EFFECT OF PHOSPHORUS ON GROWTH PERFORMANCE, SKELETAL INTEGRITY AND PHOSPHORUS UTILIZATION IN GROWING PIGS

By

Bishwo Bandhu Pokharel

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements

For the degree of

MASTER OF SCIENCE

Department of Animal Science

University of Manitoba

Winnipeg, Manitoba

Canada, R3T 2N2

Copyright © 2014 by Bishwo Bandhu Pokharel

ABSTRACT

Two experiments were conducted to determine the effects of phosphorus (P) in growing pigs. In experiment 1, fifty-four pigs were randomly allotted to three diets with available phosphorus (aP) concentrations of 0.23%, 0.17% and 0.11%. In experiment 2, twenty-eight pigs were assigned to four treatments including T1 (diet with 0.23% aP for three wks), T2 (diet with 0.11% aP for wk1 replaced by diet with 0.23% aP for wk2 and wk3), T3 (diet with 0.11% aP for wk1 and wk2 replaced by diet with 0.23% aP for wk3) and T4 (diet with 0.11% aP for all three wks). The 0.11% aP diet proved to be very low with negative effects on performance and bone quality. Reduced dietary P resulted in reduced P intake and reduced P excretion. In conclusion, growing pigs are sensitive to P and environmental pollution because of dietary P can be reduced by dietary manipulation of P.

DEDICATION

I am proud to dedicate this thesis to my dear wife Mrs. Renu Tiwari, my parents Tulsiram Pokharel and Jamuna Pokharel, my sisters Sabitri Pokharel Ghimire, Ambika Pokhrel, Ruby Tiwari and my brothers Chandra Ghimire, Khagendra Belbase and Dibya Tiwari.

ACKNOWLEDGEMENTS

I would like to express my deepest sense of gratitude, respect and appreciation to my advisor Dr. W. K. Kim for his continuous support and advice during my M.Sc. study. He did much more than just funding my research project. He was always willing to assist me and to share his knowledge with me. Special thanks to my co-advisor Dr. C. M. Nyachoti for his permanent support, advice and patience. My sincere gratitude also goes to Dr. Wole Akinremi for his encouragements and recommendations.

It would not do justice if I forget Dr. Karmin O who helped me during my stressful conditions. The long discussions we had during the study acted as stress-buster in many cases.

I would like to acknowledge Manitoba Pork Council and Agri-Food Research and Development Initiative for providing financial support to my research project. I also acknowledge University of Manitoba which made my graduate studies possible.

I would also like to thank my lab members Roshan Adhikari, Yue Shang, Dorothy Moseti and Dr. Alemu Hunde for their valuable help in my experiments. I am appreciative to Robert Stuski, Darwin Ramos and Akin Akinola who assisted my animal trials. I wish to say a word of thanks to my colleagues and friends of my department. Big thanks to Bala Jayaraman and Deepak.

I am grateful to my family, mummy and daddy, for their support and encouragement throughout my life. Finally, words would never be enough to thank my wife Renu Tiwari for her unconditional love and encouragement during the ups and downs of my study.

FOREWORD

This thesis was written in a manuscript style and is composed of two manuscripts. Manuscript I was partly presented at the ASAS-ADSA Midwest Meeting in Des Moines, IA (March 17-19, 2014) and also at ASAS-ADSA Joint Annual Meeting, Kansas City, MO (July 20-25, 2014). Both Manuscript I and II were formatted to meet the guidelines of the Journal of Animal Science.

TABLE OF CONTENTS

ABSTRACT…..ii

DEDICATION...iii

ACKNOWLEDGEMENTS..iv

FOREWORD...……....v

TABLE OF CONTENTS...vi

LIST OF TABLES..ix

LIST OF FIGURES..x

LIST OF ABBREVIATIONS...xi

1. GENERAL INTRODUCTION..1

2. LITERATURE REVIEW...4

2.1 PHOSPHORUS...4

2.1.1 Introduction...4

2.1.2 Absorption, metabolism and excretion of phosphorus...5

2.2 CALCIUM...7

2.3 PHOSPHATE TRANSPORTERS IN THE INTESTINE AND KIDNEY…………...…..9

2.4 DIGESTIBILITY STUDIES OF PHOSPHORUS..12

2.4.1 Endogenous P loss…………………………………………………………………...13

2.4.2 Determination of P digestibility……………………………………...……………14

2.4.2.1 The index method (IM)…………………………………………………………14

2.4.2.2 Total collection method……………………………………………………………..14

2.4.2.3 Index method vs. total collection method……………………………..…………15

2.5 PHOSPHORUS REQUIREMENT IN SWINE……………………………….…………16

2.6 PHOSPHORUS AND THE ENVIRONMENT……………………………………………18

2.7 ROLE OF PHOSPHORUS IN BONE MINERALIZATION OF PIGS……………...…20

2.8 SOURCES OF PHOSPHORUS……………………………………………….…..22

2.9 OPTIMIZING PHOSPHORUS FOR PIGS……………………………………………..25

2.9.1 Phase feeding……………………………………………………………………..27

2.10 CONCLUSION...30

3. MANUSCRIPT I……………………………………………………………..............31

3.1 ABSTRACT……………………………………………………………………..31

3.2 INTRODUCTION……………………………………………………...……….32

3.3 MATERIALS AND METHODS………………………………………….…….34

3.3.1 Animals and housing…………………………………………………...….34

3.3.2 Experimental diets………………………………………………………...…35

3.3.3 Growth performance and sample collection……………………………………37

3.3.4 Dual energy X-ray absorptiometry (DEXA)……………………………………37

3.3.5 Sample preparation and chemical analysis……………………………………..38

3.3.6 RNA isolation, cDNA synthesis and quantitative real-time PCR……………………40

3.3.7 Statistical analysis…………………………………………………………………41

3.4 RESULTS……………………………………………………………………………..42

3.4.1 Growth performance……………………………………………………………42

3.4.2 Bone quality………………………………………………………………………42

3.4.3 Phosphorus utilization and excretion…………………………………………...46

3.4.4 Sodium dependent phosphate transporter gene expression……………...…...….47

3.5 DISCUSSION…………………………………………………………………………49

4. MANUSCRIPT 2……………………………………………………………………….53

4.1 ABSTRACT…………………………………………………………………...53

4.2 INTRODUCTION……………………………………………………………54

4.3 MATERIALS AND METHODS…………………………………………...55

4.3.1 Animals and housing…………………………………………………………55

4.3.2 Dietary treatments……………………………………………………………56

4.3.3 Growth performance and sample collection…………………………………....59

4.3.4 Dual energy X-Ray absorptiometry (DEXA)………………………………..59

4.3.5 Sample preparation and chemical analysis…………………………………..60

4.3.6 RNA isolation, cDNA synthesis and quantitative real-time PCR……………………61

4.3.7 Statistical analysis……………………………………………………………63

4.4 RESULTS……………………………………………………………………..64

4.4.1 Growth performance…………………………………………………………64

4.4.2 Bone quality…………………………………………………………………64

4.4.3 Phosphorus utilization and NaPi transporter gene expression…………......………..67

4.5 DISCUSSION…………………………………………………………………68

5. GENERAL DISCUSSION……………………………………………………..70

6. CONCLUSION…………………………………………………………………73

7. FUTURE DIRECTIONS……………………………………………………….73

8. REFERENCES…………………………………………………………………74

LIST OF TABLES

Table 1. Dietary P requirements (% of Dietary DM) of pigs (NRC, 1998)…………….……….17

Table 2. Digestion and retention of P by different classes of pigs………………………………19

Table 3. Bioavailability of phosphorus in inorganic sources of P (NRC, 2012)………………...24

Table 4. Potential for feed management to reduce P excretion in swine manure………………..27

Table 5. Effect of different phase feeding on nutrient digestibility and excretion………………29

Table 6. Composition and analyzed values of experimental diets (as fed basis)………………...35

Table 7. NaPi cotransporters primer sequence in pigs…………………………..……………….41

Table 8. Effects of dietary treatments on growth performance of growing pigs during first wk, second wk and third wk…………………………………………………...……………..44

Table 9. Effect of dietary treatments on third (MC3) and fourth (MC4) metacarpal BMD (g/cm^2), BMC (g) and BA (cm^2) during wk1, wk2 and wk3….………………….……………….45

Table 10. Effect of dietary treatments on P utilization in growing pigs…..……………………..46

Table 11. Dietary treatments for the experimental period………………………………………57

Table 12. Composition and analyzed values of experimental diets (as fed basis)……………….57

Table 13. NaPi cotransporters primer sequence in pigs……………………..………………...63

Table 14. Effects of dietary treatments on growth performance of growing pigs……………….65

Table 15. Effect of dietary treatments on third (MC3) metacarpal BMD, BMC and BA……….66

Table 16. Effect of dietary treatments on fourth (MC4) metacarpal BMD, BMC and BA ……..66

Table 17. Effect of dietary treatments on P utilization in growing pigs…………………………67

LIST OF FIGURES

Fig 1: NaPi-IIb gene expression in jejunum……………………………………..……………...47

Fig 2: NaPi-IIa gene expression in kidney……………………………………………………….48

Fig 3: NaPi-IIc gene expression in kidney…………………………………………………...…..48

Fig 4: NaPi-IIa (SLC34A1, kidney), NaPi-IIb (SLC34A2, jejunum) and NaPi-IIc (SLC34A3, kidney) gene expression……………………………………………………………….....68

LIST OF ABBREVIATIONS

AA	Amino Acid
ADFI	Average daily feed intake
ADG	Average daily gain
ADP	Adenosine diphosphate
AMP	Adenosine monophoaphate
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
aP	Available phosphorus
ATP	Adenosine triphosphate
ATTD	Apparent total tract digestibility
BA	Bone Area
BBM	Brush border membrane
BMD	Bone Mineral Density
BMC	Bone Mineral Content
BW	Body weight
BWG	Body weight gain
Ca	Calcium
CCAC	Canadian Council on Animal Care
cDNA	Complementary deoxyribonucleic acid
CP	Crude protein
DCP	Dicalcium phosphate
DE	Digestible energy

DEXA	Dual energy x-ray absorptiometry
DM	Dry matter
DNA	Deoxyribonucleic acid
EPL	Endogenous phosphorus loss
FCR	Feed conversion ratio
FI	Feed intake
GAPDH	Glyceraldehyde 3- Phosphate Dehydrogenase
G:F	Gain to feed
GLM	General linear models
HCl	Hydrochloric acid
HNO_3	Nitric acid
ICP	Inductive Coupled Plasma Spectrometry
K	Potassium
kg	Kilogram
MC	Metacarpal
mg	milligrams
mRNA	messenger RNA
MSP	Monosodium phosphate
N	Nitrogen
Na	Sodium
NaPi	Sodium phosphate
NCBI	National Centre for Biotechnology Information
NRC	National Research Council

P	Phosphorus
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDIFF	Probability difference
PTH	Parathyroid hormone
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
SAS	Statistical Analysis Software
SBM	Soybean meal
SEM	Standard error of the mean
SLC	Solute carrier family
TiO_2	Titanium Dioxide
tP	Total phosphorus

1. GENERAL INTRODUCTION

Among 15 essential minerals required for the swine diets, P is one of the most important mineral playing roles in different vital functions of the body (Richert, 2010). Phosphorus is among the most abundant mineral in the body (McDowell, 2003). Phosphorus is involved in proper skeletal growth and mineralization as well as cellular functions. Phosphorus is involved as structural component of phospholipids and as initiation factor in protein synthesis (Crenshaw, 2001). Phosphorus is found in every cell of the body via the energy molecules AMP, ADP and ATP (Bauman, 2004). Additionally, P provides structural backbone of the nucleic acids (Anderson et al., 2006).

Although P is important for the proper functioning of the body, it has generated significant concern in the swine industry because of the cost incurred with it and its negative impact on the environment. Pigs utilize P inefficiently, excreting 60 to 80% of that consumed (Jongbloed et al. 1997; Knowlton et al., 2004). Of the nutrients excreted by pigs, P is a main contributor to impact negatively on soil and water environment (Han et al., 2001). Excessive P levels in swine diets leads to increased manure P excretion, and land application of this manure increase potential P losses from fields to water resources (Sutton et al., 2004). Due to the lack of sufficient amount of phytase enzyme in swine, phytate P cannot be utilized which in turn is excreted in the manure. On the other hand, P is the third most expensive nutrient in swine diet after energy and protein (NRC, 1998). It is important to optimize the utilization of P for pig's health as well as the environment.

Animal production within the swine industry has become intensive. A large number of pigs and large number of production facilities are concentrated within a small region, which further causes concerns regarding the impact of swine production on the environment. In many areas,

regulations are being implemented with the requirement for manure application to be based on P content (Sutton, 2008). So, it is of utmost important to optimize the utilization of P in swine diets. For this to be realized P requirements in different physiological stage should be accurately estimated and diet should be formulated on available P or digestible P basis so that maximum P ingested is utilized and less P remained in manure.

Several strategies have been introduced to optimize the nutrient utilization in swine. Phase feeding, which involves feeding of several diets for a relatively short period of time in order to closely meet the pig's nutrient requirements is one of those strategies which is shown to reduce nutrient excretion dramatically without significant negative impact on the performance of swine (Han et al., 2000). Ryan et al. (2011) studied the compensatory effects of dietary P on growth performance and bone mineral density in weaned and growing-finishing pigs. The growth performance was not significantly affected by phase feeding; however, bone mineral density and bone mineral content were affected significantly by phase feeding. Furthermore, the bone mineral density of the pigs fed high P diet was not similar with the BMD of the pigs whose low-P diet was replaced with high P diet after 28 or 35 days. This might be due to the fact that 28 or 35 days of low P treatment is too long to be compensated by the high P diet. No study has been done to see the compensatory effect of high P diet after a short duration of low P feeding in pigs.

Bone is an important indicator of P status in the body of swine (Koch and Mahan, 1985). The amount of P required for maximum bone mineralization is higher than the P required for maximum growth performance of pigs (Kanakov et al., 2004). Most of the studies on the effect of different levels of dietary P on diet are concentrated towards bone ash, bone breaking strength and several other parameters. However very few studies have used DEXA to examine the effects of P levels

in bone. It has been shown that the bone mineralization data obtained by DXA are highly correlated with the ash content (Mitchell et al., 1996; Ryan et al., 2011).

It has been found that during low intake of dietary P, there is dominance of active transport, which is linearly related to luminal sodium concentration. This active transport is regulated by sodium-dependent phosphate transporters (Saddoris et al., 2010). How these active transporters are regulated during the alteration in dietary P levels is largely unclear. Furthermore, it is still unclear how active transporters in kidney respond with alteration in dietary P levels.

The objectives of study 1 were to determine the effects of reduced dietary level of P on growth performance, skeletal integrity, P utilization and sodium-dependent phosphate cotransporter gene expression in growing pigs. The objectives of study 2 were to determine the effects of short-term phase feeding of P on growth performance, skeletal integrity, P utilization and sodium dependent phosphate cotransporter gene expression in growing pigs.

2. LITERATURE REVIEW

2.1 PHOSPHORUS

2.1.1 Introduction

Phosphorus (P) is one of the important minerals among 15 essential minerals in swine nutrition (Richert, 2010). Being found in every cell of the body, P plays vital roles in different body functions of swine. It is an important component of ATP and ADP, structural components of nucleic acids and phospholipids of cell membrane, coenzymes and is also involved in protein synthesis and acts as a buffer in acid-base balance (Crenshaw, 2001). Approximately, 20 % of total P resides in the soft tissue of an adult pig and 60-80 % of P is located in skeletal tissues (Crenshaw, 2001). The P stored in bones gives structural strength and stability to the animal. It plays an important role in the proper functioning of skeletal system and other physiological functions of the body (Crenshaw, 2001). It is not only important for body functions but also is equally important from economic and environmental point of view. It is the third most expensive nutrient after energy and protein in swine nutrition (NRC, 1998). With the application of P in excess of crop requirements, soil becomes saturated with P, increasing the potential for the runoff of P and the risk of water contamination and eutrophication (Knowlton et al., 2004). Increased concerns of the public and government bodies over the environmental impact of excess P in the excreta of pigs has driven research to investigate management strategies that can minimize the environmental effect of swine production (Baxter et al., 2003; Sutton and Richert, 2004). It is important to maximize P utilization in pigs in order to maximize animal performance and reduce P excretion. In the following review, the absorption, metabolism and excretion of P and Ca, phosphate transporters in pigs, P digestibility and its studies, P requirement in swine,

environmental aspects of P, P and bone health, sources of P and P optimization in pigs will be discussed.

