Assessment of *in vivo* calcium and phosphorus digestibility in commercial laying hens fed limestone with different particle sizes

by

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Declaration

I, Micaela Sinclair-Black hereby declare that this thesis, submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University.

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Abstract

Ascertaining calcium (Ca) and phosphorus (P) digestibility values remain a challenge in the determination of nutrient requirements of laying hens. In recent years, limited research has been conducted in this area, and there is scope to update literature to reflect the requirements of the modern hen. The present study investigated the effects of time post-oviposition (POP), limestone particle size (PS) and phytase inclusion on Ca and P digestibility, blood ionized calcium (iCa), and eggshell percentage (PE) in commercial laying hens. The treatments were comprised of two levels of phytase (0, 600 phytase units (FTU)) and two particle sizes of limestone (0.25 mm and 1.5 mm) to yield a 2x2 factorial experimental design. Digestibility values were determined at 3h and 11h POP and blood iCa values were determined at (0, 3, 6, 9, 12, 15, 18, 21) hours POP. Ca digestibility was significantly affected by time POP (P<0.01), with a higher Ca digestibility being noted at 11 hours POP during eggshell calcification than at 3 hours POP, during the inactive phase of the shell gland. There was no significant effect of limestone PS and phytase on Ca digestibility (P >0.5). However, there was a two-way interaction between the limestone PS and phytase (P<0.05). Blood iCa was also significantly affected by the time POP. Blood iCa remained high during the shell gland inactivity period and decreased rapidly during the period of eggshell calcification (P<0.01). Blood iCa was also affected by a two-way interaction between limestone PS and phytase. A significant effect of limestone PS on PE was noted, with limestone grit providing an improved PE (P<0.01). P digestibility was significantly improved by the addition of 600 FTU phytase (P<0.001). The time POP affected P digestibility (P<0.01) with P digestibility being greater at 3h POP than 11h POP. The results from this trial illustrate the presence of diurnal circadian rhythm for Ca digestibility and P digestibility. The results from the blood iCa and PE demonstrate that limestone grit improves the Ca status of the hen and that the combination of fine limestone and the addition of phytase enzyme had a positive effect on Ca and P digestibility.

Contents

List of abbreviations

AEC	Animal ethics committee
AvP	available Phosphorus
bPTH	bovine parathyroid hormone
Ca	calcium
CO_2	carbon Dioxide
Co_3O_4	cobalt Oxide
CP	crude protein
CT	calcitonin
Cu	copper
DM	dry matter
ECPD	electrochemical potential difference
EE	ether extract
EF	erlenmeyer flask
FI	feed intake
FTU	phytase unit
g	grams
GIT	gastrointestinal tract
GLY	glycine
GMD	geometric mean diameter
h	hours
HCL	hydrochloric acid
$HCLO_4$	perchloric acid

HNO_3	nitric acid
iCa	ionized calcium
ICP-OES	inductively coupled plasma optical emission spectrometry
K	potassium
KOH	potassium hydroxide
L	litre
LOD	loss on drying
MDCP	mono-dicalcium phosphate
ml	millilitre
mmol/L	millimole per litre
N	normality (Molarity)
N_2	nitrogen
Na	sodium
nm	nano Meters
NPP	non-phytate phosphorus
O	oxygen
P	phosphorus
PC	partial collection
PE	percentage eggshell
pHC	pH change method
POP	post oviposition
PP	phytate phosphorus
PS	particle Size
PTH	parathyroid hormone

rP	retained phosphorus
rpm	revelations per minute
TC	total collection
TCa	total calcium
TCO_2	total carbon dioxide
TP	total Phosphorus
WLA	weight loss method
WLA (Auburn)	weight loss Auburn university method
WLM	weight loss method
Zn	zinc
µmol	micromol

List of tables

List of figures

CHAPTER 1 Introduction

1.1 Introduction

From an economic and environmental standpoint, commercial laying hens have had their production cycle extended during recent years (Bain *et al*., 2016). Genetic selection has resulted in current strains of laying hens having hen-day egg production figures of 80% at 70 weeks (Hyline international, 2014). This has led many producers to the decision, to keep their flock for an extended period. However, as hens age, they begin to develop physiological issues such as a weakened skeletal system. Aged hens are more prone to bone breakages and a lower absorptive efficiency of nutrients, which contributes to thinner eggshells (Horikawa *et al*., 1982; Bar & Hurwitz, 1988; Albatshan *et al*., 1994; Armbrecht *et al*., 1999). Therefore, an understanding of calcium (Ca) and phosphorus (P) supply to the hen is required in order to assist the industry in maintaining these extended production cycles with limited production-based issues.

The laying hen has differing Ca demands throughout the day, depending on her physiological state. From earlier literature, it can be noted that the hen can alter her digestive capacity depending on her current Ca demand at a given period (Hurwitz & Bar 1965; Hurwitz & Bar 1973). Factors such as the time post oviposition (POP), phytase inclusion, and limestone particle size will influence how readily the hen can digest Ca from the diet. During the photophase, the hen adds Ca to her medullary bone, and during the scotophase, the eggshell is calcified (Kerschnitzki *et al*.,2014). Each of these physiological processes alters the hen's blood ionized calcium (iCa) level. Physiologically, the blood iCa acts as an active form of Ca, required for eggshell calcification and medullary bone remodelling (Parsons & Combs, 1980). Blood iCa indicates the Ca status of the hen and, dietary Ca particle size, and phytase enzyme supplementation may have an influence on the profile of the blood iCa. Currently, there is a limited supply of research-based knowledge on limestone and phytase and how they are utilized by the laying hen for Ca and P digestion.

Natural P deposits are a non-renewable resource (Van Vuuren *et al*., 2009; Hein & Leemans, 2012), and the addition of inorganic P to poultry diets is expensive (Carlos & Edwards, 1998). Furthermore, the addition of excess P to laying hen diets has a negative impact on the environment (Vaccari, 2009). Laying hen diets contain plant-based P in the form of phytate molecules that have a low P digestibility due to the presence of limestone-based Ca in the diet. The low digestibility of phytate is a consequence of the Ca from dietary limestone binding to the phytate molecule, causing the molecule to chelate and become unavailable for breakdown by the hen (Humer *et al*., 2012; Jing *et al*., 2018). Modern science has developed enzymes that aid in the breakdown of the naturally occurring plant-based P to increase P digestibility in the hen. Phytase enzymes cleave the phytate P and release available P to the hen for digestion (Selle & Ravindran, 2007), thereby reducing the need for inorganic P supplementation and limit the negative impact of P supplementation on the environment. The efficacy of phytase is also influenced by the particle size of limestone being used in poultry diets (Kim *et al*., 2018).

This study intended to gain an understanding of how dietary factors and time POP will influence the Ca and P status of the hen through the assessment of Ca and P digestibility values coupled with a blood iCa profile. Improved knowledge of the Ca and P requirements, and the corresponding Ca status of the hen would allow a feeding regime that synchronizes the Ca and P requirements of the hen to the Ca being supplied by the diet.

1.2 Aim

The aim of this study was to assess the differences in calcium (Ca) and phosphorus (P) digestibility and blood ionized Ca (iCa) levels, at different stages of the egg development cycle in laying hens as affected by limestone particle size (PS), phytase and time post oviposition (POP).

1.3 Research goals

In order to achieve the aim, the following research objectives were set.

