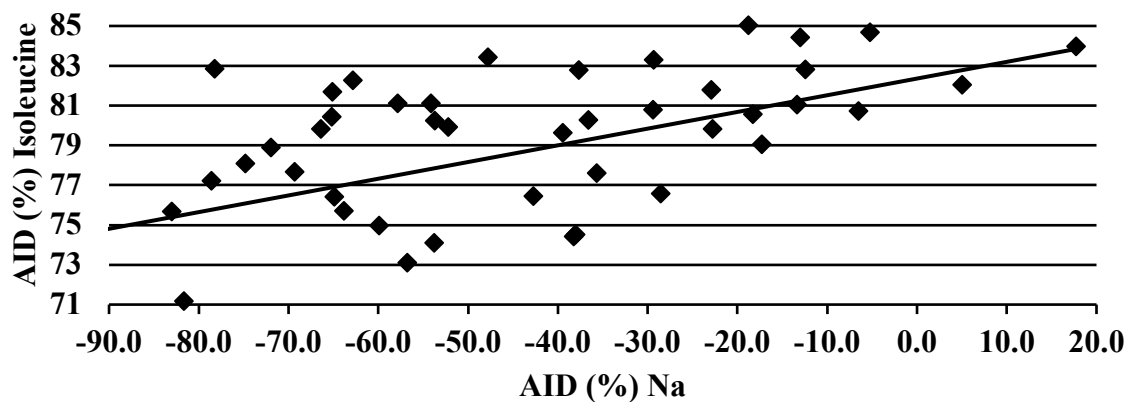


Figure 3G. Correlation between AID of Na and AID of isoleucine at 33 d of age, $P < 0.01$; $R^2 = 0.23$.

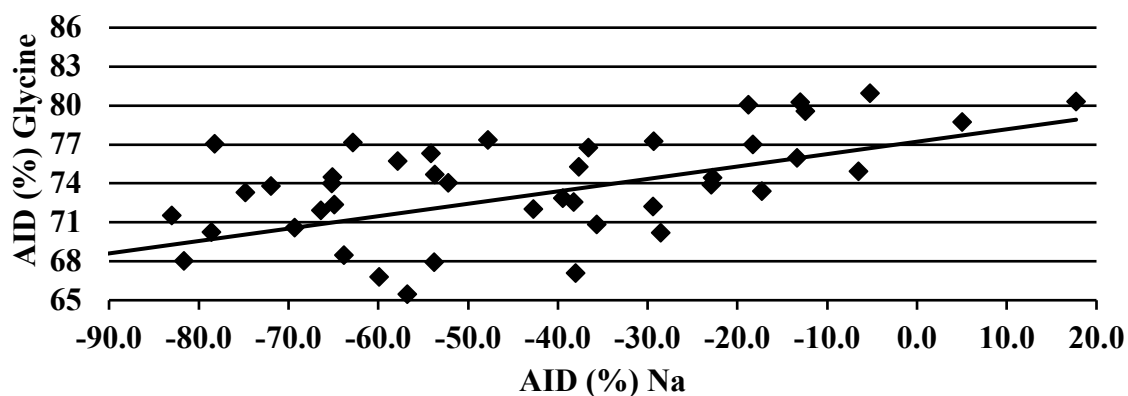


Figure 3H. Correlation between AID of Na and AID glycine at 33 d of age, $P < 0.01$; $R^2 = 0.25$.

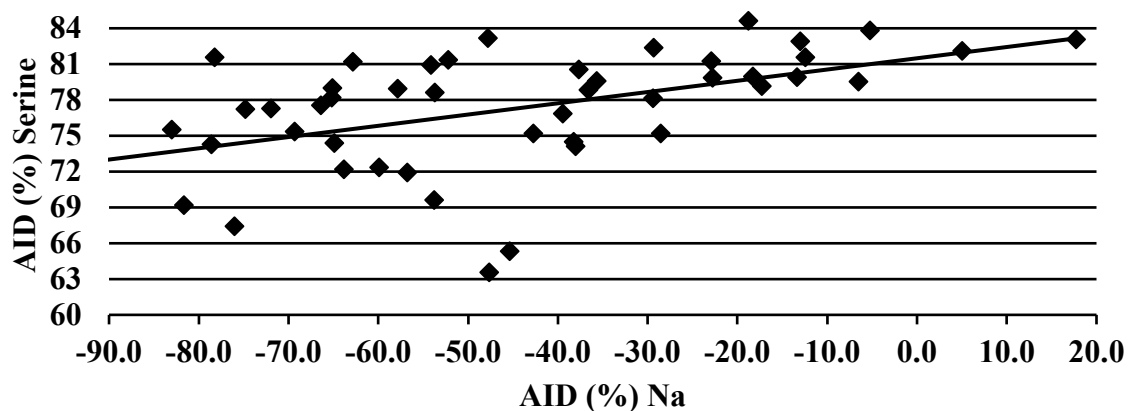


Figure 3I. Correlation between AID of Na and AID Serine at 33 d of age, $P < 0.01$; $R^2 = 0.25$.