2.1.2 Absorption, metabolism and excretion of phosphorus

In general, P is absorbed from the small intestine, particularly the jejunum (Breves and Schroder, 1991; Metzler and Mosenthin, 2008). Compared to jejunum, very little P absorption occurs from duodenum (four fold less) and ileum (Crenshaw, 2001). Phosphorus can only be absorbed from the small intestine in inorganic forms (Anderson, 1991). The absorption of P is dependent on various factors including the level of P, the level of Ca and the ratio of Ca:P in the diet (NRC, 2012). The ratio of Ca to P influences the availability of P in the body. The narrower the ratio of Ca to P, the more efficient is the utilization of P (Wu et al., 2008). Wider the ratio of Ca to P causes reduction of P utilization (Qian et al., 1996). According to NRC (2012), the ratio of Ca to P should be in the range of 1:1 to 1.25:1. Ryan et al. (2011) maintained the ratio of dietary Ca to total P as 1.3:1 so that Ca would not be the limiting factor of the study. Higher concentration of dietary Ca reduces P absorption by forming insoluble tricalcium phosphate in the intestinal tract. When the ratio of Ca to total P is wide, the extra Ca forms complex with phytic acid which is less available for degradation by phytase (Qian et al., 1996) and high dietary Ca increased the pH of the intestinal contents decreasing microbial phytase activity (Liu et al., 1998; Liu et al., 2000).

Both paracellular and transcellular pathways may be involved in the absorption of P in the intestine (Lee et al., 1986). Normally, phosphate is taken up in the jejunum in a passive, paracellular manner (Renkema et al., 2008). Transcellular absorption dominates when dietary P is below the required level (Eto et al., 2006). In contrast to the intestine, phosphate is largely absorbed

by a Na-dependent transcellular manner in the proximal convoluted tubule of kidney (Renkema et al., 2008).

The regulation of P homeostasis in the body is a result of complex interaction between intestine, kidney and bones (Berndt and Kumar, 2009; Taylor and Bushinsky, 2009). Dietary deprivation of phosphate, 1, 25-dihydroxyvitamin D3 (vitamin D3) and parathyroid hormone (PTH) are the important physiological regulators of small intestinal P absorption (Hattenhauer et al., 1999; Taylor and Bushinsky, 2009). Recently, fibroblast growth factor 23 (FGF 23) is also found to regulate serum phosphate by modulating intestinal phosphate absorption, renal phosphate reabsorption and bone metabolism (Fukumoto, 2014). Vitamin D3 increases both renal reabsorption and intestinal absorption of phosphate and works with PTH to mobilize phosphate from bone (DeLuca, 2004). Phosphate absorption in the intestine is stimulated by vitamin D3, which is independent of the presence of Ca (Chen et al., 1974). Sodium (Na) is essential for this vitamin D3 mediated P absorption. This transport system is referred as sodium phosphate cotransport system (Crenshaw, 2001).

The parathyroid hormone (PTH) secreted by the parathyroid gland works in response to the change in levels of P in the blood. The PTH acts on both bone and kidney, whereas vitamin D3 acts on bone, intestine and kidney (Jones et al., 1998). The PTH also has an indirect effect on intestine through the stimulation of vitamin D (Brown et al., 1999). When P levels are too low in the serum (hypophosphatemia), plasma Ca levels are elevated resulting in the decreased secretion of PTH. The PTH has a target receptor in the kidney that acts to decrease the renal inorganic P excretion. In addition to this, hypophosphatemia also causes an increase in vitamin D3 and calcitriol resulting in the increased mobilization of P from the bone. On the other hand,

hyperphosphatemia (increased P level in serum) leads to a decrease in plasma Ca concentration thereby causing elevated PTH and elevated renal excretion of P. The undigested P is excreted from feces and urine. Feces are the principal pathways of excretion of P while urine is the important pathway of P excretion in carnivores (McDowell, 2003). In summary, P homeostasis in blood is dependent on several factors i.e. intestinal absorption, bone catabolism and renal reabsorption and excretion (Crenshaw, 2001).

2.2 CALCIUM

In the body of pig, about 96-99% of the calcium (Ca) is found in the skeletal system where it acts as an integral part of the hydroxyapatite (Crenshaw, 2001; Ritchert, 2010). It is also found in the soft tissue and the extracellular fluid. In the body, Ca plays important role in the development of bone, blood clotting, muscle contraction and many physiological functions. It is basically absorbed from the small intestine via transcellular and paracellular routes (Bronner, 1998). Transcellular process is saturable and is mediated by vitamin D and mainly occurs in the proximal part of the small intestine. This process requires oxygen and transports Ca against a chemical gradient. In addition to this active transport, large amount of Ca is absorbed by passive transport in the lower intestine. Low Ca in diet stimulates active transport whereas abundant dietary Ca stimulates passive transport.

Calcium absorption is affected by the ratio of Ca to P in the diet (NRC, 1998). The ratio is more critical when Ca and P concentrations are inadequate in the diet (Peo, 1976). The presence of adequate vitamin D is also essential for the proper absorption of Ca. But excessively high level of vitamin D can result in excessive mobilization of calcium from bone (Jongbloed, 1987). High mobilization of calcium from bone results in weaker bones. The absorption is also affected by

dietary concentrations of sulfates, oxalates, strontium, magnesium and antibiotics (Crenshaw, 2001).

In the body, Ca is regulated by parathyroid hormone, vitamin D and calcitonin. Decreased level of Ca in the blood is immediately sensed by parathyroid gland causing release of PTH into the circulation. The PTH blocks renal P reabsorption and increase urinary P loss. The PTH also increases intestinal absorption of Ca and mobilization of Ca from bone. The PTH is also required to activate vitamin D. The activated vitamin D acts on the intestine to increase Ca absorption from the intestine. High level of Ca in blood stimulates calcitonin secretion from thyroid gland. Calcitonin enhances renal excretion of Ca, inhibits bone resorption and enhances cellular uptake of Ca.

Animals deficient in Ca show lameness, altered gait, rickets, osteomalacia and occasional fracture (Crenshaw, 2001). Rickets usually occur in younger animals whereas osteomalacia occurs in mature animals. Rickets is characterized by short and deformed limbs leading to fractures. During Ca deficiency, bones fail to be properly mineralized and are susceptible for fracture. In the meantime, accumulation of bone matrix and soft tissues occurs. Joints are found enlarged and animals suffer from spontaneous fractures and beaded ribs. In pigs, deficiencies of Ca are usually seen in sows after prolonged lactation period. Such sows show the symptoms of posterior paralysis due to vertebrae fractures and fracture in the limb bones.

2.3 PHOSPHATE TRANSPORTERS IN THE INTESTINE AND KIDNEY

The phosphate balance in the plasma is determined by the balance between intestinal P absorption, renal P reabsorption and P mobilization from the bone. The kidney is an important regulatory organ of P homeostasis. *In vivo,* the kidney should respond quickly to alteration in the extracellular environment to maintain normal homeostasis of the electrolyte in the body (Katai et al., 1997). The kidney increases or decreases the reabsorption of phosphate depending upon the phosphate needs of the body (Sewaga et al., 2009). Phosphate absorption in the intestine occurs by both active and passive transport system. Active transport system, which is dependent on luminal sodium concentration primarily, occurs in the proximal small intestine. On the other hand, passive transport, which is dependent on luminal phosphate concentration, occurs in the jejunum and ileum (Danisi and Straub, 1980). Various factors, including level of dietary P and Ca, vitamin D and PTH, influence the sodium dependent P uptake. Low intake of P causes a dominance of active transport system and high intake of P causes dominance of passive transport system. Active P transport occurs through sodium dependent phosphate transporters (NaPi) (Saddoris, 2007). Three families of Na-phosphate (NaPi) cotransporters have been identified in vertebrates. They include type I (SLC17), II (SLC34) and III (SLC20) NaPi (Saddoris et al., 2010). The Na-Pi type II cotransporter consists of three subfamilies, the type IIa, type IIb, and type IIc.

NaPi-I cotransporter has been identified in rat (Li and Xie, 1995), mouse (Chong et al., 1995) and human (Chong et al., 1993). NaPi-I is expressed in kidney, liver and brain (Murer et al., 2000). Although NaPi-I is believed to play some roles in regulating P homeostasis, phosphate uptake by NaPi-I is not regulated by the level of P in the diet or by the PTH (Murer et al., 2000). On the other hand, NaPi-II cotransporter protein plays an important role in phosphate transport

influenced by PTH and dietary P levels (Beck et al., 1998). Brush border membrane of enterocytes and apical membrane of proximal convoluted tubular (PCT) cells express NaPi-II cotransporters (Kirsten et al., 2008). NaPi-IIa (SLC34A1) and NaPi-IIc (SLC34A3) are mainly expressed in the apical membrane of PCT whereas NaPi-IIb (SLC34A2) is mainly expressed in lungs and jejunum (Miyamoto et al., 2011; Murer et al., 2000). In weaning animals, NaPi-IIc plays the most important role in renal phosphate absorption whereas in mice and rats, NaPi-IIa plays the most important role (Sewaga et al., 2009). The NaPi-IIa cotransporter plays a key role in the regulation of phosphate transport in renal brush border membrane. The study done by Beck et al. (1994) found 70% reduction in Na dependent phosphate transport and a loss of the protein in renal BBM of the mice with disruption of the NaPi-IIa gene. The NaPi-IIc mRNA is age dependent and the expression levels are almost absent in newborn and suckling mice whereas the expression is highest in weanling and growing mice which then decline with age (Segawa et al., 2002). In the adult mice fed low P diet, NaPi-IIc mRNA provided approximately 30% of the phosphate transport activity in the kidney (Ohkido et al., 2003).

Low P in the diet stimulates the increase in NaPi-II cotransporters in both jejunum and kidney. The abundance of NaPi-IIb mRNA is increased as a result of low P in the diet. In the intestine of mice, the increase in NaPi-IIb protein is paralleled by an increase in NaPi_IIb mRNA (Capuano et al., 2005). The study done by Levi et al. (1994) found that rats fed low P diets (acutely) showed 31% increase in brush border membrane phosphate transport and increase in NaPi-IIa protein but there was no increase in renal cortical NaPi-IIa mRNA. Acute deprivation of P in diet is related to the post-transcriptional event (Saddoris, 2007). Mice with no NaPi-IIa gene failed to show stimulation in renal Pi transport when fed a low P diet though there was reduced serum Pi (Hoag et al., 1999). Chronic deprivation of P (0.1%) in diet for 7 days showed 71% increase in

BBM phosphate transport, 4.9 fold increase in NaPi-IIa protein and 2.2 fold increase in NaPi-IIa mRNA compared to the rats fed high P diet (1.2%) (Levi et al., 1994). Rats fed a low P diet were found to have increased sodium-dependent phosphate transport as a result of elevated expression of NaPi-II mRNA and protein (Katai et al., 1997). The change in the level of P in the diet has a significant effect on the expression of NaPi-II gene and protein. The change in expression leads to the change in Na dependent phosphate uptake activity, which helps to balance the phosphate homeostasis in the body.

2.4 DIGESTIBILITY STUDIES OF PHOSPHORUS

Availability of P in feedstuffs and feed additives varies from 20 to 100% (Weremko et al., 1997). The amount of P available to pigs can be determined by relative bioavailability and digestibility techniques. To determine relative bioavailability, a test diet is compared to a standard P source. This technique was developed by Cromwell (1980). The method includes the use of a basal diet, 2-3 levels of a test diet and 2-3 levels of a standard diet. After the completion of the experiment, pigs are killed and bones are harvested to determine bone ash content, which is then regressed on P intake for each source. The formula to determine relative bioavailability is:

Relative bioavailability = 100 × (slope B / slope A)

Where, slope B = regression slope for the test diet and

slope A = regression slope for the standard diet

This procedure is expensive and labour intensive, and the results are variable as well as not always additive in the mixed diets (Stein, 2011).

Apparent total tract digestibility (ATTD) of P is calculated by obtaining the difference between the amount of P intake and fecal P excretion and dividing this difference by P intake (Jongbloed, 1991). The digestibility can be determined by the difference method or the direct method (Adeola, 2001). This procedure is fast and relatively inexpensive. However, there are some limitations of ATTD. It has been reported that ATTD value of P shows high variability within the same feedstuffs and depends upon the concentration of P in the diet (Fan et al. 2001). In addition, apparent P digestibility doesn't consider endogenous P losses (EPL) (Fan et al. 2001). To correct this, the digestibility of P is expressed as standard total tract digestibility (STTD) or true total tract digestibility (TTTD). It is believed that STTD and TTTD values are additive in a mixed ration

(Fan et al., 2001; Stein, 2011). The STTD and ATTD values are obtained by correcting endogenous phosphorus losses in ATTD (Peterson and Stein, 2006; Stein, 2011).

STTD of P (%) = [(P intake - (P output – basal EPL))/ P intake] × 100 (Stein, 2011)

TTTD of P (%) = [(P intake - (P output – total EPL))/ P intake] × 100 (Stein, 2011)

In this formula, P intake, P output and basal EPL are expressed as gram per day or as the gram for the entire collection period.

2.4.1 Endogenous P loss

Endogenous P losses include the P excreted from the body, which is not of dietary origin. The EPL comes from salivary, gastric and pancreatic secretion, biliary juices and sloughed mucosal cells (Jongbloed, 1987). The EPL can be measured indirectly using a regression procedure (Fan et al., 2001) and directly using diets devoid of P (Peterson and Stein, 2006). The results obtained from various studies have found that the amount of EPL seems to be variable which may be because of the factors like high fiber diets, anti-nutritional factors and animal factors (Peterson and Stein, 2006). The STTD of P is obtained by correcting basal EPL from apparent P digestibility whereas TTTD of P is obtained by correcting total EPL from apparent P digestibility. Basal EPL is obtained using P free diet. Total EPL is obtained by adding basal EPL and diet specific EPL.

2.4.2 Determination of P digestibility

2.4.2.1 The index method (IM)

Nutrient digestibility is traditionally measured either by the index method or by the total collection method (Agudelo et al., 2010). The index method, which involves the use of indigestible marker, is also called 'indicator', 'tracer', or 'marker' method or the 'ratio technique'. In this method, a marker is added to the feed and the concentration of that marker is determined in both feed and feces to calculate the digestibility of a nutrient. Calculation is done by determining the marker: nutrient ratio in both feed and in feces. Markers are considered as inert substances that are not absorbed in the intestine of an animal and can be divided into internal, representing the indigestible substance present in the diet or external, which are added intentionally to the diet (Pozza et al., 2013). Markers, in addition to being inert, should also be non-toxic and have no physiological effects on the animal and should mix well in the diets. The collection of feces can be done by a grab sampling method in the IM (Adeola, 2001). Some common markers used in IM include chromic oxide (Adeola, 2001), titanium oxide (Jagger et al., 1992), acid insoluble ash (Adeola, 2001) and dysprosium (Kennelly et al., 1980).

Using IM, digestibility is calculated as:

$$\text{Digestibility, \%} = 100 - \left[100 \times \left\{\frac{\text{\% marker in feed} \times \text{\% nutrient in feces}}{\text{\% marker in feces} \times \text{\% nutrient in feed}}\right\}\right]$$

2.4.2.2 Total collection method

The total collection method is essentially a record keeping operation where accurate record of the feed intake and output of feces and urine is required to determine the amount of component

digested and the amount of component voided via feces and urine, respectively (Adeola, 2001). This method is also referred as a quantitative collection method. For the collection of feces, individual animals are kept in special metabolic crates where they can freely lie and get up but not turn around. Feces screen and urine collection jars are placed below the crates to collect feces and urine separately. Sometimes visually distinguishable markers can be used during feeding to separate feces from feed visually. Some of those markers are ferric oxide, and indigo carmine (Agudelo-Trujillo, 2005).

Under total collection method, digestibility is calculated as:

$$\text{Digestibility, \%} = 100 \times \left\{ \frac{\text{Amt. of component consumed} - \text{Amt. of component voided in feces}}{\text{Amt. of component consumed}} \right\}$$

2.4.2.3 Index method vs. total collection method

The major advantages of IM are that it saves time, labor and cost incurred in the study. It avoids the need of quantitative records of feed intake and feces output. There is no need of keeping animal in special crates. Animals can simply be kept in normal pens though the problem of coprophagy can arise occasionally which would change the concentration of marker excreted in feces leading to the change in digestibility values (Agudelo-Trujillo, 2005). The problem with IM is that it sometimes underestimates the digestibility values of nutrients (Adeola, 2001; Agudelo et al., 2010). The recovery of indigestible markers can sometimes be a problem in index method. Total collection method is precise but involves much labor and cost (Agudelo et al. 2010). During total collection method, pigs restrained in special crates for longer period of time can have the risk of feed refusal, sickness and occasional accidents.

2.5 PHOSPHORUS REQUIREMENT IN SWINE

Phosphorus is required in relatively high amounts in the swine diets. The P requirement in swine varies according to the body weight and physiological stage (Agudelo-Trujillo, 2005). The dietary phosphorus required in swine is expressed based on the requirement for maximum bone development or maximum growth rate and performance. Phosphorus required in adequate amount for maximum performance of pig is lower than that required for maximum bone development (Kanakov et al., 2004). The requirement of P is expressed as total P (tP), available P (aP) and digestible P (NRC, 2012). Previously, P requirements of pigs used to be reported as total P (Knowlton et al., 2004).

The total P is the sum of phytate and non-phytate P. Pigs, by birth, do not have the sufficient amount of phytase enzyme required to digest phytate P in the feed (NRC, 2012). The undigested phytate P comes out of the body of pigs in the manure. Because of this, the concept of available/ digestible P has been used in the swine diet formulation. In order to reduce the over-excretion of P in manure, diets should be formulated on available or digestible P basis, which precisely meet the requirement of P in swine.