- To determine the calcium (Ca) digestibility as affected by limestone particle size (PS), phytase, and time post oviposition (POP).
- To determine P digestibility as affected by limestone PS, phytase, and time POP
- To monitor changes in the blood ionized Ca (iCa) over 24 hours.
- To investigate the effects of limestone PS and phytase on percent eggshell (PE).

1.4 Hypothesis

H_0 = Limestone particle size (PS), phytase, and time post oviposition (POP) will not have an influence on calcium (Ca) digestibility, phosphorus (P) digestibility, blood ionized Ca (iCa), and percentage eggshell (PE).

H_A = Limestone PS, phytase, and time POP will have a significant influence on Ca digestibility, P digestibility, blood iCa, and PE.

CHAPTER 2 Literature review

2.1 Introduction

The exponentially expanding global population has an ever-growing need for sustainable food production with limited resources. In the next 80 years, the world population is expected to grow within 25% to 42% (Lutz *et al.* 2014), and food production needs to meet these demands. One of the solutions to meeting the increased demand lies in making the current production more efficient and reducing wastage (Krehbiel, 2013). The South African egg industry has a vital role in providing a source of cheap, but high-quality, sustainable protein sources such as eggs for all income brackets. In 2018 there were 21.7 million laying hens in South Africa, which produced close to 5.5 billion eggs for the year (South African Poultry Association, 2018). Egg breakages form a large proportion of economic losses experienced by the egg production industry, with an average of 1 % cracked eggs costing producers R100 million (South African Poultry Association, 2018). Evaluating calcium (Ca) and phosphorus (P) digestibility values is intended to equip producers with the tools to reduce the number of eggs lost to breakages, as well as improve the welfare of the hens through optimally formulated diets (Hudson *et al.*, 1993; Cornish *et al.*, 2019).

Nutritionists have achieved this by formulating animal diets using digestibility values that closely resemble the physiological needs of the animals (Goodland, 1997). The metabolism of Ca and P are closely related, and a deficiency in either nutrient can decrease the utilization of the other (Kebreab *et al.*, 2009). Therefore, it is essential to understand the metabolism of both nutrients in order to optimize the Ca and P status of the hen for optimum production. Dietary Ca is the primary mineral essential for egg production in laying hens (Elaroussi *et al.* 1994), and hens can transfer up to 10% of their total body Ca daily to sustain egg production (Kenny 1986; Barr. 2009). However, several factors influence the movement of total body Ca into the egg, namely the time of day and the particle size of limestone provided in the diet. The literature points to significant differences in Ca digestibility values between non-shell forming and shell-forming hours in the laying hen (Hurwitz & Bar 1965; Hurwitz

& Bar 1973). In order to improve bone and egg quality, systems such as split feeding and midnight feeding have become adopted feeding strategies to provide Ca to the hen outside of the regular feeding hours (Harms *et al*., 1996).

P digestibility values are also of economic significance, as P is the most expensive mineral supplemented into poultry diets (Carlos & Edwards, 1998). P digestibility values allow producers to determine the exact P requirements for the hen, ensuring the correct inclusion levels for supplementation, thereby limiting the adverse environmental effects of inorganic P excretion. A cheaper method of meeting poultry P requirements involves the addition of exogenous phytase enzymes. These enzymes cleave the naturally bound P in cereal grains and oilseeds used in the formulation of poultry diets, making it available to the hen. Phytase enzymes have a positive influence on P digestibility values (Menezes-Blackburn *et al*., 2015). The efficacy of phytase enzymes may be influenced by the limestone particle size, level of the enzyme being supplemented into the diet, and by the type of phytase.

Increased Ca and P digestibility in laying hens has been shown to improve keel and medullary bone structures (Eusebio-Balcazar *et al*.., 2018). This improvement in overall bone integrity has benefits on animal welfare and egg production levels (Kim *et al*., 2012). Thus, a better understanding of the effects of limestone source, particle size, and the influence of phytase on Ca and P digestion will provide egg producers with the tools needed to optimize the production and maintenance of laying hens. This review investigates the factors that influence Ca and P digestibility in the modern commercial laying hen.

2.2 Calcium requirements of the laying hen

Hurwitz and Griminger (1962) were the first to show that modern-day White Leghorn hybrids require at least 3 g of Ca per day, this was higher than the recommendations for genetically unimproved breeds of laying hens which were approximately 2.6 g of Ca per hen per day (Heuser & Norris, 1945). In 2018, the recommendation for high producing commercial hens was between 4.1 g and 4.5 g Ca per hen per day (Hy-line International, 2018; Lohman International, 2018). As hens progress in their genetic capabilities, their

requirement for Ca gradually increases (Table 2.2.1). This requirement changes as the pullet transitions into a laying hen, with mature hens requiring the most Ca. However, the efficacy of these recommendations is dependent on the particle size of Ca source fed to the hen (Rao & Roland, 1989; Cufadar *et al*., 2011; Swiatkiewicz *et al*., 2015; Eusebio-Balcazar *et al*. 2018).

Table 2.2.1 Changes in calcium (Ca) recommendations for laying hens throughout history

Year	Hen age (weeks)	Ca recommendation (g/hen/day)	Study
1945	22	1.98	Heuser & Norris
1962	42 to 60	3.00	Hurwitz & Griminger
1970	20 to 52	2.86	Davidson & Boyne
1984	20 to 60	3.74	NRC
1993	20 to 64	3.74	Keshavarz & Nakajima
2004	23 to 70	4.80	Castillo *et al*.
2011	76	3.60	Cufadar *et al*.
2015	25 to 70	3.68	Swiatkiewicz *et al*.
2018	19 to 65+	4.1-4.5	Lohmann Guide

Modern laying hens in peak production require 4.10 g Ca per hen per day in a ratio of 70:30 limestone grit (1.5 mm to 3.5 mm) to fine limestone (0.01 mm to 0.05 mm) during peak production (Lohmann Tierzucht, 2018). Excessive Ca levels at a 5% inclusion level in the feed have been shown to decrease egg production and feed intake (Scott *et al*., 1971; Atteh & Leeson, 1983).

Table 2.2.2 Calcium (%) required by the hen at different feed intakes (g) at different ages in the production cycle (Lohmann Tierzucht, 2018)

Hen age (weeks)	Feed intake (g)			
	105	110	115	120
	Ca requirements (%)			
19-45	3.90	3.73	3.57	3.42
45-65	4.19	4.00	3.83	3.67
65+	4.29	4.09	3.91	3.75

Table 2.2.3 Calcium (%) requirements of laying hens at different ages (Lohmann Tierzucht, 2018)

Hen age (weeks)	Ca (g/hen/day)
19-45	4.10
45-65	4.40
65+	4.50

2.3 Phosphorus requirements of the laying hen

Phosphorus (P) in the laying hen is essential for metabolic processes and structural maintenance of the hen (Jing *et al*. 2018). P also plays an essential role in energy metabolism (Hurwitz & Bar, 1965). For many years, the NRC (1994) (Nutrient Requirements of Poultry) has been a standard reference for researchers and poultry producers alike since its first publication in 1944. The actual values for P requirements by laying hens are of considerable debate in the poultry industry, yet they have not been updated in 25 years (NRC, 1994). The NRC (1994) lowered the estimated non-phytate P requirements for layers from 350 mg/hen/d in 1984 to 250 mg/hen/d. Recent research has shown that commercially, P in poultry diets is regularly over supplemented by 20 to 100 % of the recommended phosphorus requirements (Applegate & Angel, 2014). The reason for the over-supply is due to the lack of information on digestible P values and very little information on the P concentrations in individual ingredients. Furthermore, when the NRC values for P were published, phytase was still in

its early development stages (Applegate & Angel, 2014). To further complicate matters on inaccurate P requirements for laying hens, different nomenclatures are used to describe P (Adedokun & Adeola, 2013) in poultry diets, and these are described below.