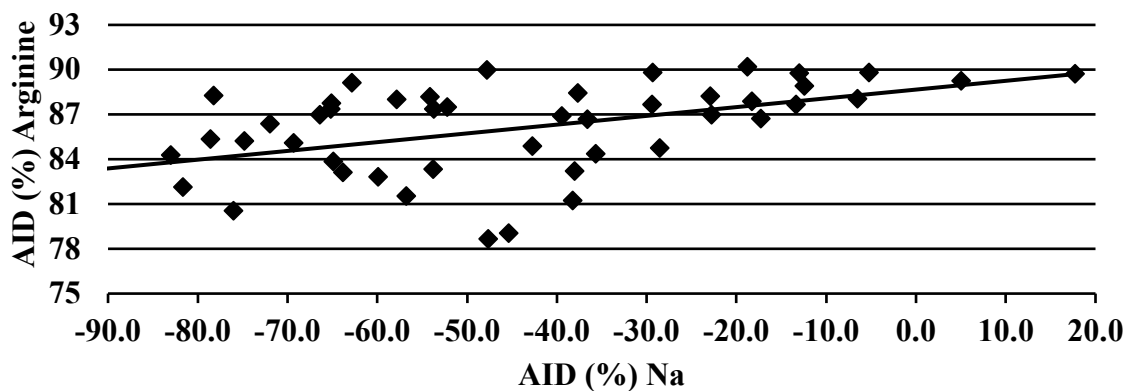


Figure 3J. Correlation between AID of Na and AID arginine at 33 d of age, $P < 0.01$; $R^2 = 0.23$.

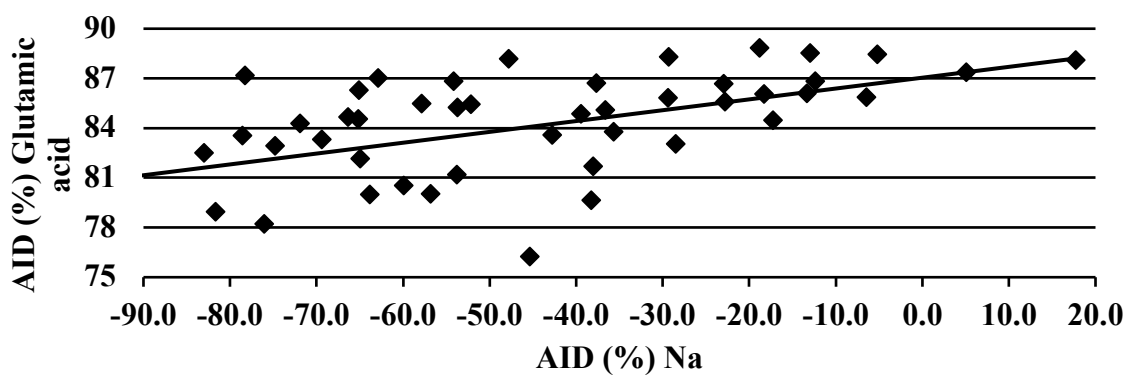


Figure 3K. Correlation between AID of Na and AID glutamine at 33 d of age, $P < 0.01$; $R^2 = 0.24$.

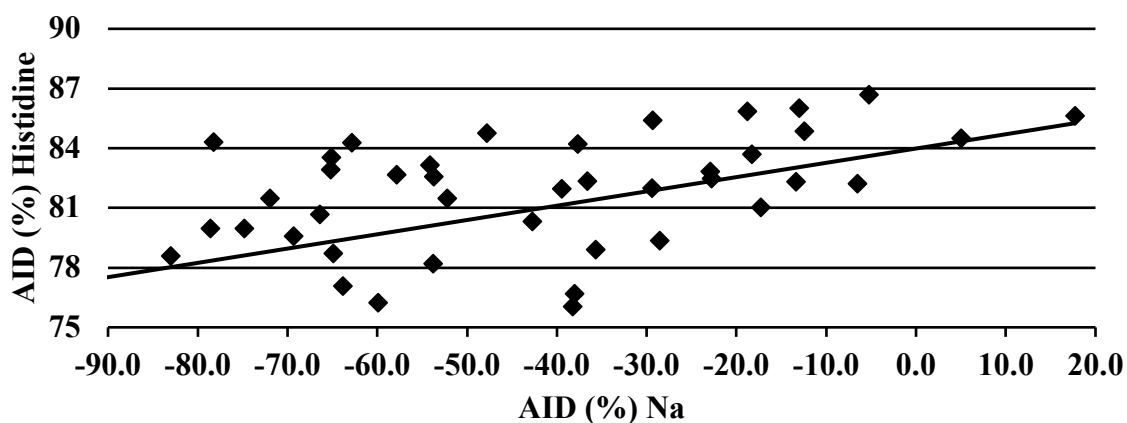


Figure 3L. Correlation between AID of Na and AID histidine at 33 d of age, $P < 0.01$; $R^2 = 0.22$.