Table 1. Dietary P requirements (% of Dietary DM) of pigs (NRC, 1998)

Item	Body weight, kg			
	10 -20	20-50	50-80	80-120
Total P	0.6	0.5	0.45	0.4
Available P	0.32	0.23	0.19	0.15

The NRC (2012) has mentioned the STTD of P requirements as 0.33, 0.31, 0.27 and 0.24 % for 11-25 kg, 25-50 kg, 50-75kg and 75-100 kg pigs, respectively.

2.6 PHOSPHORUS AND THE ENVIRONMENT

Although dietary P is essential for the growth, development, and maintenance of both muscular and skeletal tissues of pigs, public and governmental bodies are highly concerned about the potential impact of excess P in the excreta of pigs on water and soil quality (Hill et al., 2009; Prince et al. 2000). On average, finishing pigs produce 4.5 L per pig per day of manure without water wastage, which is equal to 6.8 kg of phosphorus per pig per year (Han et al., 2000). Pigs excrete 50 to 80% of P when fed diets containing common feedstuffs (Kornegay and Harper, 1997).

The composition of N and P in the swine manure is not properly balanced relative to the N and P requirement by crops (Knowlton et al., 2004). The ratio of N, P and K in the swine manure is generally 1:1:1. Corn grain, in general, requires N, P and K in the ratio of 3:1:1 (Sutton et al., 2004). As such, if manure is applied to meet the crop requirement of N, P will be applied to the soil three folds more than required, leading to excess amount of P in the soil. This can cause the saturation of soils capacity to adsorb P increasing the risk of P runoff to surface water leading to water pollution. Therefore, it is important to formulate pig diet that will reduce the amount of P that is excreted in the manure, allowing the N:P ratio of the manure to approach that of plant requirements. This is important for alleviating the problems of excess soil P and its negative impact on the environment.

Phosphorus is the nutrient limiting eutrophication in water bodies (Correll, 1999). The acceptable concentration of P in water bodies is still a subject of debate, nevertheless Correll, (1999) has indicated that the concentration of 20 µg total PL^{-1} is problematic. The author also indicated that total P concentration of 100 µg L^{-1} will be exceptionally high. A high concentration

of P in surface waters leads to eutrophication and excessive algae growth known as algal bloom (Knowlton et al., 2004). Eutrophication refers to the over enrichment of water bodies with mineral nutrients (Correll, 1998). Excessive production of phytoplankton like algae and cyanobacteria caused by excessive levels of P in the surface water may lead to decreased oxygen levels. This may be enough to kill the fish population reducing aquatic diversity and causing a major shift in species composition (Correll, 1998; Sutton et al., 2004). Another negative impact of P on water bodies is the outbreak of *Pfisteria pisidia*, a dinoflagellate, which causes lesions on fish (Sutton et al., 2004). These environmental issues resulting from high P concentration in surface waters, has led the increased pressure to the governmental authorities to keep this mineral under scrutiny and to act to restrict the swine business in many parts of the world. Since swine manure contains high level of P (Knowlton et al., 2004), it has become important for animal nutritionist to maximize P utilization in swine together with the reduced P excretion to environment.

Table 2: Digestion and retention of P by different classes of pigs

Item	Class or size of pigs			
	Young	Finishing	Gestating	Lactating
Digested, %	20 to 70	20 to 50	3 to 45	1 to 35
Retained, %	20 to 60	20 to 45	20 to 35	20

Adapted from Kornegay and Harper (1997)

2.7 ROLE OF PHOSPHORUS IN BONE MINERALIZATION OF PIGS

The major role of P involves bone and teeth mineralization, which is accomplished by its close association with Ca (Crenshaw, 2001). Phosphorus accounts for more than 25% of total body mineral matter, second to calcium. About 60-80% of P is found in the skeleton of the body where it is associated with Ca in the form of hydroxyapatite (Crenshaw, 2001; Kanakov et al., 2004). This clearly demonstrates the role of P in the proper functioning of bones. The P present in the bone acts as an important reservoir to buffer changes in blood and intracellular phosphate level. Low levels of P in blood plasma result in the elevation of vitamin D3 and calcitriol causing higher mobilization of the P from the bone. When P mobilization is more than the desired level, animal starts to show symptoms of hypophosphatemia. These symptoms include rickets, osteoporosis, soft and fragile bones and various other serious conditions (Gwendolyn, 2010). These adverse effects can be prevented by appropriate dietary intervention. Signs of P deficiency include reduced growth and bone mineralization, causing rickets in younger pigs and osteomalacia in older pigs. In sows, the deficiency can result in paralysis of the hind legs, which is called posterior paralysis.

Phosphorus has a significant effect on the rate of mineral deposition in both growing and finishing pigs. Growing pigs and market pigs are highly sensitive to dietary P and accumulate area bone mineral density (aBMD) at higher rates than weaning pigs (Ryan et al., 2011). The same authors have concluded that pigs with highest level of dietary P had the highest levels of aBMD and had the greatest rate of mineral deposition. The optimum P concentration required to maximize growth and G:F is lower than the concentration required to maximize bone mineralization and strength (Kanakov et al., 2004; NRC, 1998). Maximum bone mineralization is achieved when dietary P levels used are higher than those recommended for maximum growth response (Varley et al., 2010). Substantial bone formation occurs during the first 12 wks of life (Tanck et al., 2001).

Because of this, it is critical to optimize the P requirement during the growing stage of pigs, which provides the bone mineral reserve at a later stage of life.

The study done by Varley et al. (2011) found that pigs offered low P diets from 11 to 30 kgBW had lower concentration of bone ash, bone P and bone Ca than those offered the medium and high P diets, and lower bone density than those offered the high P diets. In weaners, pigs offered 6.4 g total P/kg had bone density value of 1.28 g/cm^3 which was significantly higher than the bone density value in pigs offered 5.5 g total P/kg (1.26 g/cm^3) and 4.5 g total P/kg (1.23 g/cm^3). Similarly in the finisher pigs, bone density was higher in the pigs fed higher P diets compared to pigs fed low P diets.

Bone parameters are good indicators of P status, as maximum bone development is required for animal longevity. Koch and Mahan (1985) found that bone ash was not influenced by Ca:P ratio but increased linearly as dietary P level increased. On the other hand, serum inorganic P decreased as Ca:P ratio was widened and increased linearly as dietary P increased. This indicates that bone ash and other bone components are more sensitive criteria than serum P level to evaluate the effects of dietary P level (Kanakov et al., 2004). The BMD and BMC are good indicators of the effects of dietary P on bone mineralization as the data obtained by DXA are highly correlated to the ash content (Mitchell et al., 1996; Ryan et al., 2011).

2.8 SOURCES OF PHOSPHORUS

Swine diet is mainly based on cereals, oilseeds and their by-products (Godoy et al., 2005). Phosphorus is found in plant, animal and inorganic sources. The availability of P to swine depends upon the sources of P and ranges from 20 -100 % (Weremko et al., 1997). Feed ingredients of animal origin and inorganic sources have high digestibility of P ranging from 67-90% (NRC, 1998; Traylor et al., 2005). However, cereals and plant sources used in swine diets have high levels of indigestible P in the form of phytate (Mroz et al., 1994). Lack of sufficient amount of phytase enzyme in mono-gastric animal is responsible for the low digestibility of P from plant origins. The digestibility of P from plant ingredients varies from 10-60% (Weremko et al., 1997).

Unlike Calcium, which is present in very low amount in cereal grains and most plant protein ingredients, P is present in the modest level in these ingredients (Adeola, 2001). The concentration of P in cereal grains ranges from 0.25 to 0.45% (Baker, 2011). About 60-85% of total P present in cereal grains and oilseed is phytate bound (Raboy, 1997). Wheat and corn have the highest percentage of phytate-P (70-75%). On the other hand, rye and oat grains have the lowest percentage of phytate-P (60%) (Weremko et al., 1997). Corn has as little as 15% digestible P (Trotter and Allee, 1979) whereas the P in wheat is more bio-available which is about 50% (Cromwell, 1992). The greater availability of P in wheat is attributed to the presence of endogenous phytase enzymes in wheat (Pointillart et al., 1984).

Due to the low digestibility of P in plant sources, swine diets are supplemented with inorganic P sources to compensate for the indigestible fraction. However, inorganic P sources should not be added in high amounts because of the negative effect on pig performance. High level of inorganic P leads to elevated gastric pH lowering efficiency of digestion and scouring problems

(Piva et al., 2002; Stein, 2002). The commonly used inorganic sources of P in swine are dicalcium phosphate (DCP) and monocalcium phosphate (MCP). These inorganic sources of P are commonly used in formulating diets to meet swine P needs. Relative bioavailability of P from various inorganic sources ranges from 20 to 100 % (Weremko et al., 1997). Petersen and Stein (2006) reported the apparent digestibility of MCP and DCP to be 81.68% - 82.55% and 81.49% respectively. Monosodium phosphate is reported to have higher digestibility than MCP and DCP (NRC, 2012).

Table 3. Bioavailability of phosphorus in inorganic sources of P (NRC, 2012)

Source of P	% P	Bioavailability of P, %
Dicalcium phosphate	12.5	80-90
Monocalcium phosphate	18.5	95-100
Monosodium phosphate	24.9	100
Rock phosphate	9.05	30-50

2.9 OPTIMIZING PHOSPHORUS FOR PIGS

Phosphorus is a costly nutrient for pigs and has potential impact on soil and water quality; therefore, it is important for swine nutritionist to optimize the utilization of P without adverse effect in the physiological status of swine. There was a trend in the swine industry whereby feeds were formulated to maximize performance with very little concerns about nutrients oversupply and nutrients excretion. Nutrients oversupply is the major cause of over excretion of nutrients. In recent times, strict legislation has been introduced to limit environmental pollution resulting from animal manures.

There are several nutritional strategies to optimize nutrients in animals especially P. The most important step is to determine the exact requirements of P during different developmental stages of swine so that the overuse of P can be avoided. On the other hand, it also helps to reduce fecal P excretion. More accurate information about ingredients is necessary to formulate feed that matches animal requirements (Kornegay and Harper, 1997).

It is well known that two third of total P in feedstuffs of vegetable origin is phytate bound. Most of the phytate bound P being indigestible is excreted in feces. The phytate bound P can be made available to swine by the use of phytase enzyme (NRC, 2012). Phytase has been found to improve P availability dramatically (Jongbloed et al., 1997). Microbial phytase supplementation of high phytate ceral grains can improve the bioavailability of phytate-P (Jongbloed et al., 1992). This strategy not only helps to reduce dietary level of P but also lowers P excretion by 30-60% (NRC, 2012). Formulating diets based on available or digestible P helps to provide accurate P requirements to pigs. This contributes to a reduction in the excretion of P from swine without negatively affecting their performance and helps to reduce the high cost of dietary P (Ekpe et al.

2002; Petersen and Stein, 2006). Formulation of diets based on apparent ileal digestibility is also helpful in reducing nutrients excreted from swine (Lenis, 1992). Feeding pigs according to gender, known as split sex feeding, is also helpful in reducing the excretion of nutrients (Han et al., 2000). Dietary requirements of nutrients can vary with gender. Cromwell et al. (1993) has mentioned that gilts require higher concentrations of dietary amino acids to maximize lean growth rate than barrows. So, different diets can be fed based on gender to closely match the animal's requirements.

Recently, a new concept, known as phase feeding, has been put forward in the swine industry to optimize nutrients utilization and to reduce nutrients excretion. Phase feeding has been shown to reduce P and nitrogen excretion by feeding pigs according to their age and physiological stage (Jongbloed and Lenis, 1992). A slight reduction in N and P excretion can be achieved in growing pigs by mixing feed rich in protein and minerals with a feed having low concentration of protein and minerals in a changing ratio (multiple phase feeding).

Table 4. Potential for feed management to reduce P excretion in swine manure[1]

Strategy	P reduction %
Formulation of diet closer to requirement of animal	10-15
Using highly digestible feeds	5
Phytase	20-30
Phytase /low P/ HAP[2] corn	40-50
Cellulase	5
Growth promoters	5
Phase feeding	5-10
Split-sex feeding	n/a[3]

[1]Adapted from Federation of Animal Science Societies (FASS) Publication "Dietary Adjustments to Minimize Nutrient Excretion from Livestock and Poultry", January. 2001

[2] High available P corn

[3] not available

2.9.1 Phase feeding

Phase feeding refers to the feeding of several diets for a relatively short period of time to closely meet the nutrient requirements of pigs (Han et al., 2000). Phase feeding helps to minimize over and under-feeding to make feeding of pigs more economical. Conventionally, pig feeding programs are based on starter, grower and finishing diets. As growing and finishing pigs excrete more pollutants than piglets, it is better to implement phase feeding in growing and finishing stages. It has been found that high nutrient diets do not always guarantee high growth rate of pigs, instead cause more nutrient excretion (Lee et al., 1999). In phase feeding, pigs are fed with slightly

altered nutrient composition of feed, which reduces nutrient excretion without the deterioration of growth performance of pigs. Phase feeding is usually applied based on the weight and/or wk. Phase feeding can be applied more efficiently in computerized feeding system.

Lee et al. (2000) studied the effect of phase feeding on performance and nutrient utilization in pigs. Pigs starting with the weight of 54 kg were kept in trial based on different phase feeding. Treatments included one phase (54 to 104kg), two phase (54 to 80 and 80 to 104 kg), three phase (54 to 70, 70 to 90 and 90 to 104 kg) and four phase (54 to 65, 65 to 80, 80 to 95 and 95 to 104 kg). Experimental diets had 16% CP for one phase feeding regimen, 16% and 12 % CP for two phase feeding regimen, 16%, 14% and 12% CP for three phase feeding regimen and 16%, 14.7%, 13.4% and 12% CP for four phase feeding regimen. The author found no significant difference in the growth performance. There was no significant difference in DM, CP and P digestibility. Regarding nutrient excretion, DM excretion of one phase feeding was significantly higher than three phase feeding regimen and daily N excretion of one phase feeding group was significantly higher than other group. For daily fecal P excretion, though not significant, one phase feeding group has numerically higher P excretion compared to other groups.

Table 5. Effect of different phase feeding on nutrient digestibility and excretion

Item	treatment 1	treatment 2	treatment 3	treatment 4	MSE[2]
Digestibilities					
DM (%)	85.3	85.8	86.7	85.9	1.32
CP (%)	83.1	82.7	83.1	83	1.79
P (%)	45.1	45.8	45.7	45.6	5.22
Fecal nutrient excretion					
DM (g/day)	282.8[a]	270.3[ab]	248.4[b]	263.4[ab]	26.6
N (g/day)	8.9[a]	8[b]	7.9[b]	7.9[b]	0.99
P (g/day)	6.29	6.43	6.03	6.18	0.58

[2] Mean Standard Error

Adapted from Lee et al., (2000)

.

2.10 CONCLUSION

Phosphorus is an important mineral of concern in the swine industry. Several studies have been done in the past to identify the requirements of P by pigs. Most of the studies are based on the performance studies. However, very few studies have been done to cover the areas of P utilization and excretion together with performance and bone studies. Due to the oversupply of P in the diets of swine leading to environmental and economic concern, the exact formulation of swine diets based on available or digestible P is necessary.

Considering all these issues, the first study was designed with the hypothesis that the reduction of dietary P will reduce P intake and increase P utilization while maintaining efficient growth performance and optimum skeletal integrity in growing pigs. The second study was designed with the hypothesis that short term phase feeding of P will improve P utilization without compromising growth performance and skeletal integrity in growing pigs.

3. MANUSCRIPT 1

Effect of reduced dietary level of available phosphorus on growth performance, phosphorus utilization, skeletal integrity and NaPi cotransporter gene expression in growing pigs

3.1 ABSTRACT

A three wk experiment was conducted with growing pigs to deterimne the effect of different dietary concentrations of available phosphorus (aP) on performance, bone parameters, P utilization and NaPi cotransporters gene expression. A total of 54 growing pigs (19.49±1.11 kgBW) were randomly allotted in six replicate pens (three pigs per pen) and provided three experimental diets with dietary aP concentrations of 0.23% (control referred as T1), 0.17% (T2) and 0.11% (T3). All diets contained 0.3% TiO_2 as an indigestible marker for P digestibility. Feed and water were provided on an *ad libitum* basis throughout the experiment. Body weight and feed intake were measured on day 0, 7, 14 and 21. At the end of each wk, one pig from each pen was housed in a metabolic crate for twenty four hours to collect fecal and urine samples and then sacrificed to obtain third (MC3) and fourth (MC4) metacarpals and jejunal and kidney samples. Bone evaluation was done using a Dual Energy X-ray Absorptiometry (DXA). Fecal and urine samples were subsampled and analyzed for P content. The expression of NaPi cotransporters (NaPi-IIb in the jejunum, NaPi-IIa and NaPi-IIc in the kidney) was analyzed using a quantitative real-time polymerase chain reaction (qRT-PCR). No significant adverse effect on performance was observed during the first wk as a result of reduction in aP, however there were 15.32% reduction in average daily gain (ADG) ($P< 0.05$) during the second wk and 15.95% reduction in ADG ($P< 0.05$) between T1 and T3 diets. Bone Mineral Density data of MC3 revealed significant reduction (*P*<

0.05) in pigs fed T3 diets compared to T1 for all three weeks, but there was no significant difference between T1 and T2 for both first and second wk except third wk. Bone mineral content of MC3 was also significantly reduced ($P < 0.05$) in T2 and T3 compared to T1 during the first and third wk but during the second wk, only T3 displayed reduced BMC compared to T1 and T2. The BMD and BMC of MC4 were not significantly different during the first wk. After the second wk, BMD was reduced in both T2 and T3 diets ($P < 0.05$ for the first wk and $P < 0.0001$ for the third wk) compared to T1. The BMC was also significantly reduced in both T2 and T3 diets ($P < 0.05$) compared to T1. The digestibility of P was lower ($P < 0.05$) in pigs fed T3 diet compared to those fed T2 and T1 diets throughout the experiment. The expression of NaPi-IIb gene in the jejunum was enhanced by up to 250% ($P < 0.01$) during the first wk in the pigs fed T3 diet compared to other dietary treatments. NaPi-IIa and NaPi-IIc gene expression in the kidney was enhanced ($P < 0.05$) by 160% and 180%, respectively, during the second wk in pigs fed T3 diets. This was accompanied by lower ($P < 0.05$) amount of P in urine of pigs fed T3 diets during wk2 and wk3. In conclusion, growing pigs are highly sensitive to dietary aP level as they displayed low ADG, bone parameters and urinary P excretion, which were profound as their age increased. Dietary P content also affected NaPi gene expression and P utilization in growing pigs.