2.3.1 Phosphorus terms in literature and application to poultry diets

There are three forms of phosphorus (P) values present in literature, namely total phosphorus (Total P), Phytate Phosphorus (PP), and Non-phytate phosphorus (NPP). Total P refers to the total amount of P in feed, including the P portion that is unavailable to the hen and bound to the phytic acid molecule. Approximately 70% of the total P in plant-based diets is in the form of PP (Jing *et al.* 2018), while the remaining 30% of P is in the NPP form. The NPP is interchanged with available phosphorus (AvP) in the literature (Li *et al*. 2017) and is assumed to be completely available to the hen. NPP can be determined by the formula: Total P - Phytate P = NPP. This terminology is internationally accepted as published in the NRC (1977). However, there are problems associated with the general assumptions put forward by these terms. For instance, NPP is not always 100% available to the hen, and only small amounts of phytate are broken down and synthesized by the microflora in the small intestine of poultry. The differences in the nomenclature used between guides are shown in Table 2.3.1.1, and the P requirements in laying hens according to literature are in Table 2.3.1.2

Table 2.3.1.1 Nomenclature on the utilization of P in the digestive tract of the hen

Parameter	Description
Digestible P	The portion of dietary total P that is not recovered in the faeces
Available P (AvP)	Proportion of total dietary P that is not recovered in the total ileum, also referred to as pre-caecal digestible or ileal P digestibility
Retainable P (rP)	Proportion of total dietary P retained in the body, also known as total tract P digestibility. In poultry, rP is not an accurate term for P utilization, since urine is included in the determination of digestible P.

(Li *et al*. 2017)

Table 2.3.1.2 Requirements of phosphorus (P) in laying hens according to previous literature

Age (weeks)	P requirements	Term used	Year	Reference
18-45	2.70 g	Retainable P (rP)	2014	Lambert *et al*. 2014
46-90	2.50 g	Retainable P (rP)	2014	Lambert *et al*. 2014
20-60	0.25 %	NonPhytate Phosphorus (NPP)	1994	NRC, 1994
18-20	0.30 %	NonPhytate Phosphorus (NPP)	1994	NRC, 1994
45	0.21%	NonPhytate Phosphorus (NPP)	2003	Keshavarz, 2003
19-45	0.6 g/hen/day	Dietary Phosphorus	2017	Lohmann Tierzucht, 2018
46-65	0.58 g/hen/day	Dietary Phosphorus	2017	Lohmann Tierzucht, 2018
65+	0.55 g/hen/day	Dietary Phosphorus	2017	Lohmann Tierzucht, 2018

2.4 Analytical methods to determine calcium and phosphorus digestibility in the laying hen

In recent years there has been an increase in ileal Ca digestibility studies completed on broilers (Hamdi *et al*., 2015; Anwar *et al*., 2016; Anwar *et al*., 2017), but there are very few reports on similar studies conducted in laying hens. Digestibility studies in laying hens have often been on an excreta-based analysis (Cheng & Coon 1990; Jalal & Schiedeler, 2001; Jing *et al*., 2018). This system has its limitations, such as contamination with feathers, feed, and intestinal mucosal cells that are sloughed off during digestion, thereby interfering with the analytical results (Siqueira *et al*., 2010).

2.4.1 Excreta collection

Excreta can be analysed as total excreta collection (TC) or as partial excreta collection with a marker (PC). TC requires total quantification of the feed consumed, as well as the total excreta by the hen over 24 hours or longer. The limitations of the TC method are that it is often contaminated with feed, feathers, and intestinal mucosal cells (Guinotte & Nys, 1991; Siqueira *et al*., 2010) and substantial labour is required to ensure total collection of the excreta. Partial excreta collection methods rely upon an indigestible marker that is added to the feed (Sales & Janssens, 2005). For a substance to be considered a marker, it needs to be non-toxic, it may not be altered during its journey through the GIT, it may not influence any physiological processes whilst in the GIT, and its rate of passage or transit time must be similar to that of the nutrient being investigated for digestibility (Sales & Janssens, 2005; Zhang & Adeola, 2017). The PC method is an alternative method used to determine digestibility values, and this method circumvents the need for total collection (TC) of faeces (Wang *et al*., 2016, Kavangh *et al*., 2001). The digestibility calculation using markers is as follows (Zhang & Adeola, 2017):

$$\text{Percentage digestibility} = 100 - [100 * \left(\frac{Mfeed * Cexcreta}{Mexcreta * Cfeed}\right)]$$

where:

M_{feed} = Concentration of marker in feed

$M_{excreta}$ = Concentration of marker in feces

C_{feed} = Concentration of component to be analysed in feed

$C_{excreta}$ = Concentration of component to be analysed in feces

The accuracy of specific markers is dependent on the recovery rate and accuracy of application to the diet. Chromic oxide (Cr_2O_3) is the most commonly used marker for digestibility studies in poultry (Sales & Janssens, 2005). The excreta that is collected in either the TC or PC method should be either oven-dried or freeze-dried to remove any residual moisture before analysis. Passing the excreta through a sieve reduces the variation in the analytical results (Sebastion *et al*., 1998).

2.4.2 Ileal collection

Ileal collection of digesta in poultry has been used as an alternative to excreta-based analysis. Ileal collection also makes use of indigestible markers to determine digestibility values. The development of ileal digestibility methods has reduced the variation caused by the microbes situated in the ceca of the hen. Calcium (Ca) and phosphorus (P) are absorbed in the proximal duodenum and jejunum (Hurwitz & Bar, 1965; Hurwitz *et al*., 1973; Bar, 2009) with minimal absorption occurring distal to these gastrointestinal tract (GIT) sections (Sturkie *et* al., 1998). Therefore, the mineral composition of the digesta located in the terminal ileum becomes an accurate estimation of the minerals that will not be further absorbed by the hen.

The ileum is defined as half of the portion of the gastrointestinal tract from the Merkel's diverticulum to 5 mm proximal to the ileocecal junction. Ileal digesta collection requires the terminal ileum to be surgically removed from the hen directly after euthanasia and placed over a cooled surface to slow the rate of microbial action on the digesta. The digesta is removed by flushing the tract with distilled water into a sterile container. The collected digesta is then pooled, freeze-dried, and ground to pass through a 60-mesh screen (Snow *et al*., 2003). Due to the dynamic Ca metabolism involved in the laying hen, the length of the period post oviposition will influence the digestibility results obtained from the digesta sample. Thus, the time of oviposition gives an indication of the phase of mineral metabolism in the hen at a specific time of day. As such, when taking ileal digesta samples in the morning hours, it is vital to slaughter the bird within seven hours of oviposition to prevent the calcification of the next egg influencing the digestibility results (Guinotte *et al*., 1995).

2.5 Factors affecting calcium digestibility

Calcium (Ca) digestibility in the modern hen is a multifactorial facet, and the hen is able to respond to hypocalcaemic conditions within minutes (De Matos, 2008). Factors affecting Ca absorption include intricate dynamics between blood ionized calcium (iCa) and its influence on bone remodelling and breakdown, the affinity of Ca transporters in the gut, and

homeostatic mechanisms such as the release of parathyroid hormone (PTH), calcitonin (CT) and vitamin 1,25-$(OH)_2$ D_3.