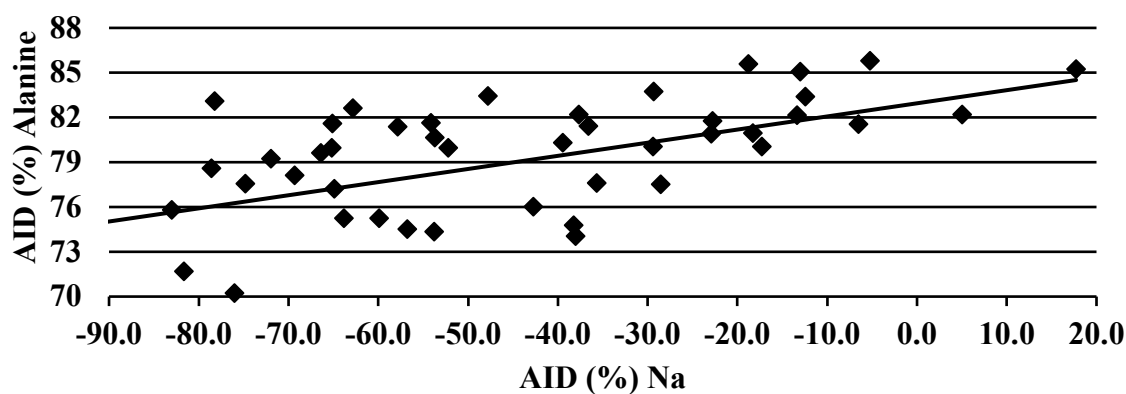


Figure 3M. Correlation between AID of Na and AID alanine at 33 d of age, $P < 0.01$; $R^2 = 0.22$.

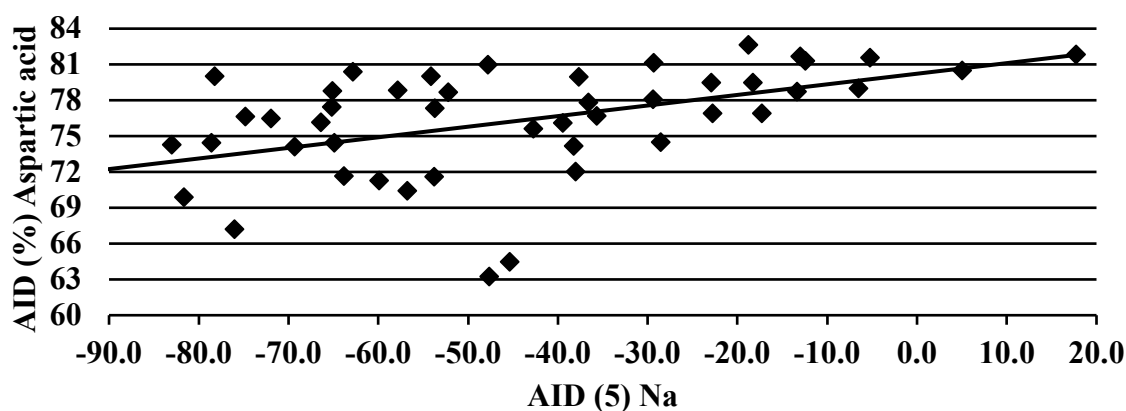


Figure 3N. Correlation between AID of Na and AID aspartic acid at 33 d of age, $P < 0.01$; $R^2 = 0.25$.

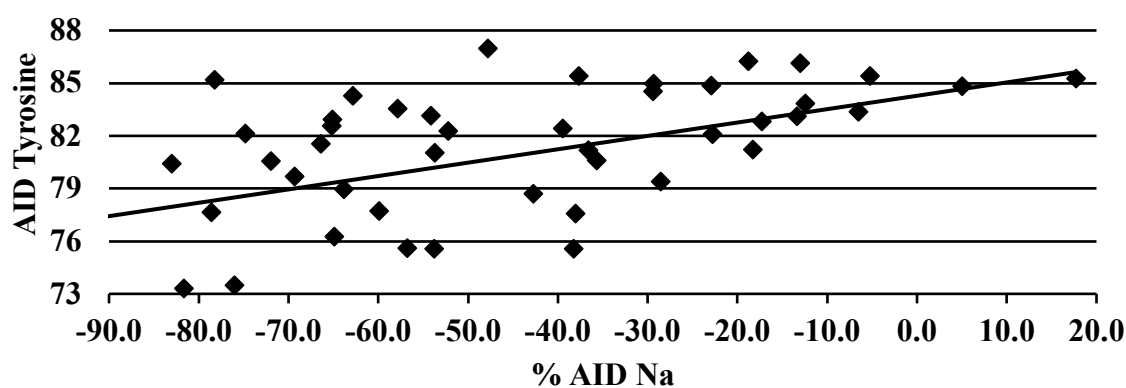


Figure 3O. Correlation between AID of Na and AID tyrosine at 33 d of age, $P < 0.01$; $R^2 = 0.23$.