Keywords: Pigs, Phosphorus, Performance, Bone parameters, P utilization.

3.2 INTRODUCTION

The swine industry is drawing attention from the public and the government due to the potential impact of the industry on soil and water quality. The improper use of swine manure poses potential environmental risk, which is mainly due to the P content of the swine manure. Excess land application of P is detrimental to the environment. This can cause the saturation of soils capacity

to adsorb P increasing the risk of P runoff to surface water leading to water pollution and subsequent eutrophication (Knowlton et al., 2004). This environmental aspect of P has kept this nutrient under scrutiny by animal nutritionists. Nevertheless, we cannot understate the importance of P in the body of swine for proper functioning of skeletal system and other physiological functions (Crenshaw, 2001). Skeletal development, leg structure and skeletal integrity in pigs are greatly associated with P reserves in the body and are directly affected by the dietary P levels (Gwendolyn, 2010). Furthermore, P is the third most expensive nutrient after energy and protein in swine nutrition (NRC, 1998).

Several aspects of P transport in the body of swine are yet to be fully understood. After enzymatic hydrolysis, phosphate is absorbed from the intestine via transcellular and paracellular pathways (Lee et al., 1986). The transcellular pathway involves the transport of phosphate by the sodium dependent phosphate cotransporters, which mainly occurs when dietary P is lower than required (Eto et al., 2006). Kidney is another important organ involved in P homeostasis where phosphate is largely absorbed by sodium-dependent transcellular pathway in the proximal convoluted tubules (Renkema et al., 2008). Few data exist regarding the effect of low dietary level of P in the gene expression of NaPi cotransporters. Reduction in the dietary P has been shown to affect intestinal phosphate uptake in rats, mice, and chickens (Hattenhauer et al., 1999; Saddoris et al., 2010). In mice, low P levels in diets increased membrane-bound NaPi-IIb cotransporter protein in the small intestine (Hattenhauer et al., 1999), but the effects of low-P diets on the expression of NaPi-IIb gene have been found to be highly variable, which is dependent on both duration and severity of reduction in dietary P level (Saddoris et al. 2010). In addition to this, the effect of low-P diets on the expression of NaPi-IIa and NaPi-IIc genes in the kidney is yet to be identified.

Growing pigs utilize P inefficiently (Jongbloed et al. 1997). It is important to supply the optimal amount of P that requires the adequate knowledge of P digestibility and requirements. Several studies have proved phytase to be a successful enzyme in improving P utilization and lowering the excretion of P in swine. However, very few studies have been done to evaluate the effects of reduced available P feeding on performance, P utilization, P excretion and bone parameters in growing pigs. This study was carried out to examine the effects of reduced dietary available P on growth performance, bone parameters, P utilization and sodium phosphate cotransporter gene expression in growing pigs.

3.3 MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee, and pigs were handled and cared according to the guidelines described by the Canadian Council on Animal Care (CCAC, 2009).

3.3.1 Animals and Housing

Fifty-four, two-month old growing pigs with an average initial BW of 19.5±1.11 kg, obtained from the University of Manitoba Glenlea Research Unit, were randomly assigned to one of three dietary treatments for 3 wks. Pigs were housed in a group of three per pen in a temperature controlled room (21±2 $^{\circ}$C) and had *ad libitum* access to feed and water. Each pen was provided with plastic covered expanded metal flooring, a nipple drinker, a stainless steel feeder and a metal wall partitioning that allowed visual contact with pigs in adjacent pens.

3.3.2 Experimental diets

Dietary treatments consisted of: 1) control diet (T1) with 0.23% aP; 2) T2 with 0.17% aP; and 3) T3 with 0.11% aP diet. The control diet was formulated to meet or exceed the National Research Council nutrient requirements for growing pigs (NRC, 1998). Calcium: total P ratio was maintained above 1.2:1 in all diets so that calcium would not be the limiting factor in the study (Ryan et al., 2011). All diets were supplemented with amino acids, mineral and vitamins to meet or exceed the recommended specification for growing pigs (NRC, 1998).

Table 6. Composition and analyzed values of experimental diets (as fed basis)

	Diet		
Item, %	T1[1]	T2[1]	T3[1]
Ingredients, %			
Corn	48.72	49.06	49.49
Wheat Hard Red Winter	23.73	23.82	23.82
Soybean meal	19.16	19.09	19.16
Vegetable oil	4.95	4.90	4.75
Limestone	0.95	0.98	0.95
Di-calcium phosphate	0.66	0.32	0.00
Iodized salt	0.35	0.35	0.35
Vitamin mineral premix[2]	1.00	1.00	1.00
Lysine-HCl	0.16	0.16	0.16
DL-Methionine	0.01	0.01	0.01
Threonine	0.01	0.01	0.01

Titanium dioxide	0.30	0.30	0.30
Calculated composition[3]			
DE, kcal/kg	3657	3665	3670
CP, %	18.03	18.05	18.12
Ca, %	0.57	0.51	0.44
tP, %	0.48	0.42	0.36
aP, %	0.23	0.17	0.11
Analyzed composition			
Dry matter, %	87.77	87.73	87.53
CP,%	18.98	19.31	19.18
Ca, %	0.59	0.52	0.45
tP, %	0.54	0.46	0.38
aP, %	0.26	0.17	0.11

[1]T1=0.23% aP diet, T2=0.17% aP diet and T3=0.11% aP diet

[2]Provided the following quantities of vitamins and minerals per kg of a complete diet: Vitamins: A, 2000 IU, D_3, 200 IU, E, 40 mg, K, 2 mg, B_1, 1.5 mg, B_2, 7 mg, B_6, 2.5 mg, B12, 25 µg, calcium pantothenate, 14 mg, folic acid, 1 mg, niacin, 21 mg, biotin, 70 µg. Minerals: Cu, 10 mg (as copper sulphate), iodine, 0.4 mg (as potassium iodine), iron, 120 mg (as ferrous sulphate), Mn, 10 mg (as manganous oxide), Se, 0.3 mg (as sodium selenite), Zn, 110 mg (as zinc oxide).

[3]Concentrations were calculated based on NRC (1998) guidelines

3.3.3 Growth performance and sample collection

The average daily gain (ADG) and average daily feed intake (ADFI) were measured weekly and feed efficiency (gain: feed ratio) was calculated as a ratio of feed intake to body weight gain. At the end of each wk, 18 pigs (6 pigs per treatment) were housed individually in adjustable metabolic crates (118 cm × 146 cm) with smooth transparent plastic sides and plastic covered expanded metal sheet flooring in a temperature controlled room for 24 hours to collect fecal and urine samples in the fecal and urine collection trays placed under the crate floor. The pigs housed in metabolic crates were then sacrificed by jugular injection of sodium pentobarbital (50 mg/kg BW) (Omogbenigun et al., 2004). Left metacarpal bones (third (MC3) and fourth metacarpal (MC4)) were collected for the analysis of bone mineralization parameters using a dual energy X-ray absorptiometry (DEXA). The bones were cleaned of attached tissues, wrapped in cheesecloth soaked with 1 × PBS and then stored at 20 °C until further analysis. The abdominal cavity was opened along the midline to remove jejunum and kidney. The jejunal (2 cm) and kidney samples were aseptically excised, immediately frozen in liquid nitrogen and preserved at -80 °C for later determination of NaPi cotransporter genes by quantitative real-time quantitative polymerase chain reaction (qRT-PCR). For performance study, individual pens were considered as the experimental unit whereas for the study of P digestibility, urine analysis, skeletal integrity and gene expression, individual pigs were considered as the experimental unit.

3.3.4 Dual energy X-Ray absorptiometry (DEXA)

The bone parameters i.e. bone mineral density (BMD), bone mineral content (BMC) and bone area (BA) of the third (MC3) and fourth (MC4) metacarpal bones were measured by a dual energy x-ray absorptiometry (pDEXA®, Norland Medical System, Inc. Fort Atkinson, WI). Calibration was

done each time before scanning for quality assurance. The MC3 and MC4 bones were placed in a standardized orientation during each scan. Both MC3 and MC4 from the same pig were scanned together. The BMD detected represents a combination of thickness of bone and density instead of a true volume and was expressed in g/cm^2 (Kim et al., 2012). The DEXA scan was obtained at a scout speed of 40 mm/sec and at a measure speed of 20 mm/sec, with the resolution of 1.0 mm × 1.0 mm.

3.3.5 Sample preparation and chemical analysis

Fecal samples were dried in a forced air oven at 60 °C to remove moisture from them and pooled per pig. Diets and fecal samples were finely ground through a 2-mm mesh screen in a Thomas Wiley Mill (Thomas model 4 Wiley Mill; Thomas Scientific) and thoroughly mixed before analysis. Each analysis was performed in duplicates. Analysis for DM in diets was carried out using the 934.01 method of AOAC (1990). For the analysis of DM, one gm of each sample was weighed in a pre-weighed silica dish and dried in an over at 104 °C for overnight. Next day, the sample was removed out of the oven, cooled down in desiccators and re-weighed. Crude protein (N× 6.25) of the diets was determined using a Leco nitrogen analyzer (model NS-2000, Leco Corp., St. Joseph, MI). Total P and Ca analyses in ingredients, feed and feces were digested according to the procedure described (method 985.01; AOAC, 1990) and were analyzed using an inductively coupled plasma (ICP) spectroscopy (Varian INC., Palo Alto, CA). For Ca and P analyses, approximately one gm of each sample was weighed in a labelled Pyrex tube without a screw and ashed overnight in a furnace at 600 °C. After ashing, tubes were allowed to cool and were removed from the furnace for acid digestion. Ten mL of 5N HCl/HNO_3 (1% v/v) was added to those tubes, capped with screws and digested for one hr in a sonication bath that was preheated to 60°C. After cooling down the samples from the bath, 2.5 mL of the sample (1mL of feces) was

pipetted and diluted with deionized water in a 100 ml volumetric flask. After that, the samples were filtered through Q5 filter paper into a 20 ml scintillation vials. Calcium and total P concentrations were measured using an inductively coupled plasma optical emission spectrometer (ICP) (AES Vista, Varian Inc., Palo Alto, CA). Phytate-P in the diet was measured as described by Haug and Lantzsch (1983). Available P was calculated by substracting phytate-P from total P. The TiO_2 in the diets and fecal samples were measured using the method of Lomer et al. (2000). The TiO_2 levels in the diets and feces were also determined using ICP. Diet and fecal TiO_2 and total P values were used in calculating ATTD of P and following equation was used for the calculation: ATTD, % = 100 - [(TiO2Diet/TiO2Feces) × (total PFeces/total PDiet) × 100]. Phosphorus excretion (kg/t of feed consumed) was determined based on the analyzed P content of the feces using the following equation:

Phosphorus in feces (%) × {1000 kg of diet – (1000 kg of diet × DM digestibility of diet)} (Omogbenigun et al., 2003)

Urine samples were thawed and pooled for each pig for analysis. P analysis in urine was done by diluting the frozen urine samples. The urine samples were thawed and from the total amount of urine, 10 ml was pipetted. The samples were then centrifuged at a relative centrifugal force (RCF) of 3,000 x g for 10 minutes so as to settle down the organic matters. Four ml of supernatant fluid was pipetted from the tube and diluted with deionized water to make it 20 ml. The samples were then filtered through Q5 filter paper into scintillation vials to get rid of any residues. Then the samples were sent for ICP analysis.

3.3.6 RNA Isolation, cDNA synthesis and Quantitative Real-Time PCR

Total RNA was extracted from jejunal and kidney samples using TRIzol reagent (Invitrogen, Life Technologies, Burlington, ON, Canada) according to the instructions provided by the manufacturer. About 100 mg of tissue samples were thawed on ice and added to 1 ml of the TRIzol reagent. The samples were then homogenized in a Mini-BeadBeater-16 homogenizer (Bio Spec Products, Bartlesville, OK) at 3450 oscillations/min for 3 min. After extraction, the RNA pellets were dissolved in 200 µl nuclease-free water (Ambion, Life Technologies, Burlington, ON, Canada) and total RNA concentrations were determined at an optical density of 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE). All RNA samples were normalized to a concentration of 2 µg/ µl and purity were verified by evaluating the optical density ratio of 260 nm to 280 nm. The normalized total RNA was then reverse transcribed using a High Capacity cDNA synthesis kit (Applied BioSystems, Life Technologies, Burlington, ON, Canada) following the manufacturer's protocol, and the synthesized cDNA were stored at -20 ºC.

Pairs of primers for each gene were designed and checked for target identity using the National Centre for Biotechnology Information (NCBI). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in duplicate reactions including nuclease free water, the forward and reverse primers of each gene, cDNA and SYBR green as a detector on Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada). Data were generated using $\Delta\Delta C_t$ method by normalizing the expression of a target gene to a housekeeping gene, Glyceraldehyde 3- Phosphate Dehydrogenase (GAPDH) and 18s ribosomal RNA and the values were reported as a fold change of the expression of the target genes in the experimental groups

compared to the control group (Regassa and Kim, 2013). Pairs of primers used for qRT-PCR assay and their sequences are presented in Table 3.

Table 7. NaPi cotransporters primer sequence in pigs

Gene[1]	Forward primer sequence	Reverse primer sequence	Annealing temperature (^{0}C)
SLC34A1	AGGAGGAACAGAAGCCAGGT	GGCACCTTGAGGAGCATTGA	60
SLC34A2	AACCTCCATCACCAACACCC	AAGAGCACCAACACGGAGAG	58
SLC34A3	GTACCACAACAGGATGCCGA	AGAGGACCCTGAACCACTGA	61
GAPDH	GGTGAAGGTCGGAGTGAACG	GGGATCTCGCTCCTGGAAGA	58
18srRNA	GTCCCGACGTGACTGCTC	CTCGACCGAGGGCACAAG	58

[1]SLC34A1= solute carrier family member 1 (type IIa sodium/phosphate cotransporter), SLC34A2 = solute carrier family member 2 (type IIb sodium/phosphate cotransporter), SLC34A3= solute carrier family member 3 (type IIc sodium/phosphate cotransporter), GAPDH= Glyceraldehyde 3-phosphate dehyadrogenase, 18sR = 18s ribosomal RNA.

3.3.7 Statistical analysis

Data were subjected to one-way ANOVA as a completely randomized design using the Proc GLM procedure of statistical Analysis Systems (SAS) Institute version 9.2. Differences between selected means were tested using the PDIFF (Probability difference) statement. For growth performance, individual pen served as the experimental unit and for P digestibility, urine analysis, skeletal integrity and gene expression, individual pigs served as the experimental unit. The data are presented as least-square means ±SEM. Significant difference were accepted if $P < 0.05$ and trends were considered at $P < 0.10$.

3.4 RESULTS

All pigs remained visibly healthy and readily consumed their daily feed allowance throughout the experimental period. The analyzed diet compositions of the Ca, P and crude protein of the three diets are listed in Table 3. All values were within the acceptable ranges and were in agreement with the calculated composition of the diets. The analyzed values for the dietary P were closer to the calculated for all diets (Table 3).

3.4.1 Growth performance

The ADG was not different statistically (Table 5) among diets during the first wk. During the second wk, pigs fed T3 diet had lower ($P< 0.05$) ADG than the pigs fed T1 and T2 diets. The ADFI was not significantly different (Table 5) among the treatments during all the wks. However, pigs fed T3 diet had numerically lowest ADFI compared to the pigs fed higher aP diets (T1 and T2) in all the wks. Gain to feed ratio (G: F) was similar (Table 5) among diets during first and third wk. During the second wk, pigs fed T3 diet had the lowest ($P< 0.05$) G: F than those fed T1 and T2 diets.