2.5.1 Blood ionized calcium

Extracellular Ca is located in the blood plasma, which contains three forms of Ca, namely, ionized Ca, protein-bound Ca, and anion-bound Ca (De Matos, 2008; Bar, 2009; Adedokun & Adeola, 2013). The blood ionized calcium (iCa) determines the activation of homeostatic mechanisms such as the release of PTH and bone remodelling (Parsons & Combs, 1980). The level of PTH and bone remodelling has a significant influence on the Ca digested by the hen. The blood iCa fraction is influenced by the level of bone demineralization, the retention of Ca by the kidney, and the level of dietary Ca absorption from the gut. Scott *et al*. (1971) found that hens fed a blend of limestone that contained a ratio of 66.6% limestone grit and 33.3% fine limestone had blood iCa levels that were 17% higher in the evening hours than the hens supplemented only with fine limestone. The fluctuations in blood iCa coincide with the metabolic events occurring during the egg formation cycle (Parsons & Combs, 1980).

There are various devices used to measure blood Ca and P levels in poultry. The i-STAT has been validated for use in poultry (Steinmetz *et al*., 2007) and provides a convenient method to analyse blood parameters rapidly. Previous blood research relied upon atomic absorption techniques (Guinotte & Nys 1991) and required that the blood be stored following withdrawal; the blood storage process has the potential to increase variation between samples. The primary blood collection point for analysis is located at the brachial wing vein; however, the medial metatarsal and jugular veins may also be used as blood collection sites (Owen, 2011). Previous studies have shown that the average iCa level in the blood of a hen is 1.3 mmol/L to 1.69 mmol/L (Angel, 2004; Schaal *et al*., 2016) and Martin *et al*. (2010) established similar reference ranges for breeder hens as 1.20 mmol/L to 1.73 mmol/L with a mean of 1.47 mmol/L. Parsons & Combs (1980) found that 72-week-old hens showed a sinusoidal pattern of blood iCa relative to the position of the egg in the oviduct. This pattern was noted during calcification of the eggshell, followed by a rapid drop in the blood iCa

levels to 1.23 mmol/L. However, soon after oviposition of the calcified egg, the blood iCa levels rose back up to 1.4 mmol/L (Figure 2.5.1).

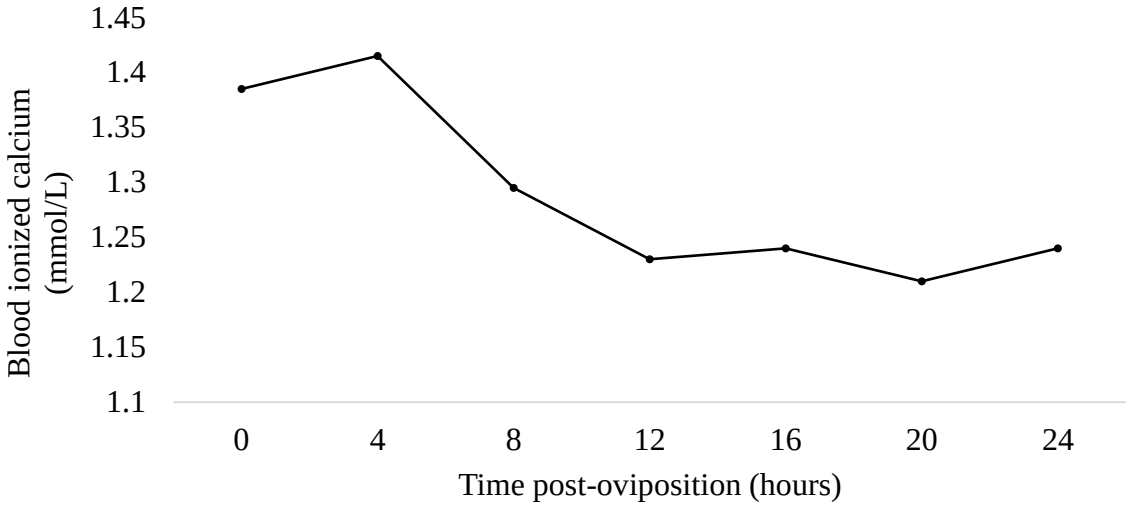


Figure 2.5.1 Blood ionized calcium (iCa) of 18-month-old laying hens (Parsons & Combs, 1980)

During peak eggshell formation, the transfer of Ca from the blood to the eggshell is 100 mg/h to 200mg/h. This rapid depletion reduces the plasma levels to 0 within seven to 15 minutes if there is no other alternative Ca source available. This is where it is essential to optimize Ca nutrition to ensure that there is sufficient ionized calcium available to the hen.

During the period of low blood iCa, the hen's Ca homeostasis is disrupted, and the hen elevates PTH hormone secretion. The primary function of PTH in the hen's body is to increase blood iCa levels by breaking down bone and decreasing renal Ca excretion (Singh *et al.*,1986).

In the past, research focused on the total plasma Ca levels (TCa), which had limitations due to blood pH fluctuations, as well as ionic strengths of the Ca and protein concentrations within the blood (Moore, 1970). These factors influence the equilibrium between bound Ca and free Ca, leading to inaccurate estimation of the Ca^{2+} levels when Ca binds to these fractions. Recent research focused on the use of blood iCa to replace the TCa levels. The

advantage with blood iCa levels is that it provides an indication of the stage of egg production and the state of bone breakdown. The unique bone structure found in avian species is further discussed below.

2.5.2 Laying hen bone structure and calcium utilization

Approximately 99% of the calcium (Ca) in the hen's body is in the form of skeletal hydroxyapatite crystals (Veum, 2010). The formation of hydroxyapatite crystals is dependent on the presence of sufficient Ca and P (Whitehead, 2004). If Ca or P digestibility is inadequate, the hen will be unable to deposit Ca or P into her bone.

During the process of egg formation, the bone acts as a major Ca source to the hen, and it can be rapidly utilized when there is a Ca shortfall. Within the laying hen, three types of bone exist, namely, the cortical bone, the trabecular bone/cancellous bone, and the medullary bone (Kim *et al*., 2012).

Both the cortical bone and trabecular bone have a structural role in the skeleton and are developed until the sexual maturity of the hen (Hudson *et al*., 1993). Cortical bone has a compact, highly organized structure (Kim *et al*., 2012), and the trabecular bone has a honeycomb or a lattice-type structure. Trabecular bone is usually located towards the ends of developing bones as it plays a role in bone formation (Odgaard, 1997). In the absence of sufficient Ca for extended periods, the structural bone (cortical and trabecular bone) is broken down, resulting in a decrease in the structural integrity of the skeleton as the hen ages (Fleming *et al.*, 1998). If extreme, it may lead to osteoporosis and bone fractures as the hen ages (Whitehead, 2004). Studies done by Taylor *et al*. (2010) show that hens with osteoporosis result in an 18% decrease in egg production, compared to hens with a healthy bone structure.

Medullary bone development is initiated by elevated levels of circulating oestrogen at sexual maturity (Fisher & Schraer, 1982), at a rapid rate at first, then slowly as the hen ages

(Whitehead, 2004). The medullary bone is comprised of hydroxyapatite crystals that are orientated around an irregular type I collagen matrix in the pneumatic cavities of the humerus in spicule formation (Whitehead, 2004). The medullary bone acts as a labile Ca source that can be rapidly demineralized when the hen requires Ca for eggshell formation.