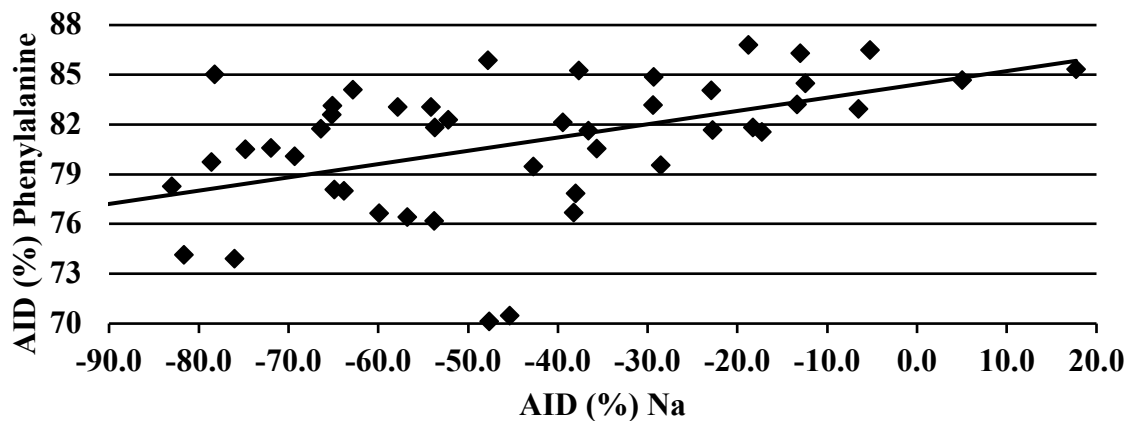


Figure 3P. Correlation between AID of Na and AID phenylalanine at 33 d of age, $P < 0.01$; $R^2 = 0.24$.

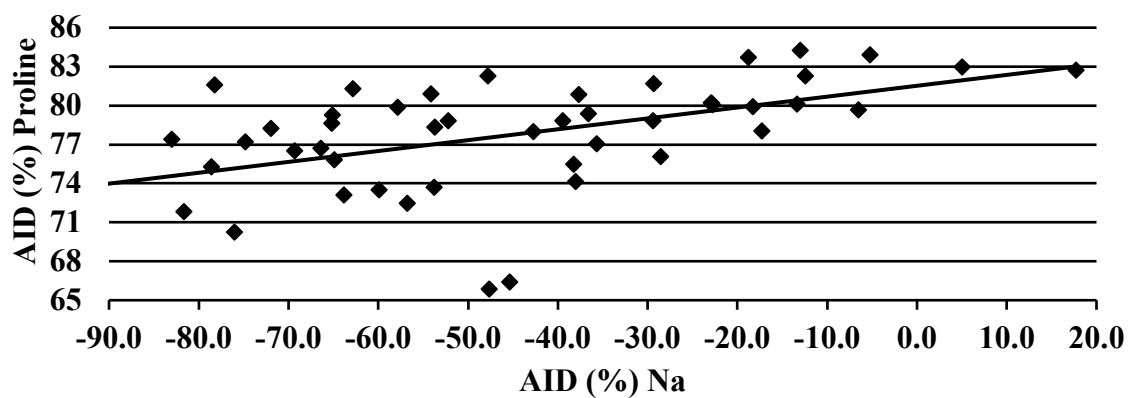


Figure 3Q. Correlation between AID of Na and AID of proline at 33 d of age, $P < 0.01$; $R^2 = 0.26$.