3.4.2 Bone quality

The results of the bone mineralization parameters (especially BMD and BMC) of third and fourth metacarpal bones showed significant differences between the treatments (Table 6). For the third metacarpal bone, pigs had significantly higher BMD and BMC ($P< 0.05$) in the T1 compared to T3 group during all the wks. Pigs fed T1 and T2 diets had similar BMD during the first and second wk whereas during third wk, pigs in the T1 diet had significantly higher ($P< 0.05$) BMD compared to T2. BMC in the pigs fed T2 diet was significantly lower compared to T1 during the first and

third wk. The bone area was similar among all treatments in all wks. For the fourth metacarpal bones, bone parameters were not significantly different among treatments during the first wk. During the second and third wk, BMD was significantly higher ($P < 0.05$) in T1 compared to T2 and T3. BMC was significantly higher in T1 compared to T3 during both second and third wk whereas in second wk, pigs fed T1 and T2 diets had similar BMC. Bone area was not different among treatments during the entire period.

Table 8. Effects of dietary treatments on growth performance of growing pigs

Item	Treatment			SEM	P
	T1	T2	T3		
wk 1					
ADFI (g)	966	970	928	32.61	0.861
ADG (g)	488	528	453	27.03	0.574
gain:feed	0.5	0.54	0.47	0.015	0.237
wk 2					
ADFI (g)	1,494	1,447	1,418	31.29	0.635
ADG (g)	886[a]	812[ab]	750[b]	23.35	< 0.05
gain:feed	0.59[a]	0.56[ab]	0.52[b]	0.01	< 0.05
wk 3					
ADFI (g)	1,796	1,626	1,591	51.94	0.235
ADG (g)	895	850	752	27.9	0.095
gain:feed	0.52	0.5	0.47	0.01	0.274
overall					
ADFI (g)	1418	1371	1312	46.21	0.295
ADG (g)	756[a]	745[a]	652[b]	22.88	< 0.05
gain:feed	0.53[a]	0.54[a]	0.49[b]	0.009	< 0.05

[1] T1=0.23% aP diet, T2=0.17% aP diet and T3= 0.11% aP diet

[2]Standard error of the mean

Values with different superscripts within a same row are different (P< 0.05)

Table 9. Effect of dietary treatments on third (MC3) and fourth (MC4) metacarpal BMD (g/cm^2), BMC (g) and BA (cm^2) during wk1, wk2 and wk3

wk	Item	MC3			SEM[1]	*P*-value	MC4			SEM[1]	*P*-value
		T1	T2	T3			T1	T2	T3		
wk1	BMD	0.211^a	0.205^{ab}	0.197^b	0.003	< 0.05	0.224	0.226	0.210	0.006	0.266
	BMC	1.961^a	1.775^b	1.752^b	0.043	< 0.05	1.832	1.755	1.653	0.053	0.147
	BA	9.227	8.871	8.697	0.188	0.136	8.176	7.85	7.751	0.160	0.200
wk2	BMD	0.221^a	0.210^a	0.193^b	0.004	< 0.05	0.237^a	0.22^b	0.204^b	0.005	< 0.05
	BMC	2.132^a	2.085^a	1.838^b	0.066	< 0.05	2.039^a	2.000^a	1.730^b	0.081	< 0.05
	BA	10.137^a	9.498^b	9.420^b	0.174	< 0.05	8.970	8.437	8.432	0.172	0.069
wk3	BMD	0.248^a	0.213^b	0.201^b	0.005	< 0.001	0.263^a	0.228^b	0.210^b	0.006	< 0.001
	BMC	2.649^a	2.250^b	2.128^b	0.086	< 0.05	2.500^a	2.140^b	1.969^b	0.076	0.001
	BA	10.662	10.556	10.520	0.219	0.899	9.456	9.381	9.350	0.158	0.893

[1] Standard error of the mean.

Values with different superscripts of the same row are significantly different (*P*< 0.05)

3.4.3 Phosphorus utilization and excretion

The dietary treatments had significant effects on the level of urinary P during the second and third wk but not in the first wk. During the second wk, urinary P from the pigs fed T3 diets had significantly lower ($P< 0.05$) level of urinary P compared to control whereas during the third wk, pigs fed T2 and T3 diets had significantly lower ($P< 0.05$) urinary P compared to control. Regarding the effect of dietary treatments on P excretion in feces, we found no significant effect of the treatment on P excretion in feces during all the wks but the value of P in feces were numerically lower in the pigs fed T2 and T3 diets compared to pigs fed T1 diet. The digestibility of P was found to be significantly lower in the pigs fed T3 diets compared to T1 during all the wks however, the digestibility of P was found to be similar among T1 and T2 diets.

Table 10: Effect of dietary treatments on P utilization in growing pigs

Item		Treatment			SEM[1]	*P*
		T1(control)	T2	T3		
P intake (g/day)	wk1	5.22[a]	4.48[b]	3.52[c]	0.25	0.001
	wk2	8.07[a]	6.74[b]	5.38[c]	0.26	<0.001
	wk3	9.05[a]	6.98[b]	6.99[b]	0.33	<0.001
Fecal P excretion (kg/ton of feed)	wk1	1.81	1.56	1.8	0.18	0.561
	wk2	1.89	1.84	1.58	0.23	0.640
	wk3	1.89	1.67	1.41	0.24	0.424
Apparent P digestibility (%)	wk1	35.55[a]	33.34[a]	23.23[b]	1.69	<0.001
	wk2	34.73[a]	26.73[ab]	22.31[b]	2.75	< 0.05
	wk3	38[a]	29.49[a]	18.33[b]	3.09	0.001
Urinary P (mg/L)	wk1	58.42	44.81	51.43	19.42	0.883
	wk2	92.90[a]	48.13[ab]	37.66[b]	15.09	< 0.05
	wk3	85.30[a]	32.61[b]	44.42[b]	12.42	< 0.05

[1] Standard error of the mean. Values with different superscripts of the same row are significantly different ($P< 0.05$)

3.4.4 Sodium dependent phosphate cotransporter gene expression

Low aP diets had a significant effect on the relative expression of sodium-dependent phosphate cotransporter genes in the jejunum and kidney. Earlier stage of P deprivation in diets showed a significant effect of NaPi cotransporter gene expression in the jejunum followed by the significant effect of cotransporter, gene expression in kidney. During the first wk, NaPi-IIb gene expression was significantly up regulated in the jejunum in pigs fed T3 (lowest aP) diet compared to those fed T1 and T2. During the second and third wk, the effect of diet on NaPi-IIb gene expression was not significant in the jejunum. During the second wk, NaPi-IIa and NaPi-IIc gene expressions were significantly upregulated in the kidney of pigs fed T3 diet compared to those fed higher aP diets. However, during the third wk, no significant effect was seen on NaPi cotransporters gene expression as a result of low aP diets.

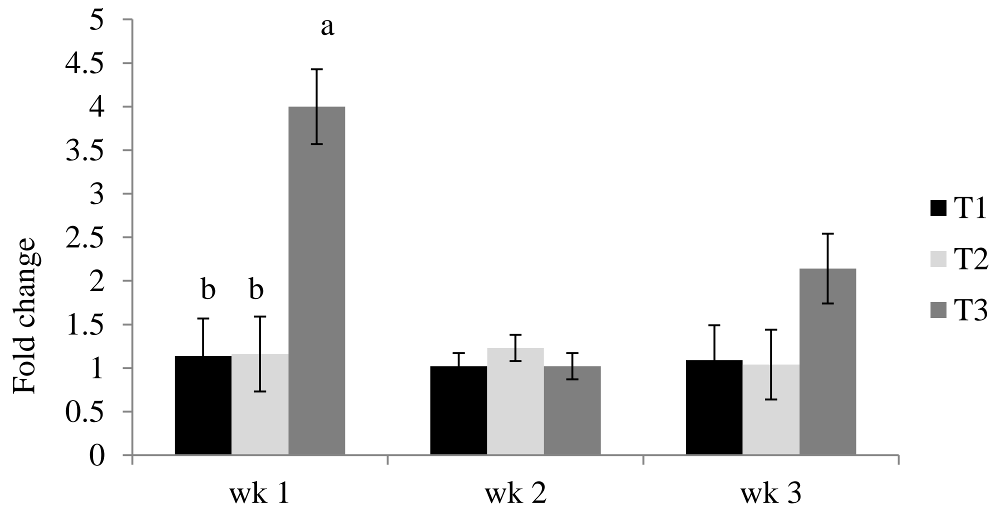

Fig 1: NaPi-IIb gene expression in the jejunum

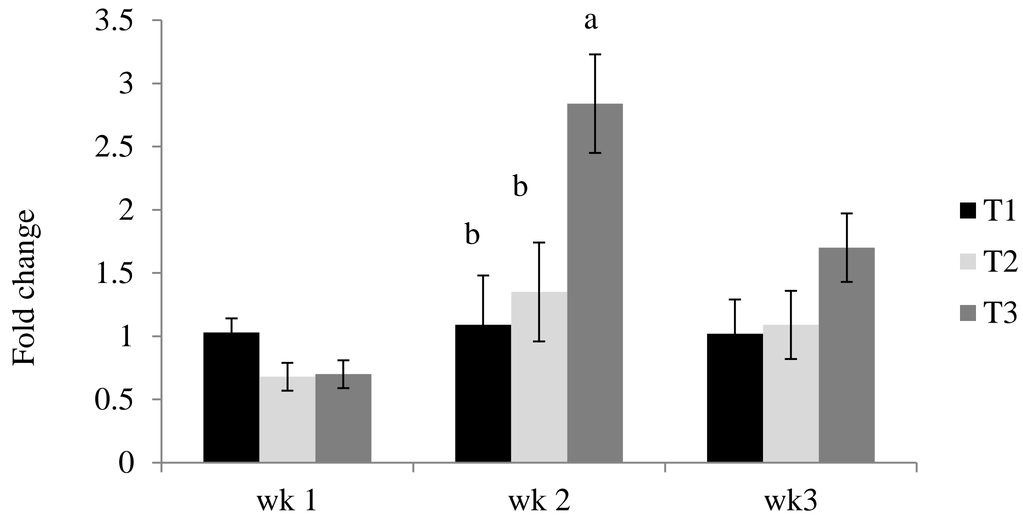

Fig 2: NaPi-IIa gene expression in the kidney

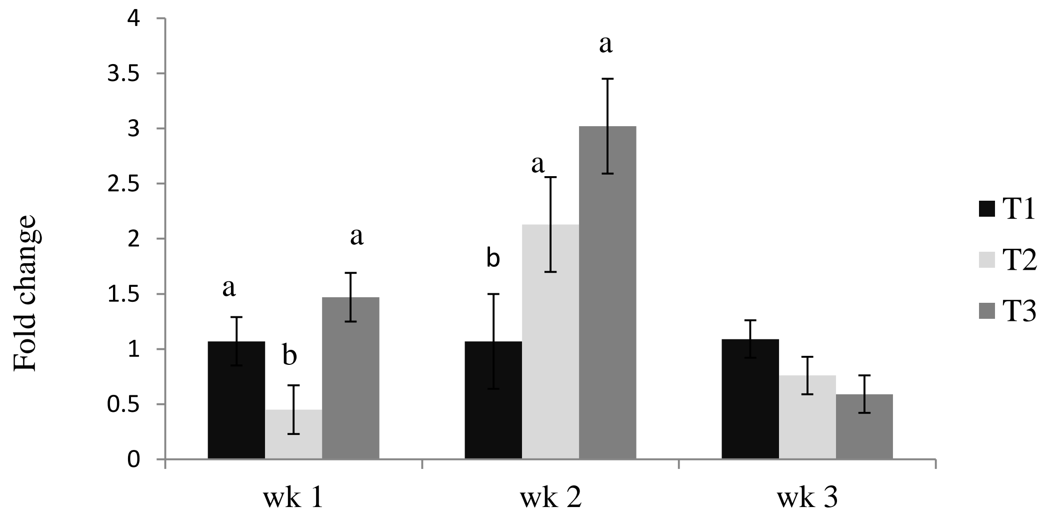

Fig3: NaPi-IIc gene expression in the kidney

3.5 DISCUSSION

The effects of lower aP diets on growth performance of pigs in the present study were in agreement with previous findings (Hall et al., 1991; Ryan et al., 2010; Varley et al., 2010). Hall et al. (1991) found that pigs fed P deficient diets (0.3% tP) had a slower growth rate and lower feed efficiency than the pigs fed adequate P diets. Varley et al. (2010) found higher ($P < 0.05$) ADG in finishing pigs offered high and medium available P diets (0.862 and 9.832 vs. 0.766 kg/day) compared to pigs offered low available P diets. The study conducted by Eeckhout et. al. (1995) on 180 growing pigs showed that a dietary level of 0.11% aP had a negative impact on feed efficiency and daily gain during the first five wks compared to 0.14% and 0.17% aP. Ryan et al., (2010) reported improved ADG and feed conversion ratio in pigs fed high and medium digestible P diets compared to those fed low digestible P diets. On the other hand, the study done by Ketaren et al., (1993) found that feed intake and live weight gain were similar in the pigs fed different levels of aP diets i.e. 1g aP/kg, 2 g aP/kg, 3 g aP/kg and 4 g aP/kg feed. However, FCR was initially improved and then depressed with increased dietary P level.

The results of bone mineralization parameters in the present study showed a negative impact of the reduced dietary aP on the skeletal integrity especially during the second and third wk. Similar effects were found in previous studies done by Eeckhout et al. (1995), Ketaren et al. (1993) and Ryan et al. (2010). The analysis of bone parameter was done on the third and fourth metacarpal bones that have been used for studies by several authors (Shaw et al., 2006; Varley et al., 2011).

Varley et al. (2011) found that pigs offered low P diets from 11 to 30 kgBW had lower bone density than those offered the high P diets. In weaner pigs, pigs offered 6.4 g total P/kg of diet had bone density value of 1.28 g/cm^3 which was significantly higher than the bone density value in pigs offered 5.5 g total P/kg of diet (1.26 g/cm^3) and 4.5 g total P/kg of diet (1.23 g/cm^3). Similarly in the finisher pigs, bone density was higher in the pigs fed higher P diets compared to pigs fed low P diets. Ryan et al. (2010) observed higher ($P < 0.001$) metacarpal apparent density from those fed high digestible P diets (2.8 g/kg) than pigs fed medium (2.2 g/kg) or low P (1.6 g/kg) diets. The metacarpal BMD was higher ($P < 0.001$) in pigs fed the high and medium P diets than the low P diets. Ketaren et al. (1993) found increased ash concentration in the radius/ulna and femur bones with increasing dietary aP indicating the increase in bone mineralization. The same author also observed gait difficulty in two pigs fed the diet containing 1g available P/kg at the end of the experiment. The result of the bone parameters showed that bone parameters are more important indicators of reduction of dietary P than performance parameters. The negative effect on bone parameters may be because substantial bone formation occurs during first 12 wks of age (Tanck et al., 2001).

Conflicting results have been reported on the effect of low P-diets on NaPi-IIb mRNA expression. The expression is found to be dependent on both the severity and length of dietary P deprivation. Saddoris et al. (2010) observed higher sodium-dependent phosphate transport up to 46% as dietary aP was decreased but this was not accompanied by increased NaPi-IIb mRNA expression. Weanling pigs fed low P diets (0.23% aP) were not found to have greater expression of NaPi-IIb compared to those fed adequate P diets (0.4% aP). Studies in mice regarding the effect of low P diets on NaPi-IIb mRNA expression have shown widely variable results with no effect to

highly significant effect. Katai et al. (1997) found significant increase in sodium dependent phosphate transport activity in rats fed chronically low P diets possibly due to the elevated expression of NaPi-II mRNA and protein.

Though the result was not significant, fecal P output was increased with the increase of P in the diet. The result was comparable to the findings of Varley et al. (2010). The author also found higher ($P < 0.05$) urinary output of P in pigs fed medium (2.0 g/kg aP) and high P diets (2.5 g/kg aP) than the pigs fed low P diets (1.5 g/kg aP). The reduction in P digestibility of pigs fed low aP diet is attributed to greater reduction in non-phytate P in the low aP diets compared to the high aP diets. Even though the expression of intestinal NaPi transporter gene was increased by lowering dietary aP levels, higher portion of non-digestible phytate-P in the low aP diets might have reduced total P digestibility.

The need to reduce the amount of P in swine manure has demanded a new perspective about the formulation of diets to meet the minimum requirement of pigs without compromising their production performance. Strict environmental regulations about the level of P to be discharged in swine effluent have led to the dietary manipulation to reduce P level in the diets and to make enzymatic use to improve P utilization (NRC, 1998). Based on this study, the diets with 0.11% aP level was not advantageous to pigs in terms of growth performance and bone parameters, however, P excretion in urine was reduced significantly. NaPi-II cotransporter genes were highly expressed in pigs fed 0.11% diets. This has certainly improved our understanding of P utilization in the growing pigs. Still, there are many issues to be resolved at the protein level of those cotransporters, which will enable nutritionist to find ways to improve P utilization in the pigs. The

0.17% aP had less severe effect on the performance and bone parameters of growing pigs compared to 0.11% aP. Because there was no difference among performance, bone parameters, P utilization and P excretion among pigs fed control diets and moderately low level of aP diet, nutritionist can go for phase feeding with the manipulation of dietary level of P during different wks to see compensatory effect of higher level of aP on those parameters.