Kim *et al*. (2007) reported that after nine days of feed withdrawal, the medullary bone was completely absent, indicating its use as a labile Ca source. This bone can be broken down twice as fast as the cortical bone due to its organic matrix structure, highly vascularized channels, and elevated osteoclast activity (Etches 1987; Whitehead, 2004). The release of Ca from the medullary bone is dependent on osteoclast structures and their activity to breakdown the bone.

It has been shown that the population of osteoclasts within the medullary bone does not change, but their structure does over a 24-hour egg-laying cycle (Kim *et al*., 2012). When the egg transits down the infundibulum, magnum, or isthmus, the osteoclasts lose their ruffled border, which is needed for bone breakdown, causing a cessation in bone resorption. Subsequently, during the active period, when the egg reaches the shell gland, the ruffled borders of the osteoclasts actively breakdown the medullary bone for eggshell calcification (Kim *et al*., 2012). The activity of osteoclasts in the medullary bone is an indication of Ca supply to the hen (Fleming, 2008). Further evidence of bone breakdown during eggshell calcification is supported by the higher collagenolytic activity in the bone (Bannister *et al*., 1973) and the elevated levels of inorganic P that is released from the breakdown of the hydroxyapatite crystals (Sauveur, & Mongin, 1983).

Consequently, the medullary bone is greatest in mass before the initiation of eggshell calcification (Candlish, 1971; Castillo *et al*., 1979). Bar (2009) suggested that between 20% and 40% of the Ca required for eggshell formation originates from the medullary bone reserves; and earlier studies such as those by Buss & Guyer (1984) and Wilson *et al*. (1998) reported this figure to be between 35 to 40 %. However, this figure will change depending on the particle size and solubility of the dietary Ca source (Guinotte & Nys, 1991).

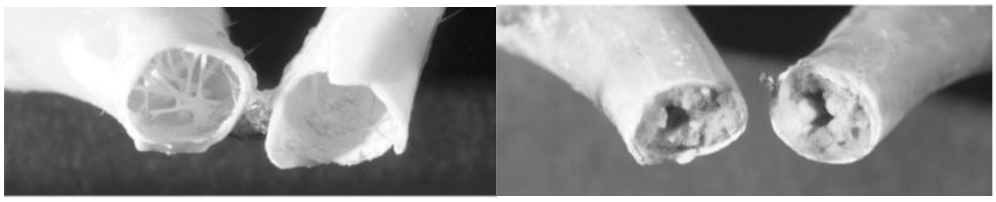


Figure 2.5.2.1 Laying hen bone showing (left) pneumatized internal cavity of humerus and (right) humerus cavity filled with medullary bone (Whitehead *et al*., 2004)

2.5.3 Absorption of calcium from the gut

As the hen ages, there is an increasing demand for Ca. The efficiency with which the hen absorbs Ca from the body, is regulated by hormonal shifts that occur at sexual maturity. The ability to absorb Ca increases gradually from the onset of lay (Bar, 2009) and the primary site of Ca absorption in the laying hen is the proximal intestine (Hurwitz, 1965; Bar, 2009). Most Ca is absorbed before it reaches the lower ileum due to the lower electrochemical potential difference (ECPD) (Bar, 2009). The level of Ca absorbed from the gut depends on the level of Ca in the diet, the physical form of Ca, and the efficiency of absorption across the gut and is not directly related to the amount of Ca ingested (Hurwitz, 1965). It has been shown that a diet that contains more than 0.6% Ca allows Ca to be transported from the gut into the blood, but absorption across the gut differs according to differences in transport mechanisms in the epithelial lining of the gut (Bar, 2009). Some of these transport mechanisms may be rate-limited, whilst others rely on an ECPD between the gut lumen and the blood (Bar, 2009).

Several transport proteins play an essential role in maintaining Ca movement across the gut. The epithelial Ca channels, TRPV5 and TRPC6, are located in the proximal intestine in large quantities, and they are regulated by vitamin D and oestrogen. These channels allow the transcellular movement of Ca between intestinal cells (Sturkie, 1998; Hoenderop *et al*., 2003). Calbindins are the second group of transport proteins involved in the transcellular movement of Ca across the gut (Bar, 2009), and there are three forms of calbindins with Calbindin 28k being the most prevalent in the hen (Hurwitz, 1988). Calbindins are also

vitamin D dependent and may act as a buffer by maintaining a low concentration of Ca close to the epithelial Ca channel pores, thereby ensuring a downhill movement of Ca into the cell. The epithelial channels and the calbindins allow passive transport of Ca across the gut wall. However, transporters such as the plasma membrane adenosine triphosphate (ATP)ases and the sodium–calcium exchange proteins allow the active transport of Ca across the gut in the absence of the correct ECPD. Plasma membrane ATPases play the most vital role in transporting Ca actively across the gut.

The level of Ca intake from the diet changes throughout the day (Etches, 1987) as hens have a greater Ca appetite towards the late evening hours. Previous research has shown that the rate of Ca absorption from the gut changes throughout the day; there is a circadian rhythm that increases Ca absorption during the period of shell calcification. It is noteworthy that dietary Ca is only available from the diet during the photophase when there is feed available to the hen, whereas, during the scotophase, the gut empties for four to five hours after the cessation of feed availability (Bar, 2009). Following the emptying of the gut, the hen must rely on coarse Ca particles retained in the gizzard as a Ca supply during eggshell calcification (Lichovnikova, 2007).

During shell formation, the cellular transport mechanisms in the intestine account for the net absorption of approximately 67% of dietary intake, but where the eggshell gland is inactive, this absorption rate drops to 39% (Hurwitz & Bar, 1965; Hurwitz & Bar, 1973). The increase in Ca absorption during the shell calcification process corresponds to the drop-in plasma iCa (Parsons & Combs 1980) and increased plasma PTH levels (Van de Velde *et al*., 1984). In addition, during eggshell formation, the soluble Ca increases (Mongin, 1976; Guinotte & Nys, 1991) due to increased hydrochloric acid (HCL) secretion from the proventriculus. This increase in the soluble Ca fraction in the gut increases the passive transport of Ca into the blood during eggshell formation. According to Rao & Roland (1990), hens fed a calcium-deficient diet can absorb more Ca from their gut. Each of the mechanisms described above illustrates how the hen is able to modify her Ca absorption according to the physiological demands and increase Ca absorption from the gut during eggshell calcification.

2.5.4 Parathyroid hormone

Located within each of the two thyroid glands are two smaller glands that are known as the parathyroid glands. The chief cells in these glands are responsible for regulating both Ca and P levels through the secretion of parathyroid hormone (PTH) (Simkiss, 1961; Kennedy, 1986). PTH is secreted in response to hypocalcaemic conditions in order to increase the blood plasma Ca levels (Singh *et al.*, 1986). PTH is responsible for bone remodelling through the regulation of Ca resorption in the gut and kidneys and inhibition of P excretion, but increased Ca levels in the blood inhibit its secretion. In addition, PTH is further responsible for the conversion of 25-(OH)-D_3 to the active form of vitamin D_3 (1,25-$(OH)_2$-D_3) via 1-α-hydroxylase in the proximal tubules of the kidney (Fraser & Kodicek 1973; Proszkowiec-Weglarz & Angel, 2013).