REFERENCES

- Anderson J. O., D. C. Dobson, O. K. Jack. 1984. Effect of particle size of the calcium source on performance of broiler chicks fed diets with different calcium and phosphorus levels. *Poult. Sci.* 63:311-316.
- Anwar, M. N., V. Ravindran, P. C. H. Morel, G. Ravindran, and A. J. Cowieson. 2016. Effect of limestone particle size and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. *Br. Poult. Sci.* 57:707-713.
- Anwar, M. N. 2017. Measurement of true ileal calcium digestibility of feed ingredients for broiler chicks. Dissertation. Massey University, Palmerston north, New Zealand.
- Bueno, I. J. M., D. Surek, C. Rocha, V. G. Schramm, K. Muramatsu, F. Dahlke, and A. Maiorka. 2016. Effects of different limestone particle sizes in the diet of broiler breeders post molting on their performance, egg quality, incubation results, and pre-starter performance of their progeny. *Poult. Sci.* 95:860-866.
- Bradbury, E. J., S. J. Wilkinson, G. M. Cronin, C. L. Walk, and A. J. Cowieson. 2018. Effects of phytase, calcium source, calcium concentration and particle size on broiler performance, nutrient digestibility and skeletal integrity. *Anim. Prod. Sci.* 58:271-283.
- Broer, S. 2008. Amino acid transport across mammalian intestinal and renal epithelia. *Physiol. Rev.* 88:249-286.
- Broz, J., P. Oldale, A. -H. Perrin-Voltz, G. Rychen, J. Schulze, and C. S. Nunes. 1994. Effects of supplemental phytase on performance and phosphorus utilization in broiler chickens fed a low phosphorus diet without addition of inorganic phosphates. *Br. Poult. Sci.* 35:273-280.
- Cabahug, S., V. Ravindran, P. H. Selle, and W. L. Bryden. 1999. Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-

- phytate phosphorus contents. I. Effects on bird performance and toe ash. *Br. Poult. Sci.* 40:660-666.
- Cheng, T. K., and C. N. Coon, 1990a. Comparison of various in vitro methods for the determination of limestone solubility. *Poult. Sci.* 69:2204-2208
- Cheng, T. K., and C. N. Coon, 1990b. Effect of Ca source, particle size, limestone solubility in vitro, and Ca intake level on layer bone status and performance. *Poult. Sci.* 69:2228-2230.
- Cowieson, A.J., T. Acamovic, and M. R. Bedford. 2004. The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. *Br. Poult. Sci.* 45:101–108.
- Cowieson, A. J., T. Acamovic, and M. R. Bedford. 2006. Phytic acid and phytase: implications for protein utilization by poultry. *Poult. Sci.* 85:878-885.
- Cowieson, A. J., and N. P. Cowieson. 2011. Phytate and the thermodynamics of water. *Proc. Aust. Poult. Sci. Symp.* 22:22-25.
- Cowieson, A. J., M. R. Bedford, V. Ravindran, and P. H. Selle. 2011. Increased dietary sodium chloride concentrations reduce endogenous amino acid flow and influence the physiological response to the ingestion of phytic acid by broiler chickens. *Br. Poult. Sci.* 52:613-624.
- Cowieson, A. J., J. -P. Ruckebusch, I. Knap, P. Guggenbuhl, and F. Fru-Nji. 2016. Phytate-free nutrition: A new paradigm in monogastric animal production. *Anim. Feed Sci. Technol.* 222:180-189.
- Cowieson, A. J., J. -P. Ruckebusch, J. O. B. Sorbara, J. W. Wilson, P. Guggenbuhl, and F. F. Roos. 2017. A systematic view on the effect of phytase on ileal amino acid digestibility in broilers. *Anim. Feed Sci. Technol.* 225:182-194.

- Dilger, R. N., E. M. Onyango, J. S. Sands, and O. Adeola. 2004. Evaluation of microbial phytase in broiler diets. *Poult. Sci.* 83:962-970.
- Ekmay, R. D., and C. N. Coon. 2010. The effect of limestone particle size on the performance of three broiler breeder purelines. *Int. J. Poult. Sci.* 9:1038-1042.
- Guinotte, F., Y. Nys, and F. de Monredon. 1991. The effects of particle size and origin of calcium carbonate on performance and ossification characteristics in broiler chicks. *Poult. Sci.* 70:1908-1920.
- Guinotte, F., and Y. Nys. 1991. Effects of particle size and origin of calcium sources on eggshell quality and bone mineralization in egg laying hens. *Poult. Sci.* 70:583-592.
- Hurwitz, S., and A. Bar. 1971. Calcium and phosphorus interrelationships in the intestine of the fowl. *J. Nutr.* 101:677-685.
- Hu, Y., X. Liao, Q. Wen, L. Lu, L. Zhang, and X. Luo. 2018. Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. *Br. J. Nutr.* 119:1346-1354.
- Leske, K.L., Coon, C.N., 1999. A bioassay to determine the effect of phytase on phytate phosphorus hydrolysis and total phosphorus retention of feed ingredients as determined with broilers and laying hens. *Poult. Sci.* 78, 1151-1157.
- Li, W., R. Angel, S. -W. Kim, E. Jiménez-Moreno, M. Proszkowiec-Weglarz, and P. W. Plumstead. 2015. Impact of response criteria (tibia ash weight vs. percent) on phytase relative non phytate phosphorus equivalence. *Poult. Sci.* 94:2228-2234.
- Liu, S. Y., R. M. Bold, P. W. Plumstead, and P. H. Selle. 2015. Effects of 500 and 1000FTU/kg phytase supplementation of maize-based diets with two tiers of nutrient specifications on performance of broiler chickens. *Anim. Feed Sci. Technol.* 207:159-167.