4. MANUSCRIPT 2

Effect of short-term phase feeding of available phosphorus on growth performance, phosphorus utilization, skeletal integrity and NaPi cotransporter gene expression in growing pigs

4.1 ABSTRACT

A three wk experiment was conducted with growing pigs to investigate the effect of short term phase feeding of reduced dietary concentrations of available phosphorus (aP) on growth performance, bone parameters, P utilization and NaPi cotransporter gene expression. A total of 28 pigs (22.28 ± 2.08 kgBW) were used for this feeding trial. Experimental pigs were randomly allotted as one pig per pen to provide 7 pigs per treatment. Treatments included T1 (a diet with 0.23% aP for all three wks), T2 (a diet with 0.11% aP for first wk replaced by diet with 0.23% aP for next two wks), T3 (a diet with 0.11% aP for first two wks replaced by diet with 0.23% aP for last wk) and T4 (a diet with 0.11% aP for all the wks). All diets contained 0.3% TiO_2 as an indigestible marker. Feed and water were provided on an *ad libitum* basis. Body weight and feed intake were measured on day 0, 7, 14 and 21. Feces were collected by grab sampling at the end of each wk. At the end of the experiment, 6 pigs from each treatment were sacrificed to obtain third (MC3) and fourth (MC4) metacarpals and the jejunal and kidney samples. Dual energy X-ray absorptiometry (DXA) was used to evaluate skeletal integrity. Fecalsamples were subsampled and analyzed for P content. Expression of NaPi cotransporters (NaPi-IIb in the jejunum, NaPi-IIa and NaPi-IIc in the kidney) was analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). Average daily gain and gain: feed was lower ($P < 0.05$) during the first wk in the pigs provided T2, T3 and T4 treatments compared to pigs provided T1 treatment; however, during the

second and third wk, no significant differences were observed in pigs among different treatments. The BMD was lower in (*P*< 0.05) in T3 and T4 compared to T1 and T2 whereas BMC was lower in pigs with T4 treatment compared to pigs provided T1, T2 and T3 treatments. The digestibility of P was found to be lower (*P*< 0.05) in the pigs fed lower aP diets during all the wks but digestibility improved with increase in dietary aP. No significant difference was observed in the gene expression of sodium dependent phosphate cotransporters as a result of dietary treatments. In conclusion, growing pigs are sensitive to dietary P levels and the negative effect of low-P diets on skeletal integrity can be compensated by normal-P in the diet provided that the low-P diet is for short duration.

Keywords: Pigs, Phosphorus, Growth performance, Bone parameters, P utilization.

4.2 INTRODUCTION

Traditionally, swine diets have been formulated in order to provide excess of nutrients to maximize the performance of pigs. This has led to increased concern on the negative impact of the swine production system on the environment. The dietary P is of particular concern because of its involvement in soil and water pollution (Knowlton et al., 2004). Phosphorus is an essential nutrient for both plants and animals. Thus, optimal utilization of dietary P is important for both the environmental and normal physiological functions of swine including bones. Several strategies have been found to be effective in combating these issues: 1) improving feed efficiency, 2) accurate estimation of nutrient requirement of animals, 3) feeding for optimum performance rather than maximum performance, 4) phase feeding and separate sex feeding, 5) improving availability of P and 6) reduced feed wastage (Kornegay and Harper, 1997). Phytase enzyme has been successfully used in swine diets to increase P utilization and to reduce P excretion. Very few studies have been

done to examine the effects of available P phase feeding on P utilization, growth performance and skeletal integrity in swine.

The study done by Ryan et al. (2011) found that production indices (ADG, ADFI and FCR) were not affected by dietary P phase feeding; however, reduced P in diet negatively affected bone quality. Replacing a low-P diet with a high P diet after 4 or 5 wk didn't result in similar bone mineral density to those pigs that were fed a high P diet throughout the experimental period. The reason might be that 4 or 5 wk period of low P treatment is too long to be compensated for. Instead, if the low P treatment is reduced to 1 or 2 wk, the negative effect may be compensated by a high P treatment thereafter. In addition to the aforementioned issues, few studies have been done to evaluate the effects of dietary P phase feeding on Na-dependent P transporters. Therefore, this study was carried out to examine the effects of short-term phase feeding of reduced dietary concentrations of available phosphorus (aP) on growth performance, bone parameters, P utilization and NaPi cotransporter gene expression.

4.3 MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee, and pigs were handled and cared according to the guidelines of Canadian Council on Animal Care (CCAC, 2009).

4.3.1 Animals and housing

A total of 28 two-months old growing pigs with an average initial BW of 22.28 ± 2.08 kg were acquired from the University of Manitoba Glenlea Research Unit. Pigs were randomly

allotted to one of the four treatments for 3 wks. Pigs were individually housed in the pen in a temperature controlled room (21±2^{0}C) and had an *ad libitum* access to feed and water. Each pen was provided with plastic covered expanded metal flooring, a nipple drinker, and a stainless steel feeder and a metal wall partitioning that allowed visual contact with pigs in adjacent pens.

4.3.2 Dietary treatments

Dietary treatments (Table 12) consisted of: 1) control (T1) - pigs were provided with 0.23 % aP diet for whole three wk; 2) T2 – pigs were provided with a low aP diet for the first wk and a normal aP diet for last two wks; 3) T3 – pigs were provided with a low aP diet for the first two wk and a normal aP diet for last wk; 4) T4 – pigs were provided with a low aP diet for whole three wk. Calcium: total P ratio was maintained above 1.2:1 in all diets so that calcium would not be the limiting factor in the study (Ryan et al., 2011). All the diets were supplemented with amino acids, mineral and vitamins to meet or exceed the recommended specification for growing pigs (NRC, 1998).

Table 11. Dietary treatments for the experimental period

Treatment \ Wk	wk 1	wk 2	wk 3
T1	C	C	C
T2	L	C	C
T3	L	L	C
T4	L	L	L

L (low aP diet) = diet with 0.11% aP

C (normal aP diet) = diet with 0.23% aP

Table 12. Composition and analyzed values of experimental diets (as fed basis)

	Diet	
Item, %	C (normal aP diet)	L (low aP diet)
Ingredient, %		
Corn	43.11	44.10
Wheat hard red winter	26.00	26.00
Soybean meal 44% CP	22.4	22.26
Vegetable Oil	4.95	4.75
Limestone	0.95	0.95
Dicalcium Phosphate	0.65	0.00
Iodized salt	0.35	0.35
Vitamin mineral premix [1]	1.00	1.00

Lysine-HCl	0.18	0.18
DL-Methionine	0.08	0.08
Threonine	0.03	0.035
Titanium dioxide	0.30	0.30
Total	100	100
Calculated composition[2]		
DE, kcal/kg	3626	3639
CP, %	18.01	18.02
Ca, %	0.69	0.55
tP, %	0.52	0.4
aP, %	0.23	0.11
Analyzed composition		
CP, %	18.98	18.31
Ca, %	0.60	0.55
tP, %	0.55	0.41
aP, %	0.28	0.13

[1]Provided the following quantities of vitamins and minerals per kg of a complete diet: Vitamins: A, 2000 IU, D_3, 200 IU, E, 40 mg, K, 2 mg, B_1, 1.5 mg, B_2, 7 mg, B_6, 2.5 mg, B12, 25 µg, calcium pantothenate, 14 mg, folic acid, 1 mg, niacin, 21 mg, biotin, 70 µg. Minerals: Cu, 10 mg (as copper sulphate), iodine, 0.4 mg (as potassium iodine), iron, 120 mg (as ferrous sulphate), Mn, 10 mg (as manganous oxide), Se, 0.3 mg (as sodium selenite), Zn, 110 mg (as zinc oxide).

[2]Concentrations were calculated based on NRC (1998) guidelines.

4.3.3 Growth performance and sample collection

The BWG and FI were measured weekly, and feed efficiencies were calculated as a ratio of feed intake to body weight gain. At the end of experiment, 24 pigs (6 pigs per treatment) were sacrificed by jugular injection of sodium pentobarbital (50 mg/kg BW) (Omogbenigun et al., 2004). Left metacarpal bones (third and fourth metacarpal) were collected for the analysis of bone mineralization parameters. The bones were cleaned of attached tissues, wrapped in cheesecloth soaked with 1 × PBS and then stored at -20 ^{0}C until further analysis. The abdominal cavity was opened along the midline to remove the jejunum and the kidney. The jejunal (2 cm) and kidney samples were aseptically excised, immediately frozen in liquid nitrogen and preserved at -80 ^{o}C for later determination of NaPi cotransporter genes by quantitative real-time polymerase chain reaction (qRT-PCR).

4.3.4 Dual energy X-Ray absorptiometry (DEXA)

The bone parameters i.e. BMD, BMC and BA of the third (MC3) and fourth (MC4) metacarpal bones were measured by a dual energy x-ray absorptiometry (pDEXA®, Norland Medical System, Inc. Fort Atkinson, WI). Calibration of the machine was done each time before scanning for the quality assurance. The MC3 and MC4 bones were placed in a standardized orientation during each scan. Both MC3 and MC4 from the same pig were scanned together. The BMD detected represents a combination of thickness of bone and density instead of a true volume and was expressed in g/cm^2 (Kim et al., 2012). The DEXA scan was obtained at a scout speed of 40 mm/sec and at a measure speed of 20 mm/sec, with the resolution of 1.0 mm × 1.0 mm.

4.3.5 Sample preparation and chemical analysis

Fecal samples were dried in a forced air oven at 60^0 C to remove moisture from them and pooled per pig. Diets and fecal samples were finely ground using 2-mm mesh screen in a Thomas Wiley Mill (Thomas model 4 Wiley Mill; Thomas Scientific) and thoroughly mixed before analysis. Each analysis was performed in duplicates. Analysis for DM in diets was carried out using the 934.01 method of AOAC (1990). For the analysis of DM, 1 gm of sample was weighed in a pre-weighed silica dish and dried in an over at 104^0 C for overnight. Next day, the sample was removed out of oven, cooled down in desiccators and re-weighed. Crude protein ($N\times 6.25$) of the diets was determined using a Leco nitrogen analyzer (model NS-2000, Leco Corp., St. Joseph, MI). Total P and Ca analyses in ingredients, feed and feces were digested according to the procedure described (method 985.01; AOAC, 1990) and were analyzed using inductively coupled plasma (ICP) spectroscopy (Varian INC., Palo Alto, CA). For Ca and P analyses, approximately 1 gm sample was weighed in a labelled Pyrex tube without screw and ashed overnight in a furnace with 600 ^{0}C. After ashing, tubes were allowed to cool and were removed from the furnace for acid digestion. 10 mL of 5N HCl/HNO_3 (1% v/v) was added to those tubes, capped with screw and were digested for 1 hour in a sonication bath that was preheated to 70 ^{0}C. After cooling down the samples from the bath, 2.5 mL of sample (1mL of feces) was pipetted and diluted with deionized water in 100 ml volumetric flask. After that, the samples were filtered through Q5 filter paper into a 20 ml scintillation vials. Calcium and total P concentrations were measured using an inductively coupled plasma optical emission spectrometer (ICP) (AES Vista, Varian Inc., Palo Alto, CA). Phytate P in the diet was measured as described by Haug and Lantzsch (1983). Available P was calculated by subtracting phytate P from total P. The TiO_2 in the diets and fecal samples were measured using the method of Lomer et al. (2000). The TiO_2 levels in the diets and feces were also

determined using ICP. Diet and fecal TiO_2 and total P values were used in calculating ATTD of P and following equation was used for the calculation: ATTD, % = 100 - [(TiO2Diet/TiO2Feces) × (total PFeces/total PDiet) × 100].

4.3.6 RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from jejunal and kidney samples using TRIzol reagent (Invitrogen, Life Technologies, Burlington, ON, Canada) according to the instruction provided by manufacturer. About 100 mg of tissue samples were thawed on ice and added to 1 ml of the TRIzol reagent. The samples were then homogenized in a Mini-BeadBeater-16 homogenizer (Bio Spec Products, Bartlesville, OK) at 3450 oscillations/min for 3 min. After extraction, the RNA pellets were dissolved in 200 µl nuclease-free water (Ambion, Life Technologies, Burlington, ON, Canada) and total RNA concentrations were determined at an optical density of 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE). All RNA samples were normalized to a concentration of 2 µg/ µl and purity were verified by evaluating the optical density ratio of 260 nm to 280 nm. The normalized total RNA was then reverse transcribed using a High Capacity cDNA synthesis kit (Applied BioSystems, Life Technologies, Burlington, ON, Canada) following the manufacturer's protocol, and the synthesized cDNA were stored at -20 ºC.

Pairs of primers for each gene were designed and checked for target identity using the National Centre for Biotechnology Information (NCBI). Quantative real-time polymerase chain reaction was performed in duplicate reactions including nuclease free water, the forward and reverse primers of each gene, cDNA and SYBR green as a detector on Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada). Data were generated

using $\Delta\Delta C_t$ method by normalizing the expression of a target gene to a housekeeping gene, Glyceraldehyde 3- Phosphate Dehydrogenase (GAPDH) and 18s ribosomal RNA and the values were reported as a fold change of the expression of the target genes in the experimental groups compared to the control group (Regassa and Kim, 2013). Pairs of primers used for qRT-PCR assay and their sequences are presented in table 3.

Table 13. NaPi cotransporters primer sequence in pigs

Gene[1]	Forward primer sequence	Reverse primer sequence	Annealing temperature (^{0}C)
SLC34A1	AGGAGGAACAGAAGCCAGGT	GGCACCTTGAGGAGCATTGA	60
SLC34A2	AACCTCCATCACCAACACCC	AAGAGCACCAACACGGAGAG	58
SLC34A3	GTACCACAACAGGATGCCGA	AGAGGACCCTGAACCACTGA	61
GAPDH	GGTGAAGGTCGGAGTGAACG	GGGATCTCGCTCCTGGAAGA	58

[1]SLC34A1= solute carrier family member 1 (type IIa sodium/phosphate cotransporter), SLC34A2 = solute carrier family member 2 (type IIb sodium/phosphate cotransporter), SLC34A3= solute carrier family member 3 (type IIc sodium/phosphate cotransporter), GAPDH= Glyceraldehyde 3-phosphate dehydrogenase.

4.3.7 Statistical Analysis

Data were subjected to one-way ANOVA as a completely randomized design using the Proc GLM procedure of statistical Analysis Systems (SAS) Institute version 9.2. Differences between selected means were tested using the PDIFF statement. The data are presented as least-square means ±SEM. Significant difference were accepted if $P < 0.05$ and trends were considered between $P \geq 0.05$ and $P < 0.10$.

4.4 Results

All pigs were found to be visibly healthy and readily consumed their daily feed allowance throughout the experimental period. The analyzed content of Ca, P and CP of the diets are listed in table 13. All values were within the acceptable ranges and are in agreement with the calculated composition of the diet. The analyzed values for the dietary P were closer to the calculated values for all diets (Table 3).

4.4.1 Growth performance

The ADFI was not different statistically (Table 11) among treatments during all the wks. The ADG and gain to feed ratio were lower ($P < 0.05$) in T2, T3 and T4 compared to T1 during the first wk. During second and third wk, ADG and gain to feed ratio were not different among treatments.

4.4.2 Bone Quality

The result of the bone mineralization parameters of the third and fourth metacarpal bones obtained from the DXA showed quiet interesting results. For both third and fourth metacarpal bones, the BMD was lower ($P < 0.05$) in T3 and T4 compared to T1 whereas pigs in T1 and T2 were found to have similar BMD. The BMC was not different among T1, T2 and T3 but pigs in T4 were found to have lower ($P < 0.05$) BMC compared to T1. The bone area was similar among all treatments.