The action through which PTH remodels bone structure is through increasing the ruffled borders of the osteoclasts (Miller, 1978). The remodelling of bone increases the available Ca during eggshell calcification. In previous studies, the level of PTH was found to be ten times higher (5.96 pg/ml) during active eggshell calcification compared to periods of inactivity of the eggshell (0.55 pg/ml) (Van de Velde *et al*., 1984; Singh *et al*., 1986). Other studies show that parathyroidectomy resulted in hypocalcaemia, tetany, and death (Kenny, 1986). Low Ca diets in laying hens caused the parathyroid glands to increase six-fold (Simkiss, 1961), while the PTH levels in the blood became twice that of the hens calcifying eggs (12.85 pg/ml) (Singh *et al*., 1986).

Kenny (1986) also found that an injection of bovine parathyroid hormone (bPTH) in the laying hen caused a blood plasma Ca response within 10 minutes of administration. The injection caused a rapid rise of the ionized fraction of blood plasma Ca levels, while the total blood Ca levels rose slowly and then later dropped slowly in comparison to the ionized blood plasma level. Increased levels of administered bPTH also caused an increase of urinary Ca and P excretion. Kenny (1986) speculated that the increase in urinary phosphate was a result of increased renal tubular secretion and decreased tubular resorption of phosphate.

Lastly, PTH has also been shown to activate cellular cyclic adenosine monophosphate (AMP) production and promote absorption of Ca in the thick ascending limb of Henle, thereby increasing Ca digestibility. This action occurs through increased apical permeability or through the active transport of Ca in the basolateral membrane (Pines *et al*., 1981). Both PTH and 1,25-$(OH)_2$-D_3 act to ensure that Ca is one of the most efficiently regulated plasma constituents (Sturkie, 1998).

2.5.5 Calcitonin

While PTH is responsible for increasing blood ionized calcium (iCa), the hormone responsible for decreasing blood iCa levels is calcitonin (CT) (Dacke *et al*., 1972; Luck *et al*., 1980). Caudal to the parathyroid glands are the ultimobranchial glands that produce CT (De Matos, 2008). CT is released in response to hypercalcaemic and hyperphosphatic conditions in the body of the hen. However, the exact mechanism of CT in regulating Ca is controversial (Kenny, 1971). CT may act on the bone by inhibiting the action of osteoclastic cells and increasing Ca excretion from the nephron (Milhaud *et al*., 1977; De Matos, 2008). These actions increase iCa levels back to set point. Klandorf *et al*. (1997) found that hens that were fed a non-restricted diet had a nocturnal surge of CT that had a role in the Ca homeostasis of the bone and kidney. However, hens that were fasted or had a continuous lighting system had no significant spike in CT levels. This illustrated CT release from the ultimobranchial glands may be regulated by feeding and lighting stimuli. Whilst PTH and CT act to tightly regulate Ca levels in the hen's body, vitamin D in its different forms play an integral part of Ca metabolism in the laying hen.

2.5.6 Vitamin D metabolism

Vitamin D metabolism is essential in regulating Ca homeostasis in the body of the hen (Bar, 2009). Before the hen can utilize vitamin D from the body or the diet, vitamin D must first be transformed into a form that is detectable by receptors. The biologically active form of vitamin D is 1,25-$(OH)_2$-D_3, and the activation process is shown in Figure 2.5.6.1. The

production of 1,25-$(OH)_2$-D_3 is part of a negative feedback mechanism, and increased levels of the active vitamin inhibit the production of 1-α-hydroxylase and stop the overproduction of 1,25-$(OH)_2$-D_3. Other factors inhibiting the overproduction of 1,25-$(OH)_2$-D_3 include elevated plasma Ca levels and elevated levels of fibroblast growth factor 23 (FGF 23) (Bar, 2009; Bikle, 2014). An alternative metabolite pathway to 1,25-$(OH)_2$-D_3 is the production of 24,25-$(OH)_2$-D_3. This alternative pathway occurs under eucalcaemic conditions (De Matos, 2008). Vitamin D also plays a role in bone remodeling, and either enhances or reduces bone breakdown depending on the physiological requirements of Ca from the hen, and it is the presence of parathyroid hormone (PTH), which also regulates the negative feedback loop on the production of biologically available Vitamin D_3.

Depending on the Ca status of the hen, vitamin D has different essential roles in calcium metabolism in the body of the hen (Table 2.4.6.1).

Table 2.5.6.1 The effect of calcium status on vitamin D metabolism in the hen.

Blood mineral status	Effect of vitamin D
Eucalcaemic	• Osteocalcin created in order to increase osteoblast proliferation which rebuilds bone matrix • Increases Ca absorption from the gut through the synthesis of calcium-binding proteins such as calbindin D_{28k}
Hypocalcaemic	• Osteoclast proliferation breaks down bone matrix • Increases tubular resorption of Ca and decreases renal Ca excretion • Increases Ca absorption from the gut through the synthesis of calcium-binding proteins such as calbindin D_{28k}
Hypophosphatemic	• Osteoclast proliferation breaks down bone matrix

Evidently, Ca levels and Ca absorption have a direct relationship with vitamin D production in the hen; this highlights the importance of feeding the correct vitamin D levels in the feed to ensure efficient Ca digestibility.