- Manangi, M. K., and C. N. Coon. 2006. Evaluation of Phytase Enzyme with Chicks Fed Basal Diets Containing Different Soybean Meal Samples. *J. Appl. Poult. Res.* 15:292-306.
- Manangi, M. K., and C. N. Coon. 2007. The effect of calcium carbonate particle size and solubility on the utilization of phosphorus from phytase for broilers. *Int. J. Poult. Sci.* 6:85-90.
- McNaughton, J. L., B. C. Dilworth, and E. J. Day. 1974. Effect of particle size on the utilization of calcium supplements by the chick. *Poult. Sci.* 53:1024-1029.
- McNaughton, J. L. 1981. Effect of calcium carbonate particle size on the available phosphorus requirement of broiler chicks. *Poult. Sci.* 60:197-203.
- Mohanna, C., and Y. Nys. 1999. Changes in zinc and manganese availability in broiler chicks induced by vegetal and microbial phytases. *Anim. Feed Sci. Technol.* 77:241-253.
- Namkung, H., and S. Leeson. 1999. Effect of phytase enzyme on dietary nitrogen-corrected apparent metabolizable energy and ileal digestibility of nitrogen and amino acids in broiler chicks. *Poult. Sci.* 78:1317-1319.
- National Research Council. 1994. *Nutrient Requirements of Poultry*. 9th rev. ed. National Academy of Science, Washington, DC.
- Olukosi, O. A., C. Kong, F. Fru-Nji, K. M. Ajuwon, and O. Adeola. 2013. Assessment of a bacterial 6-phytase in the diets of broiler chickens. *Poult. Sci.* 92:2101-2108.
- Olukosi, O. A., and F. Fru-Nji. 2014a. The interplay of dietary nutrient specification and varying calcium to total phosphorus ratio on efficacy of a bacterial phytase: 1. Growth performance and tibia mineralization. *Poult. Sci.* 93:3037-3043.
- Olukosi, O. A., and F. Fru-Nji. 2014b. The interplay of dietary nutrient level and varying calcium to phosphorus ratios on efficacy of a bacterial phytase: 2. Ileal and total tract nutrient utilization. *Poult. Sci.* 93:3044-3052.

- Onyango, E. M., Bedford, M. R. and Adeola, O. 2004. The yeast production system in which *Escherichia coli* phytase is expressed may affect growth performance, bone ash, and nutrient use in broiler chicks. *Poult. Sci.* 83:421-427.
- Onyango, E. M., E. K. Asem, and O. Adeola. 2009. Phytic acid increases mucin and endogenous losses from the gastrointestinal tract of chickens. *Br. J. Nutr.* 101:836-842.
- Peter C. M., and D. H. Baker. 2001. Microbial phytase does not improve protein-amino acid utilization in soybean meal fed to young chickens. *J. Nutr.* 131:1792-1797.
- Phillippy, B. Q., and J. M. Bland. 1988. Gradient ion chromatography of inositol phosphates. *Anal. Biochem.* 175, 162-166.
- Pirgozliev, V., O. Oduguwa, T. Acamovic, and M. R. Bedford. 2008. Effects of dietary phytase on performance and nutrient metabolism in chickens. *Br. Poult. Sci.* 49:144-154.
- Pirgozliev, V., O. Oduguwa, T. Acamovic, and M. R. Bedford. 2007. Diets containing *Escherichia coli*-derived phytase on young chickens and turkeys: effects on performance, metabolizable energy, endogenous secretions, and intestinal morphology. *Poult. Sci.* 86:705-713.
- Plumstead, P. W., A. B. Leytem, R. O. Maguire, J. W. Spears, P. Kwanyuen, and J. Brake. 2008. Interaction of calcium and phytate in broiler Diets. 1. Effects on apparent prececal digestibility and retention of phosphorus. *Poult. Sci.* 87:449-458.
- Proszkowiec-Weglarz, M., and R. Angel. 2013. Calcium and phosphorus metabolism in broilers: Effect of homeostatic mechanism on calcium and phosphorus digestibility. *J. Appl. Poult. Res.* 22:609-627.
- Qian, H., H. P. Veit, E. T. Kornegay, V. Ravindran, and D. M. Denbow, 1996. Effects of supplemental phytase and phosphorus on histological and other tibial bone