Table 14. Effects of dietary treatments on growth performance of growing pigs

Item	Treatment				SEM[1]	*P*
	T1	T2	T3	T4		
wk 1						
ADFI(g)	1,675	1,584	1,540	1,646	72.50	0.571
ADG(g)	1021[a]	827[b]	771[b]	805[b]	63.48	< 0.05
Gain:feed	0.6[a]	0.5[b]	0.5[b]	0.5[b]	0.03	< 0.05
wk 2						
ADFI(g)	2,012	1,784	1,548	1,869	115.97	0.075
ADG(g)	835	798	786	794	51.20	0.890
Gain:feed	0.56	0.46	0.51	0.49	0.04	0.224
wk 3						
ADFI(g)	1,892	2,057	2,190	1,990	140.45	0.521
ADG(g)	1033	957	1033	996	38.81	0.464
Gain:feed	0.56	0.46	0.49	0.51	0.04	0.453
overall						
ADFI(g)	1,837	1,808	1,749	1,835	78.00	0.483
ADG(g)	946	876	878	919	35.14	0.445
Gain:feed	0.5	0.5	0.51	0.5	0.02	0.970

[1] Standard error of the mean. Values with different superscripts within a row are significantly different ($P < 0.05$)

Table 15. Effect of dietary treatments on third (MC3) metacarpal BMD, BMC and BA

Item	Treatment				SEM[1]	*P*
	T1(control)	T2	T3	T4		
BMD (g/cm^2)	0.27[a]	0.25[ab]	0.24[b]	0.21[c]	0.007	< 0.001
BMC (g)	2.77[a]	2.56[ab]	2.55[ab]	2.28[b]	0.105	0.029
Area (cm^2)	10.19	10.22	10.58	10.39	0.290	0.776

[1] Standard error of the mean. Values with different superscripts of the same row are significantly different ($P < 0.05$)

Table 16. Effect of dietary treatments on fourth (MC4) metacarpal BMD, BMC and BA

Item	Treatment				SEM[1]	*P*
	T1(control)	T2	T3	T4		
BMD (g/cm^2)	0.27[a]	0.25[ab]	0.24[bc]	0.22[c]	0.007	< 0.001
BMC (g)	2.6[a]	2.46[ab]	2.4[ab]	2.2[b]	0.105	0.092
Area (cm^2)	9.45	9.62	9.97	9.8	0.277	0.588

[1] Standard error of the mean. Values with different superscripts of the same row are significantly different ($P < 0.05$)

4.4.3 Phosphorus utilization and NaPi transporter gene expression

The digestibility of P was found to be lower in the pigs fed lower aP diets during all the wk. During the first wk, P digestibility was lower in pigs with T2, T3 and T4 treatments compared to T1. During the second wk, the digestibility improved in pigs with T2, which was similar with T1 showing the effect of increasing P level in the diet. In the third wk, digestibility of P in pigs with T1, T2 and T3 treatments were similar suggesting the improvement in digestibility with increased level of available phosphorus. Dietary treatments were found to have no significant effects on urinary P excretion although T3 and T4 had numerically lower value than T1 and T2 treatments. Dietary treatments were found to have no significant effect on sodium dependent phosphate cotransporter gene in jejunum and kidney.

Table 17. Effect of dietary treatments on P utilization in growing pigs

Item		Treatment				SEM[1]	*P*
		T1(control)	T2	T3	T4		
P intake (g/day)	wk1	8.98^{a}	6.59^{b}	6.64^{b}	6.86^{b}	0.37	< 0.001
	wk2	11.8^{a}	9.97^{b}	6.35^{c}	7.76^{c}	0.50	< 0.001
	wk3	12.24^{a}	11.5^{a}	10.57^{a}	8.26^{b}	0.73	< 0.05
P excretion (% of diet)	wk1	0.32	0.28	0.27	0.29	0.01	0.120
	wk2	0.32	0.31	0.29	0.28	0.01	0.265
	wk3	0.35	0.36	0.35	0.34	0.01	0.751
P digestibility (%)	wk1	41.97^{a}	31.52^{b}	32.62^{b}	29.12^{b}	3.10	< 0.05
	wk2	41.33^{a}	43^{a}	24.88^{b}	30.35^{b}	3.18	0.001
	wk3	36.2^{a}	34.76^{a}	33.73^{a}	16.85^{b}	2.58	< 0.001

[1] Standard error of the mean.

Values with different superscripts within a same row are significantly different ($P < 0.05$)

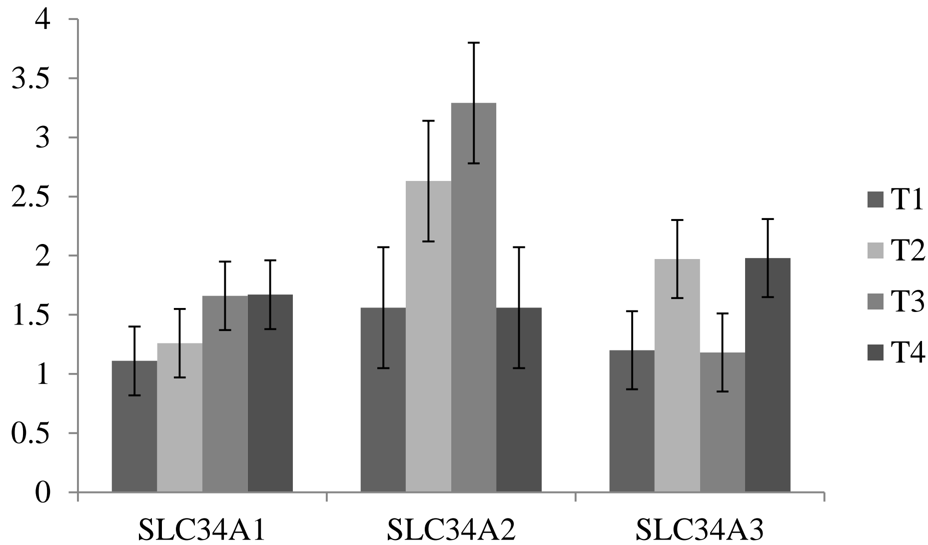

Fig 4: NaPi-IIa (SLC 34A1, kidney), NaPi-IIb (SLC34A2, jejunum) and NaPi-IIc (SLC34A3, kidney) gene expression

4.5 DISCUSSION

During the first wk, the pigs that were provided with lower aP diets had reduced ADG and gain to feed ratio. The result was in agreement with the findings (Eeckhout et al., 1995; Ryan et al., 2010). Eeckhout et al. (1995) found negative impact on daily gain and feed efficiency of growing pigs fed 0.11% aP diets compared to pigs fed 0.14% and 0.17% aP diets. Hall et al. (1991) reported slower growth rate and lower feed efficiency in the pigs fed deficient P (0.3% tP) compared to pigs fed adequate P diets. The lack of significant effects on performance of pigs among the treatments during second and third wk may be either due to the compensatory effect of high P diet or acclimatization of pigs to the diet up to the threshold level.

The data of bone parameters obtained from DEXA showed interesting results regarding the compensatory effect of higher level of P after supplementation of lower level of P than required

for 1 week. Pigs with T1 and T2 displayed similar BMD and BMC suggesting that the negative effect of providing lower level (0.11% aP) for one wk was compensated by providing the required level of aP (0.23%) for the next two wks. Pigs with T3 had lower BMD compared to T1 suggesting that one wk of compensatory period (providing required level of aP) was not enough to combat the negative effect on bone density produced by lower level of aP (0.11%) for two wk. The reduced BMD and BMC in the pigs with T4 was in agreement with previous studies done by Eeckhout et al. (1995), Ketaren et al. (1993) and Ryan et al. (2010). The metacarpal bone has been the subject of research for the bone mineral studies carried out by several authors (Shaw et al., 2006; Varley et al., 2011, Ryan et al., 2011).

The Phosphorus digestibility was found to be reduced in the pigs fed low aP diets. The digestibility improved with the increase in level of aP in the diet. The reduced digestibility is due to the fact that the relative ratio of phytate P was increased in the pigs fed low aP diets.

Based on the study, it was found that pigs fed a low aP diet (0.11% aP) had lowest growth performance and bone mineralization. Phosphorus was found to have a significant effect on the rate of mineral deposition in grower pigs. However, the compensatory effect was seen when the growing pigs fed low P diets earlier were fed abundant P diet. Pigs which were provided a low P diet for one wk and an adequate P diet for next two wks were found to perform as well as the pigs provided the adequate P diet for all 3 wks in terms of both growth performance and bone quality at the end of trial. This suggests that short term phase feeding should be explored in greater detail in the future to gain maximum performance from pigs as well to make the pig industry sustainable.

5. 0 GENERAL DISCUSSION

Three aspects are mainly important regarding the sustainability of swine industry: 1) maximum performance, 2) high profit margin and 3) low impact on the environment. Accurate estimation of the dietary P requirement of pigs is very critical to all three aspects. Optimizing the utilization of P in growing pigs requires a better understanding of P metabolism in the body and how the body responds to the alteration in dietary level of P. This understanding is required to match the pig's body requirement with dietary P as well as to minimize the negative impacts from the swine industry. These experiments were designed to address some of these issues to improve their understanding so that the contribution can be done to make swine industry environmentally safe and economically viable in the future.

Although several studies have been done in the past to better understand the effects of different dietary levels of P on the performance of pigs, more detailed information is still lacking on aspects of P utilization and P metabolism. The main objective of this thesis was to better understand the effects of different P levels on both performance and P utilization of growing pigs. In the first experiment, growing pigs were fed low P diets to evaluate the effects on production parameters, skeletal parameters and P utilization so that a level of P could be determined which improves P utilization in the body without compromising production performance and skeletal integrity. In the second experiment, pigs were fed low P diet in different phases (short term phase feeding) to evaluate the compensatory effect of feeding high P diet on production parameters, bone quality and P utilization of growing pigs after feeding low-P diet for a short duration.

In the initial experiment, growing pigs were fed three different levels of available P, one being 0.23% aP as recommended by NRC (1998) and other two being 0.17% and 0.11% aP. As the level of P in diet declined, growing pigs displayed reduced daily gain and gain to feed ratio. In the later experiment also, the reduction in aP has resulted in reduced daily gain and gain to feed ratio during the first wk. Some of the previous studies (Eeckhout et al., 1995; Hall et al., 1991) also found reduced growth performance in the pigs fed low level of P in diet compared to higher level of P. The low-P diet resulted in reduced bone mineral density and mineral content in the pigs. In the second experiment, the pigs with the lowest level of P had the lowest bone mineral density and bone mineral content but the pigs whose low-P diet were replaced after one wk with normal P diet displayed similar bone parameters with those fed normal P diet throughout the experiment. This suggests that providing high P in the diet can compensate for a reduction in dietary P for a period of one wk time. In contrast, Ryan et al. (2011) replaced low-P diet with high-P diet after 28 or 35 days and observed significantly low bone density even after replacing low-P diet with high-P diet. This suggests that in phase feeding, the low P diet should be changed after a relatively short period of time so that the negative effects of low nutrient diet would be compensated by high nutrient diet.

The digestibility of P was found to be significantly low in the pigs fed low aP diet which is attributed to the presence of high amount of phytate phosphorus in the low aP diets. The digestibility was improved with increase of aP in the diet. In the initial experiment, it was found that with the reduction of P in the diet, the urinary P output was reduced which was comparable to the findings of Varley et al. (2010) who also found higher urinary output of P in pigs fed high-P diets. This is valuable in understanding the regulation of P status in the body especially during the

situations where diet is deficient in P. During such conditions, the kidney plays a vital role in reducing the urinary P output ultimately trying to save the amount of P in the blood.

The effect of alteration in dietary P level on NaPi-mRNA gene expression was found to be significant during the first one or two wk; however as the wk progressed the effect was non-significant. The alteration in expression of NaPi gene expression is dependent on the length and severity of P deprivation (Saddoris et al., 2010).

6. CONCLUSION

- 0.11% aP level in the diet has negative impact on the growth performance and skeletal integrity of growing pigs.
- Reduction in dietary aP causes up regulation of NaPi cotransporters in jejunum during the earlier stage of P deprivation whereas during the later stage, NaPi cotransporters are up regulated in the kidney as a result of P deprivation.
- Reduction of dietary phosphorus diet is helpful in reducing P excretion, which should be taken into consideration with the performance of pigs.
- Replacing low-P diet with high-P diet after short duration (a wk) may be helpful in compensating the negative effects of low-P diet in growing pigs.

7.0 FUTURE DIRECTIONS

- Evaluate the protein expression of NaPi cotransporters in the pigs fed low phosphorus diets
- Use phytase in the pigs fed low available phosphorus diet to better understand the utilizations of non-digested phytate phosphorus
- Conduct the trials to evaluate the effect of multiple short term phase feeding of phosphorus on growth performance, bone quality and P utilization
- Evaluate the effect of different level of vitamin D and Ca in the pigs fed low P diets
- Use of DEXA to determine bone quality of whole body rather than post-mortem metacarpal assessment
- Conduct similar trials for longer duration and in different age groups to understand issue in better way.

8. REFERENCES

Adeola, O. 2001. Digestion and balance techniques in pigs Swine Nutrition. CRC Press LLC, Boca Raton, FL.

Agudelo-Trujillo, J., H. 2005. An examination of dietary amendments to affect phosphorus utilization in growing pigs. Ph. D. thesis, University of Kentucky, Lexington, USA.

Agudelo, J. H., M. D. Lindemann, and G. L. Cromwell. 2010. A comparison of two methods to assess nutrient digestibility in pigs. Livest. Sci. 133: 74-77.

Anderson, J. J. B. 1991. Nutritional biochemistry of calcium and phosphorus. J. Nutr. Biochem. 2: 300-307.

AOAC International. 1990. Official Methods of Analysis. 15th ed. AOAC Int. Washington, DC.

Baker, S. R. 2010. Aspects of phosphorus nutrition in swine. M S. Thesis, University of Illinois at Urbana-Champaign, Urbana, Illinois.

Bauman, B. V. 2004. Microbial metabolism. p 125-127 in Microbiology. Pearson Custom Publishing, Boston, MA.

Baxter, C. A., B. C. Joern, D. Ragland, J. S. Sands, and O. Adeola. 2003. Phytase, High-Available-Phosphorus Corn, and Storage Effects on Phosphorus Levels in Pig Excreta. J. Environ. Qual. 32: 1481-1489.

Beck, L., A. C. Karaplis, N. Amizuka, A. S. Hewson, H. Ozawa, and H. S. Tenehouse. 1998. Targeted inactivation of Npt2 in mice leads to sever renal phosphate wasting, hypercalciuria, and skeletal abnormalities. Proc. Natl. Acad. Sci. USA. 95: 5372-5377.

Berndt, T., and R. Kumar. 2009. Novel Mechanisms in the Regulation of Phosphorus Homeostasis. Physiol. 24: 17-25.

Breves, G., and B. Schroder. 1991. Comparative aspects of gastrointestinal phosphorus metabolism. Nutr. Res. Rev. 4:125-140.

Bronner F. 1998. Calcium absorption–a paradigm for mineral absorption. J. Nutr. **128**: 917-920.

Brown, A. J., A. Dusso, and E. Slatopolsky. 1999. Vitamin D. Am. J. Physiol. 289: 8-28.

Capuano, P., T. Radanovic, C. A. Wagner, D. Bacic, S. Kto, Y. Uchiyama, R. St.- Arnoud, H. Murer, and J. Biber. 2005. Intestinal and renal adaptation to a low-Pi diet of type II NaPi cotransporters in vitamin D receptor- and 1αOHase-deficient mice. Am. J. Physiol. 288: 429-434.

CCAC, 2009. CCAC guidelines on: the care and use of farm animals in research, teaching and testing. Ottawa, ON, Canada.

Chen, T. C., L. Castillo, M. Korycka-Dahl, and H. F. DeLuca. 1974. Role of vitamin D metabolites in phosphate transport of rat intestine. J. Nutr. 104: 1056-1060.

Chong, S. S., C. A. Kozak, L. Liu, K. Kristjansson, S. T. Dunn, J. E. Bourdeau, and M. R. Hughes. 1995. Cloning, genetic mapping and expression analysis of a mouse renal sodium-dependent phosphate cotransporter. Am. J. Physiol. 268: 1038-1045.

Chong, S. S., K. Kristjansson, H. Y. Zoghbi, and M. R. Hughes. 1993. Molecular cloning of the cDNA encoding a human renal sodium phosphate transport protein and its assignment to chromosome 6p21.3-p23. Genomics. 18: 355-359.

Correll, D. L. 1998. The role of phosphorus in the eutrophication of receiving waters: A review. J. Env. Qual. 27: 261-266.

Correll, D. L. 1999. Phosphorus: a rate limiting nutrient in surface waters. Poult. Sci. 78:674-682.

Crenshaw, T., 2001. Calcium, phosphorus, vitamin D and vitamin K in swine nutrition. In: Lewis, A.J., Southern, L.L. (Eds.), swine nutrition. CRC Press, London, p 187-212.

Cromwell, G. L. 1980. Biological availability of phosphorus for pigs. Feedstuffs. 52: 38-42.

Cromwell, G. L. 1992. The biological availability of phosphorus in feedstuffs for pigs. Pig News Info. 13: 75-78.

Cromwell, G.L., T. R. Cline, J. D. Crenshaw, T. D. Crenshaw, R. C. Ewan, C. R. Hamilton, A. J. Lewis, D. C. Mahan, E. R. Miller, J. E. Pettigrew, L. F. Tribble, and T. L. Veum. 1993. The dietary protein and (or) lysine requirements of barrows and gilts. J. Anim. Sci. 71:1510-1519.

Danisi, G., and R. W. Straub. 1980. Unidirectional influx of phosphate across the mucosal membrane of the rabbit small intestine. Pflugers Arch. 385: 117-122.

DeLuca, H. F. 2004. Overview of general physiologic features and functions of vitamin D1–4. Am. J. Clin. Nutr. 80: 1689-1696.

Eeckhout W., M. de Paepe, N. Warnants, and H. Bekaert. 1995. An estimation of the minimal P requirements for growing-finishing pigs, as influenced by the Ca level of the diet. Anim. Feed Sci. Technol. 52: 29-40.

Ekpe, E.D., R. T. Zijlstra, and J. D. Patience. 2002. Digestible phosphorus requirement of grower pigs. Can. J. Anim. Sci. 82: 541-549.

Eto, N., M. Tomita, and M. Hayashi. 2006. NaPi-mediated transcellular permeation in the dominant route in intestinal inorganic phosphate absorption in rats. Drug Metab. Pharmacokinet. 21: 217-221.