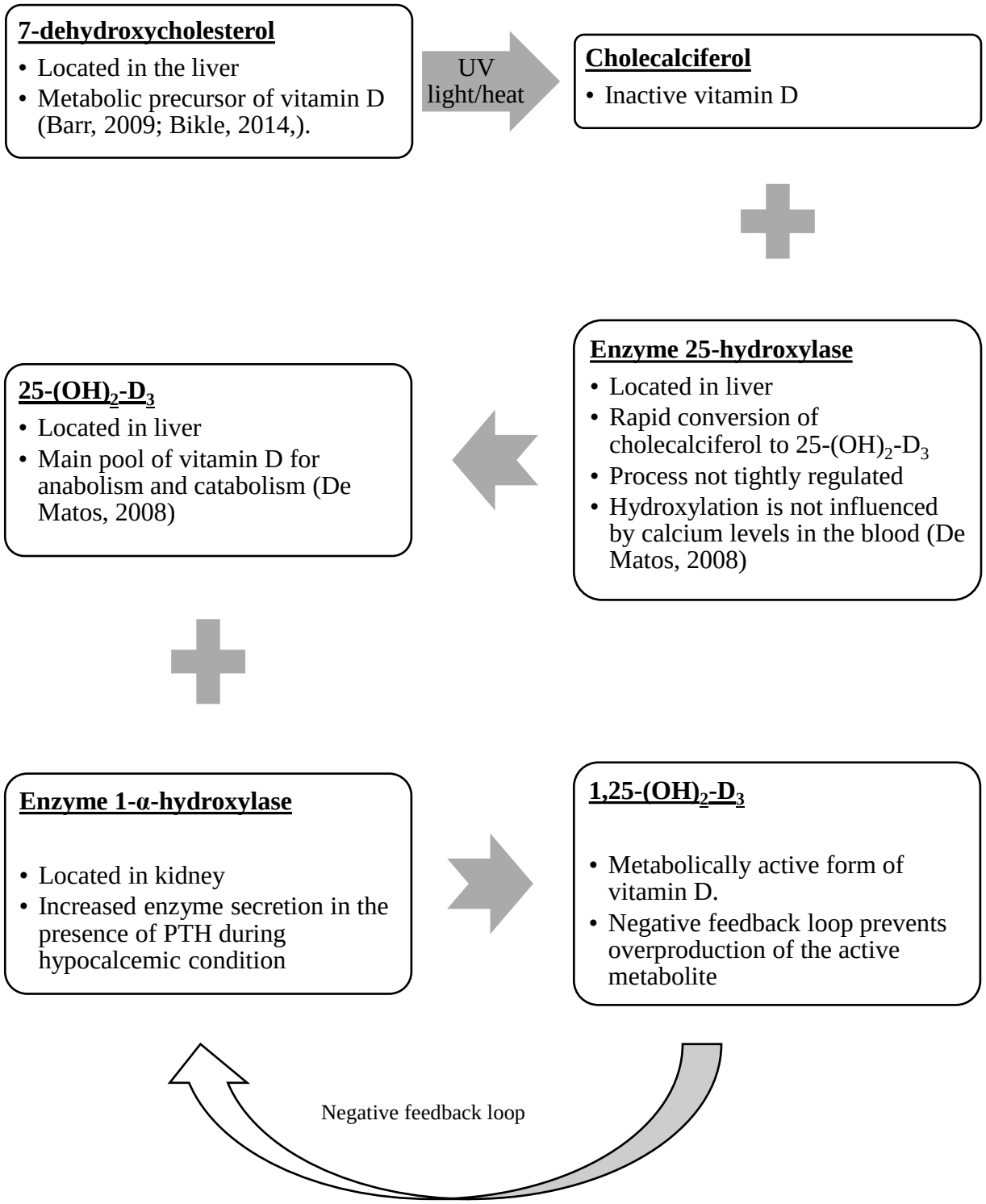


Figure 2.5.6.1 The production of biologically active vitamin D_3 (1,25-$(OH)_2$-D_3)

2.6 Phosphorus digestibility

Global P reserves are an increasingly finite resource, and the addition of excess inorganic P to poultry diets is contributing to a dwindling P supply. These P reserves are likely to reach depletion by 2100 (Van Vuuren *et al*., 2009; Hein & Leemans, 2012). The addition of inorganic P to poultry diets has had negative impacts on natural water systems and soil due to the oversupply of P from poultry manure (Vaccari, 2009). It is essential that laying hen diets be formulated using digestible P values in order to limit the P excretion into the environment. The accurate determination of P digestibility values has a sizeable economic benefit, as P is an expensive mineral in poultry diets (Costa *et al*., 2008).

P in the laying hen diet is mostly unavailable as it is bound in the phytate molecule. In response to this, nutritionists either add a source of available P to the diet such as non-phytate P or available P, which infers excess feed costs (Jing *et al.*, 2018) or supplement exogenous phytase enzymes into the diet. Recent advances in enzyme technology have allowed the development of exogenous phytase enzymes that drastically improve the availability and digestibility of plant-based phytate P. The dynamics of phytate and phytase enzymes in laying hen feed are discussed below.

2.6.1 Phytate and the effects of phytase enzyme supplementation

Phytate is also known as phytic acid (myo-inositol hexaphosphate [IP6]) and is the main organic form of P in plants. This molecule contains between 70% and 80% of the total P in the plant and is present at a volume of roughly 10 g/kg feed (Selle *et al*., 2009; Jing *et al*., 2018). The IP6 molecule is polyanionic with the ability to bind cations such as Ca, zinc (Zn), and copper (Cu) (Humer *et al*., 2012). Due to these effects, the phytate molecule has the ability to bind six Ca ions at any given time (Maddaiah *et al*., 1964).

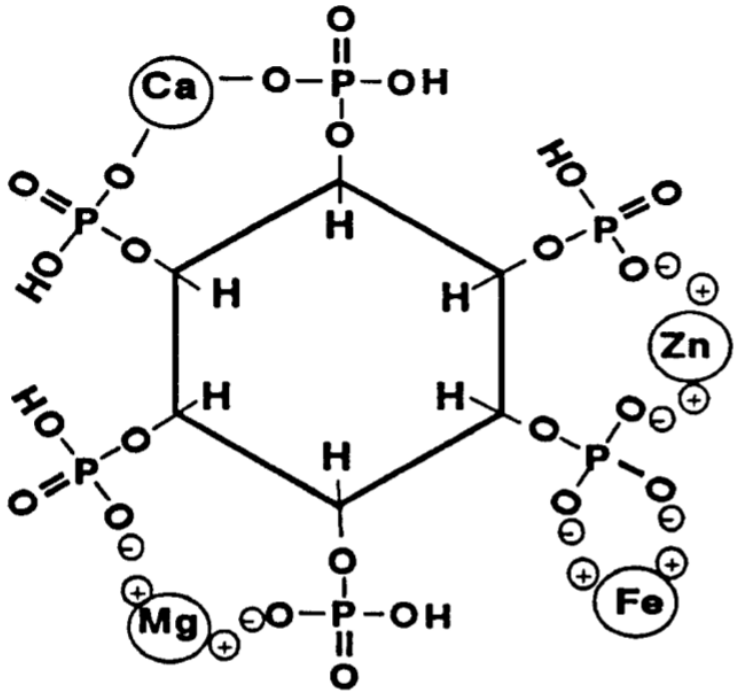


Figure 2.6.1.1 The structure of phytate (phytic acid) and its interactions with Ca and other divalent minerals (Sebastion *et al*., 1998)

Upon the binding of Ca to phytate, the molecule forms an insoluble complex (Selle & Ravindran, 2007). This complexed molecule is able to resist degradation by phytase enzymes (Selle *et al*., 2009), decreasing ileal phytate breakdown from 33% to 9% (Mohammed *et al*., 1991; Van der Klis *et al*., 1997). The binding of Ca to the phytate molecule occurs optimally within specific pH ranges and molar concentrations depending on the type of phytase enzyme being used (Selle *et al*., 2009). The pH in the intestine of the laying hen changes from highly acidic in the gizzard and proventriculus, approaching a neutral PH in the small intestine. The phytate–calcium complex is soluble below pH 4 and starts to precipitate above pH 5 (Nash *et al*., 1998). In the laying hen, the gizzard plays an important role in phytase activity as the digesta typically has a pH of approximately 4.89, which suggests that there is a significant effect of Ca binding to phytate (Shafey *et al*., 1991; Juanpere & Brufau, 2004).

Some studies suggest that, due to the absence of endogenous phytase enzymes in the digestive tracts of poultry, phytate P in plants is poorly digested (Sebastion *et al*., 1998).

Other studies, however, have identified the presence of sufficient endogenous phytase secretion in the gut to digest plant-based P in the absence of high dietary Ca levels (Maenz & Classen, 1997, Sommerfeld & Schollenberger, 2018).