- characteristics and performance of broilers fed semi-purified diets. *Poult. Sci.* 75:618-626.
- Qian, H., E. T. Kornegay, and D. M. Denbow. 1997. Utilization of phytate phosphorus and calcium as influenced by microbial phytase, cholecalciferol, and the calcium: total phosphorus ratio in broiler diets. *Poult. Sci.* 76:37-46.
- Ravindran, V., S. Cabahug, G. Ravindran, P. H. Selle, and W. L. Bryden. 2000. Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorous levels. II. Effects on apparent metabolizable energy, nutrient digestibility and nutrient retention. *Br. Poult. Sci.* 41:193-200.
- Ravindran, V., P. C. H. Morel, G. G. Partridge, M. Hruby, and J. S. Sands. 2006. Influence of an *Escherichia coli*-derived phytase on nutrient utilization in broiler starters fed diets containing varying concentrations of phytic acid. *Poult. Sci.* 85:82-89.
- Ravindran, V., A. J. Cowieson, and P. H. Selle. 2008. Influence of dietary electrolyte balance and microbial phytase on growth performance, nutrient utilization, and excreta quality of broiler chickens. *Poult. Sci.* 87:677-688.
- Reeds, P. J., and D. G. Burrin. 2000. The gut and amino acid homeostasis. *Nutrition* 16:666-668.
- Rodehutsord, M., A. Dieckmann, and M. Witzig. 2012. A note on sampling digesta from the ileum of broilers in phosphorus digestibility studies. *Poult. Sci.* 91:965-971.
- Rutherford, S. M., T. K. Chung, P. J. Moughan. 2002. The effect of microbial phytase on ileal phosphorus and amino acid digestibility in broiler chickens. *Br. Poult. Sci.* 44:598-606.
- Rutherford, S.M., T. K. Chung, P. C. H. Morel, and P. J. Moughan. 2004. Effect of microbial phytase on ileal digestibility of phytate phosphorus, total phosphorus, and amino acids in a low-phosphorus diet for broilers. *Poult. Sci.* 83:61-68.

- Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Lague. 1996. The effects of supplemental microbial phytase on the performance and utilization of dietary calcium, phosphorus, copper and zinc in broiler chickens fed corn-soybean diets. *Poult. Sci.* 75:729-736.
- Sebastian, S., Touchburn, S.P., Chavez, E.R., Lague, P.C., 1997. Apparent digestibility of protein and amino acids in broiler chickens fed a corn–soybean diet supplemented with microbial phytase. *Poult. Sci.* 76, 1760-1769.
- Selle, P. H., V. Ravindran, G. Ravindran, and W. L. Bryden. 2007. Effects of dietary lysine and microbial phytase on growth performance and nutrient utilization of broiler chickens. *Asian-Aust. J. Anim. Sci.* 20:1100-1107.
- Selle, P. H., A. J. Cowieson, N. P. Cowieson, and V. Ravindran. 2012. Protein–phytate interactions in pig and poultry nutrition: a reappraisal. *Nutr. Res. Rev.* 25:1-17.
- Short, F. J., P. Gorton, J. Wiseman, and K. N. Boorman. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. *Anim. Feed Sci. Technol.* 59:215-221.
- Skoglund, E., Carlsson, N.-G., Sandberg, A.-S., 1998. High-performance chromatographic separation of inositol phosphate isomers on strong anion exchange columns. *J. Agric. Food Chem.* 46, 1877-1882.
- Sklan, D., Noy, Y., 2000. Hydrolysis and absorption in the small intestines of post hatch chicks. *Poult. Sci.* 79,1306-1310.
- Skou, J. C. 1965. Enzymatic basis for active transport of Na⁺ and K⁺ across cell membrane. *Physiol. Rev.* 45:596-617.
- Silk, D. B., G. K. Grimble, and R. G. Rees. 1985. Protein digestion and amino acid and peptide absorption. *Proc. Nutr. Soc.* 44:63-72.

- Simons, P. C. M., H. A. J. Versteegh, A. W. Jongbloed, P. A. Kemme, P. Slump, K. D. Bos, M. G. E. Wolters, R. F. Beudeker, and G. J. Verschoor. 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. *Br. J. Nutr.* 64:525-540.
- Singh, A., C. L. Walk, T. K. Ghosh, M. R. Bedford, and S. Haldar. 2013. Effect of a novel microbial phytase on production performance and tibia mineral concentration in broiler chickens given low-calcium diets. *Br. Poult. Sci.* 54:206-215.
- Sommerfeld, V., M. Schollenberger, I. Kühn, and M. Rodehutschord. 2018. Interactive effects of phosphorus, calcium, and phytase supplements on products of phytate degradation in the digestive tract of broiler chickens. *Poult. Sci.* 97:1177-1188.
- Tamim, N. M., R. Angel, and M. Christman. 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. *Poult. Sci.* 83:1358-1367.
- Truong, H. H., S. Yu, A. Peron, D. J. Cadogan, A. Khoddami, T. H. Roberts, S. Y. Liu, and P. H. Selle. 2014. Phytase supplementation of maize-, sorghum- and wheat-based broiler diets with identified starch pasting properties influences phytate (IP6) and sodium jejunal and ileal digestibility. *Anim. Feed Sci. Technol.* 198:248-256.
- Truong, H. H., R. M. Bold, S. Y. Liu, and P. H. Selle. 2015. Standard phytase inclusion in maize-based broiler diets enhances digestibility coefficients of starch, amino acids and sodium in four small intestinal segments and digestive dynamics of starch and protein. *Anim. Feed Sci. Technol.* 209:240-248.
- Viveros, A., A. Brenes, I. Arija, and C. Centeno. 2002. Effects of microbial phytase supplementation on mineral utilization and serum enzyme activities in broiler chicks fed different levels of phosphorus. *Poult. Sci.* 81:1172-1183.