Fan, M. Z., T. Archbold, W. C. Sauer, D. Lackeyram, T. Rideout, Y. Gao, C. F. M. de lange, and R. R. Hacker. 2001. Novel methodology allows simultaneous measurement of true phosphorus digestibility and the gastrointestinal endogenous phosphorus outputs in studies with pigs. J. Nutr. 131: 2388-2396.

FASS, 2001. Dietary adjustments to minimize nutrient excretion from livestock and poultry. http://www.fass.org/facts/livestockpoultry.htm. On 10/11/2014.

Fukumoto, S. 2014. Phosphate metabolism and vitamin D. http://www.nature.com/bonekeyreports/2014/140205/bonekey2013231/full/bonekey2013231.html. On 11/5/ 2014.

Godoy, S., C. Chicco, F. Meschy, and F. Requena. 2005. Phytic phosphorus and phytase activity of animal Feed Ingredients. Interciencia 30: 24-28.

Gwendolyn, J. 2010. Enhancing sow longevity – A nutritional approach. http://www.pigprogress.net/Home/General/2010/12/Enhancing-sow-longevity--A-nutritional-approach-PP006922W/. On 10/4/2014.

Hall, D., G. Cromwell, and T. Stahly. 1991. Effects of dietary calcium phosphorus, calcium phosphorus ratio and vitamin K on performance, bone strength and blood. J. Anim. Sci. 69: 646-655.

Han, I. K., J. H. Lee, J. H. Kim, Y. G. Kim, J. D. Kim, and I. K. Paik. 2000. Application of phase feeding in swine production. J. Appl. Anim. Res. 17: 27-56.

Han, I. K., J. H. Lee, X. S. Piao, and D. Li. 2001. Feeding and management system to reduce environmental pollution in swine production. Asian-Aust. J. Anim. Sci. 14: 432-444.

Hattenhauer, O., M. Traebert, H. Murer, and J. Biber. 1999. Regulation of small intestinal Na-Pi IIb cotransporter by dietary phosphate intake. Am. J. Physiol. 277: 756-762.

Hill, B. E., A. L. Sutton, and B. T. Richert. 2009. Effects of low-phytic acid corn, low-phytic acid soybean meal, and phytase on nutrient digestibility and excretion in growing pigs. J. Anim. Sci. 87: 1518-1527.

Hoag, H. M., J. Martel, C. Gauthier, and H. S. Tenehouse. 1999. Effects of Npt2 gene ablation and low-phosphate diet on renal Na/phosphate cotransporter gene expression. J. Clin. Invest. 104: 679-686.

Huag, W., and H. J. Lantzsch. 1983. Sensitive method for the rapid determination of phytate in cereals and cereal products. J. Sci. Food Agr. 34: 1423-1426.

Jagger, S., Wiseman, J., Cole, D.J.A. and Craigon, J. 1992. Evaluation of inert markers for the determination of ileal and fecal apparent digestibility values in the pig. Br. J. Nutr. 68:729

Jones, G., S. A. Strugnell, and H. F. DeLuca. 1999. Current understanding of the molecular actions of vitamin D. Physiol. Rev. 78: 1194-1231.

Jongbloed, A. W. 1987. Phosphorus in the feeding of pigs: effect of diet on the absorption and retention of phosphorus by growing pigs. Ph. D. Diss., Wageningen Agricultural Univ., Wageningen, The Netherlands.

Jongbloed, A.W., and N. Lenis. 1992. Alteration of nutrition as a means to reduce environmental pollution by pigs. Lives. Prod. Sci. 31: 76-95.

Jongbloed, A. W., N. P. Lenis, and Z. Mroz. 1997. Impact of nutrition on reduction of environmental pollution by pigs: An overview of recent research. Vet. Q. 19: 130-134.

Jongbloed, A. W., H. Everts, and P. A. Kemme. 1991. Phosphorus availability and requirements in pigs. Recent Advances in Animal Nutrition. p 65-80, E. R. Heinemann Butterworth, London, UK.

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. 70:1159-1168.

Kanakov, D., P. Petkov, and K. Stojanchev. 2004. Changes of bone parameters of growing pigs to different phosphorus levels in the diet. Trakia J. Sci. 2:58-63.

Katai, K., H. Segawa, H. Haga, K. Morita, H. Arai, S. Tatsumi, Y. Taketani, K. Miyamoto, S. Hisano, Y. Fukui, and E. Takeda. 1997. Acute regulation by dietary phosphate of the sodium-dependant phosphate transporter (NaPi-2) in rat kidney. J. Biochem. 121: 50-55.

Kennelly, J. J., Aherne, F.X. and Apps, M.J. 1980. Dysprosium as an inert marker for swine digestibility studies. Can. J. Anim. Sci. 60:441.

Ketaren, P. P., E. S. Batterham, E. White, D. J. Farrell, and B. K. Milthorpe. 1993. Phosphorus studies in pigs. Br. J. Nutr. 70: 249-268.

Kim, W. K., S. A. Bollmfield, T. Sugiyama, and S. C. Ricke. 2012. Concepts and methods for understanding bone metabolism in laying hens. World's Poult. Sci. 68: 71-82.

Kornegay, E. T. and Harper, A.F. 1997. Environmental nutrition: nutrient management strategies to reduce nutrient excretion of swine. Prof. Anim. Sci. 13: 99-111.

Knowlton, K. F., J. S. Radcliffe, C. L. Novak, and D. A. Emmerson. 2004. Animal management to reduce phosphorus losses to the environment. J. Anim. Sci. 82: 173-195.

Koch, M. E., and D. C. Mahan. 1985. Biological characteristics for assessing low phosphorus intake in growing swine. J. Anim. Sci. 60: 699-708.

Lee, D. B., M. W. Walling, and D. B. Corry. 1986. Phosphorus transport across rat jejunum: Influence of sodium, pH, and 1,25-$(OH)_2D_3$. Am. J. Physiol. 250: 369-373.

Lee, D. B., W. Walling, and N. Barautbar. 1986. Intestinal phosphate absorption: influence of vitamin D and non-vitamin D factors. Am. J. Physiol. 250: 369-373.

Lee, J. H., Kim, J.D., Kim, J.H., Jin, J. and Han, In K. 2000. Effect of phase feeding on the growth performance, nutrient utilization and carcass characteristics in finishing pig. Asian-Aus. J. Anim. Sci. 13: 1137-1146.

Levi, M., M. Lotscher, V. Sorribas, M. Custer, M. Arar, B. Kaissling, H. Murer, and J. Biber. 1994. Cellular mechanisms of acute and chronic adaption of rat renal Pi transporter to alterations in dietary Pi. Am. J. Physiol. 267: 900-908.

Li, H., and Z. Xie. 1995. Molecular cloning of two rat Na/Pi cotransporters: evidence for differential tissue expression of transcripts. Cell Mol. Biol. Res. 41: 451-460.

Liu, J., D. W. Bollinger, D. R. Ledoux, and T. L. Veum. 1998. Lowering the dietary calcium to total phosphorus ratio increases phosphorus utilization in low-phosphorus corn-soybean meal diets supplemented with microbial phytase for growing-finishing pigs. J. Anim. Sci. 76: 808-813.

Liu, J., D. W. Bollinger, D. R. Ledoux, and T. L. Venum. 2000. Effects of dietary calcium:phosphorus ratios on apparent absorption of calcium and phosphorus in the small intestine, cecum, and colon of pigs. J. Anim. Sci. 78: 106-109.

Lomer, M. C. E., R. P. H. Thompson, J. Commisso, C. L. Keen, and J. J. Powell. 2000. Determination of titanium dioxide in foods using inductively coupled plasma optical emission spectrometry. Analyst. 125: 2339-2343.

Metzler, B. U., and R. Mosenthin. 2008. A review of interactions between dietary fiber and the gastrointestinal microbiota and their consequences on intestinal phosphorus metabolism in growing pigs. Asian-Aust. J. Anim. Sci. 4: 603-615.

McDowell, L.R. 2003. Minerals in animal and human nutrition, Second Ed. Elsevier science. The Netherlands, p 33-146.

Mitchell, A. D., J. M. Conway, and A. M. Scholz. 1996. Incremental changes in total and regional body composition of growing pigs measured by dual-energy x-ray absorptiometry. Growth Dev. Aging. 60: 95-105.

Miyamoto, K., S. Haito-Sugino, S. Kuwahara, A. Ohi, K. Nomura, M. Ito, M. Kuwahata, S. Kido, S. Tatsumi, I. Kaneko, and H. Segawa. 2011. Sodium-dependent phosphate cotransporters: lessons from gene knockout and mutation studies. J Pharm. Sci. 100: 3719–3730.

Mroz, Z., A. W. Jongbloed, and P. A. Kemme. 1994. Apparent digestibility and retention of nutrients bound to phytate complexes as influenced by microbial phytase and feeding regimen in pigs. J. Anim. Sci. 72: 126-132.

Murer, H., N. Hernando, I. Forster, and J. Biber. 2000. Proximal tubular phosphate reabsorption: molecular mechanisms. Physiol. Rev. 80: 1373–1409.

NRC. 1998. Nutrient Requirements of Swine. 10th rev. ed. Natl. Acad. Press, Washington, DC.

NRC. 2012. Nutrient Requirements of Swine. 11th rev. ed. Natl. Acad. Press, Washington, DC.

Ohkido, I.,H. Segawa, R. Yanagida, M. Nakamura, and K. Miyamoto. 2003. Cloning, gene structure and dietary regulation of the type IIc Na/Pi cotransporter in the mouse kidney. Pflugers Arch. Eur. J. Physiol. 446: 106-115.

Omogbenigum, F. O., C. M. Nyachoti, and B. A. Slominski. 2003. The effect of supplementing microbial phytase and organic acids to a corn-soybean based diet fed to early-weaned pigs. J. Anim. Sci. 81:1806-1813.

Omogbenigum, F. O., C. M. Nyachoti, and B. A. Slominski. 2004. Dietary supplementation with multienzyme preparations improves nutrient utilization and growth performance in weaned pigs. J. Anim. Sci. 82: 1053-1061.

Peo, E. R., Jr. 1976. NFIA literature review on calcium in swine nutrition, National Feed Ingredients Association, West Des Moines, IA. In Crenshaw, T., 2001. Calcium, phosphorus, vitamin D and vitamin K in swine nutrition. In: Lewis, A.J., Southern, L.L. (Eds.), swine nutrition. CRC Press, London, p 187-212.

Petersen, G. I., and H. H. Stein. 2006. Novel procedure for estimating endogenous losses and measurement of apparent and true digestibility of phosphorus by growing pigs. J. Anim. Sci. 84: 2126-2132.

Piva, A., G. Casadei, and G. Biagi. 2002. An organic acid blend can modulate swine intestinal fermentation and reduce microbial proteolysis. Can. J. Anim. Sci. 82: 527–532.

Pointillart, A., A. Fourdin, and N. Fontaine. 1987. Importance of cereal phytase activity for phytate phosphorus utilization by growing pigs fed diets containing triticale or corn. J. Nutr. 117: 907-913.

Pozza, P.C., Gomes, P.C., Pozza, M.S.S., Nunes, R.V., Ferreira, R. A. and Rodrigueiro, J.B. 2013. Use of markers to determine the ileal digestibility of amino acids for swine. *Ciênc. agrotec.* 37: 259-265.

Prince, T.J., Sutton, A.L., von Bernuth, R.D. and Verstegen, M.W.A. 2000. Application of nutritional knowledge for developing econutrition feeding programs on commercial swine farms. J. Anim. Sci. 77:1-10.

Qian, H., E. T. Kornegay, and D. E. Conner Jr. 1996. Adverse effects of wide calcium:phosphorus ratios on supplemental phytase efficacy for weanling pigs fed two dietary phosphorus levels. J. Anim. Sci. 74: 1288-1297.

Raboy, V. 1997. Accumulation and Storage of Phosphate and Minerals. In: B. Larkins and I. Vasil (eds.) Cellular and Molecular Biology of Plant Seed Development. Advances in Cellular and Molecular Biology of Plants No. 4. p 441-477. Springer Netherlands.

Regassa, A., and W. K. Kim. 2013. Effects of oleic acid and chicken serum on the expression of adipogenic transcription factors and adipogenic differentiation in hen preadipocytes. Cell Bio. Int. 37: 961-971.

Renkema, K.Y., R. T. Alexander, R. J. Bindels, and J. G. Hoenderop. 2008. Calcium and phosphate homeostasis: Concerted interplay of new regulators. Annl. Med. 40:82-91.

Richert, B. T. 2010. Macro minerals for swine diets. National Swine Nutrition Guide. D. J. Meisinger, ed. U.S. Pork Center of Excellence, Ames, IA, p 52-56.

Ryan, W. F., P. B. Lynch, and J. V. O. Doherty. 2011. Effect of dietary phosphorus on the development of bone mineral density of pigs assessed using dual energy X-ray absorptiometry. Livest. Sci. 137: 101–107.

Saddoris, K. L. 2007. Dietary factors affecting sodium-dependent phosphate uptake and gene expression of the NaPi-IIb in the small intestine of weanling pigs. Ph. D. thesis, Purdue University, Indiana, USA.

Saddoris, K. L., J. C. Fleet, and J. S. Radcliffe. 2010. Sodium-dependent phosphate uptake in the jejunum is post-transcriptionally regulated in pigs fed a low-phosphorus diet and is independent of dietary calcium concentration. J. Nutr. 140: 731-736.

Segawa, H., A. Onitsuka, M. Kuwahata, E. Hanabusa, J. Furutani, I. Kaneko, Y. Tomoe, F. Aranami, N. Matsumoto, M. Ito, M. Matsumoto, M. Li, N. Amizuka, and K. Miyamoto. 2009. Type IIc sodium-dependent phosphate transporter regulates calcium metabolism. J Am. Soc. Nephrol. 20: 104-113.

Segawa, H., I. Kaneki, A. Takahashi, M. Kuwahata, M. Ito, I. Ohkido, S. Tatsumi, and K. Miyamoto. 2002. Growth-related renal type II Na/Pi cotransporter. J. Biol. Chem. 277:19665-19672.

Shaw, D.T., D. W. Rozeboom, G. M. Hill, M. W. Orth, D. S. Rosenstein, and J. E. Link, 2006. Impact of supplement withdrawal and wheat middling inclusion on bone metabolism, bone strength, and the incidence of bone fractures occurring at slaughter in pigs. J. Anim. Sci. 84: 1138–1146.

Stein, H. H. 2002. Experience of feeding pigs without antibiotics: a European perspective. Anim. Biotechnol. 13: 85–95.

Stein, H. H. 2011. Standardized total tract digestibility (STTD) of phosphorus. In: Proceedings of Midwest Swine Nutrition Conference, Des Moines, IA, USA. p 47-52.

Sutton, A. L. 2008. Auditing and assessing nutrient management for water quality on animal feeding operations. Prof. Anim. Sci. 24: 107-113.

Sutton, A. L., and B. T. Richert. 2004. Nutrition and feed management strategies to reduce nutrient excretions and odors from swine manure. Water Sci. Technol. 49:397-404.

Sutton, A.L., S. Radcliffe, and B. C. Joern. 2004. Overview of phosphorus issues in swine feeding. http://livestocktrail.illinois.edu/sowm/paperDisplay.cfm?ContentID=6509. On 9/4/2014.

Tanck, E., J. Homming, G. Van Lenthe, and R. Huiskies. 2001. Increase in bone volume fraction precedes architectural adaptation in growing bone. Bone. 28: 650-654.

Taylor, J. G., and D. A. Bushinsky. 2009. Calcium and phosphorus homeostasis. Blood purification. 27: 387-394.

Traylor, S. L., G. L. Cromwell, and M. D. Lindemann. 2005. Bioavailability of phosphorus in meat and bone meal for swine. J. Anim. Sci. 83: 1054-1061.

Varley, P. F., J. J. Callan, and J. V. O'Doherty. 2010. Effect of phosphorus level and phytase inclusion on the performance, bone mineral concentration, apparent nutrient digestibility, and on mineral and nitrogen utilization in finisher pigs. Irish J. Agric. Food. Res. 49: 141-152.

Varley, P. F., J. J. Callan, and J. V. O'Doherty. 2011. Effect of dietary phosphorus and calcium level and phytase addition on performance, bone parameters, apparent nutrient digestibility, mineral and nitrogen utilization of weaner pigs and the subsequent effect on finisher pig bone parameters. Anim. Feed Sci. Technol. 165: 201-209.

Weremko, D., H. Fandrejewski, T. Zebrowska, I. K. Han, J. H. Kim, and W. T. Cho. 1997. Bioavailability of phosphorus in feeds of plant origin for pigs. Asian J. Anim. Sci. 10:551-566.

Wu, X., Z. Ruan, Y. G. Zhang, Y. Q. Hou, Y. L. Yin, T. J. Li, R. L. Huang, X. F. Kong, W. Y. Chu, L. X. Chen, Y. Q. Hou, B. Gao, and L. X. Chen. 2008. True digestibility of phosphorus in different resources of phosphorus ingredients in growing pigs. Asian-Aust. J. Anim. Sci. 21: 107-119.