- Walk, C. L., E. K. Addo-Chidie, M. R. Bedford, and O. Adeola. 2012. Evaluation of a highly soluble calcium source and phytase in the diets of broiler chickens. *Poult. Sci.* 91:2255-2263.
- Williams, B., D. Waddington, D. H. Murray, and C. Farquharson. 2004. Bone strength during growth: Influence of growth rate on cortical porosity and mineralization. *Calcif. Tissue Int.* 74:236-245.
- Wise, A., 1983. Dietary factors determining the biological activity of phytates. *Nutr. Abstr. Rev. Clin. Nutr.* 53,791-806.
- Wu, G. 1998. Intestinal mucosal amino acid catabolism. *J. Nutr.* 128:1249-1252.
- Xu, Y., C. R. Stark, P. R. Ferket, C. M. Williams, W. J. Pacheco, and J. Brake. 2015. Effect of dietary coarsely ground corn on broiler live performance, gastrointestinal tract development, apparent ileal digestibility of energy and nitrogen, and digesta particle size distribution and retention time. *Poult. Sci.* 94:53-60.
- Zhang, B., and C. N. Coon. 1997. Improved in vitro methods for determining limestone and oyster shell solubility. *J. Appl. Poult. Res.* 6:94-99.

SUMMARY AND CONCLUSIONS

The objective of this research was to develop better understanding of the effect of levels of dietary available phosphorus and potassium on the fecal moisture and egg characteristics of individual broiler breeder hens during the period of onset of lay and in broilers the effect of potassium and limestone particle size as effected by blood physiology, mineral and nutrient availability.

Effect of Available Phosphorus and Potassium in Broiler Breeder during Onset of Lay

An individual bird model was chosen to investigate the effect of two levels (0.3% and 0.5%) of the dietary available phosphorus (AvP) and its interaction with short-term supplementation (with or without 0.2%) of potassium (K) on fecal moisture (FM) and fecal liquid portion (LP) and second and tenth egg characteristics as physiological time points. The dietary calcium concentration of 2.7% was held constant across the treatments. The experiment thus, designed and subsequently reported has been exploratory in nature. The change from a grower diet to the experimental diets caused hens to generally increase FM. The lower rate of AvP (0.3%) interacted with (0.2%) K to cause a reduction in FM while higher rate of AvP (0.5%) interacted with (0.2%) K to cause an increase in FM. The nature of FM was dependent on the rate of AvP supplementation in the lay diets. There was a large impact of individual bird genetic predisposition that was observed on the individual bird LP output, which perhaps has reflected the individual bird metabolism and renal handling of P excretion. However, the positive linear relationship between the FM and LP was established and the variation in FM explained by LP improved as the hens progressed in the laying cycle as was evidenced by the improved R^2 . In addition, as the birds attained peak egg production, there was a general decline in the FM which suggested a period of physiological adjustment impacting FM. There were variations in the rate of lay during the early phase of onset of egg production, however, there

were no differences in egg production after 27 wk of age. With the exception of transient decrease in egg weight of second egg laid caused by grower AvP (0.5%) diets, there were no negative effects of added AvP and K on the egg characteristics.

Based on the present results, it was concluded that under the conditions of this experiment the addition of K (0.2%) had a transitory effect on reduction of fecal moisture with lower levels of dietary phosphorus (0.3%) when fed constant level of dietary Ca (2.7%) and the adjustments in dietary phosphorus levels may be considered during the onset of laying period in broiler breeder hens.

Effect of Limestone Particle Size and Potassium in Broilers

This experiment revealed that the dietary K supplementation as K_2CO_3 affected the DEB of the diets. However, added K decreased the live performance, where an effect of potassium source can't be ruled out. The added K maintained the acid-base status and the blood gas parameters appeared to be within a physiological homeostatic range.

Effect of Limestone Particle Size, Available Phosphorus and Phytase in Broilers

This experiment revealed that the phytase supplementation to 0.30% AvP diets improved broiler live performance and bone mineralization, suggested that the phytase enzyme matrix allowed the reduction up to 0.15% dietary AvP. The dietary fine limestone particle size was beneficial for the live performance in the starter phase. The dietary coarse limestone improved the Ca, P and AA digestibility during the grower phase feeding. However, further research will be required to study Na digestibility in relation to nutrient retention. The limestone particle size should be considered when evaluating or optimizing diet formulations with supplementation of phytase.