The addition of exogenous phytase enzymes is aimed at releasing the bound phytate P to the bird. As such, phytase is routinely supplemented into diets of laying hens to hydrolyze phytate and create available P for digestion in the hen (Selle & Ravindran, 2007). Different forms of naturally occurring phytase enzymes from sources such as microbiota, plants, fungus, and animals are extensively available. The enzymes that are commercially added to poultry feeds are from fungal and microbial origins (Menezes-Blackburn *et al*., 2015). Phytase enzyme activity is measured in phytase units per kilogram (FTU) with the definition of a phytase unit being the amount of enzyme that releases one µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37°C (Afify *et al*., 2011). The phytase enzyme has been shown to increase P digestibility and utilization in poultry (Selle & Ravindran, 2007). Phytase enzymes, therefore, reduce the amount of inorganic P excreted into the environment (Van Vuuren *et al*., 2009; Hein & Leemans, 2012). Several factors, however, such as source and particle size of limestone, can influence the efficacy of phytase (Selle & Ravindran, 2007). Fine limestone rapidly dissolves in the gizzard, and this may reduce phytase enzyme efficacy due to calcium–phytate interactions. As a result, by increasing the Ca levels in the diet, less Ca is digested by the bird due to the binding of Ca to the phytate molecule. Young broiler chicks up to three weeks of age require about 0.45% of available P. However, with the correct phytase and Ca inclusion levels, the total P requirements of the bird can be reduced by 0.2% (Mitchell & Edwards, 1995). Consequently, the inclusion of phytase into poultry diets has a beneficial effect on P digestibility that benefits the bird, the producer, and the environment.

2.7 Hen age effects on calcium and phosphorus absorption

As the laying hen ages, the quality of eggs produced decreases rapidly (Izat *et al*., 1985; Bar *et al*., 1998; Hurwitz *et al*., 1998). The decrease in eggshell quality is likely due to the

decreased efficacy of the intestine to absorb nutrients such as Ca and P (Horikawa *et al*., 1982; Bar & Hurwitz, 1987; Al-Batshan *et al*., 1994; Armbrecht *et al*., 1999; Driver, 2004). Horikawa *et al*. (1982) proposed that the decrease in eggshell quality as hens age is due to a decrease in cholecalciferol metabolism; however, Bar and Hurwitz (1987) suggested that the production of cholecalciferol does not change in aged hens but rather that the hens lose their ability to adapt to changes in Ca intake and Ca requirement. Bar *et al*. (2002) found that aged laying hens (450 d to 650 d) had a higher Ca requirement for optimal eggshell production above 3.6 g/day and that there is a need to increase diet P if the diet Ca increases. By increasing dietary Ca and P supply, the effect of the decreased mineral absorption from the small intestine can be limited; however, when considering Ca and P digestibility values, the age of the hen must be considered.

2.8 The influence of housing systems on calcium and phosphorus requirements

Consumer preferences have put pressure on large poultry production systems to shift from conventional cage systems to alternative enriched cages that contain perches, nest boxes, and scratching areas (Holt *et al*., 2011). The efficiency of nutrient absorption in hens in the enriched cages differs, as they utilize nutrients at different levels (Lay *et al*., 2011). Neijat *et al*. (2011) found that hens contained within enriched cages deposited more phosphorus (P) into their eggs and would have a higher dietary P requirement (Miles *et al*.,1983). Hens in conventional cages have a tendency towards osteoporosis, while hens in enriched cage systems tend to have a higher incidence of bone fractures (Lay *et al*., 2011). Keel injuries in enriched cage systems can range from 52% to 73% (Nicol *et al*., 2006); hence, these hens have an elevated calcium (Ca) requirement needed for bone strength and eggshell quality (Neijat *et al*., 2011). It was also noted that enriched cage hens deposited less Ca into eggshell (Neijat *et al*., 2011). When assessing the Ca and P requirements of laying hens, the type of housing system needs serious consideration.

2.9 Limestone in the laying hen diet

Limestone is the primary source of Ca in the laying hen diet, providing more than 90% of the total Ca requirement of the hen. Limestone also plays a significant role in P digestibility due to its interactions with phytate. Pure limestone or Ca carbonate ($CaCO_3$) has a Ca percentage of 40.40%. The American Association of Feed Control Officials (AAFCO) states that limestone should not be less than 33% Ca in animal feed. True limestone has a Ca content of more than 36%. A high-quality grit should have a Ca percentage of between 38% and 39% (Roland, 1986). The overall availability of Ca from each limestone source will differ due to differences in particle size, rock composition, and solubility. It is, therefore, essential to evaluate each of these characteristics before deciding on the limestone suitability for a poultry production system. Due to limestone's significant influence on both Ca and P digestibility values, it is discussed in detail below.

2.9.1 Particle size

The particle size (PS) of limestone can be defined as the total surface area of a material per unit of mass or solid volume. It is derived as a scientific value that can be used to determine the properties of a material (Tiggelaar *et al*., 2009). The particle size of limestone plays an important role in the availability of Ca and P to the hen during different periods of the production cycle due to its surface area available for digestion by hydrochloric acid (Guinotte and Nys, 1991). Large limestone grit has a smaller surface area than that of fine limestone per unit mass. Particle size influences the retention of particles by the gizzard. Limestone with a particle size greater than 1 mm remains in the gizzard for a longer duration of time than that of limestone particle sizes smaller than 1mm (Scott *et al*., 1971; Zhang & Coon, 1997; Koreleski & Swiatkiewicz, 2004). The gizzard does not retain particles that are smaller than 1 mm; they enter directly into the digestive tract. The retention of limestone grit in the gizzard ensures that there is Ca available during the periods of shell formation during which there is no feed intake (Roland *et al*., 1971). The consequence is that if the particle size is not large enough, the gizzard will not retain the particles, and Ca reserves from the skeletal system will be used (Scott, 1971; Roland *et al*., 1973). It is recommended to feed

66% grit and 33% fine limestone (Lichovnikova, 2007) depending on the age of the hen (Scott *et al*., 1971). Using a blend of fine limestone to limestone grit had positive effects on bone mineral density, and egg quality in the reported studies by Scott *et al*. (1971), Cheng & Coon, (1990a) and Eusebio-Balcazar *et al*. (2018), and no effect in the studies reported by Guinotte & Nys (1991) and Safaa *et al*. (2008). When using regression equations to model shell quality traits, a greater accuracy is achieved when particle size coupled with limestone solubility is modelled into the regression equation (Cheng & Coon, 1990b). The effects of limestone solubility on Ca supply are discussed further below.

2.9.2 Solubility

Limestone must be dissolved in hydrochloric acid (HCL) for the ionic Ca to be absorbed by the small intestine (Guinotte *et al*., 1995). This process occurs through the gastric acid secretion of the proventriculus. Zhang & Coon (1997) postulated that the solubility of the limestone may be more important than the particle size of the limestone for improving bone-breaking strength and eggshell thickness. The pH of the digestive tract influences limestone solubility, with a low pH increasing the proportion of soluble Ca, while a high pH decreases the level of soluble Ca available to the hen (Guinotte *et al*., 1995). It has been found in some studies that the hen increases the level of HCL secretion during eggshell formation, therefore increasing the level of soluble Ca in the gut (Mongin *et al*. 1976; Guinotte *et al*., 1995; Zhang & Coon, 1997).

Limestone that has a higher solubility will increase the overall Ca digestibility to the hen due to increased Ca ion availability. It is important to note that a limestone source with a high solubility point will also lower the phosphorus (P) digestibility value, due to the interactions with phytate. The physical and chemical composition of limestone sources differs depending on the location of origin. These differences in crystal structure within the rock will influence the solubility and availability of the Ca to the hen.

Limestone PS influences limestone solubility, but there are other several factors at play. The composition of the limestone rock, as well as the porosity of the rock, will influence how

readily the HCL will break down the limestone (Guinotte & Nys, 1991). Cheng and Coon (1990a) illustrated that limestone from different sources at the same PS had varying solubility rates ranging from 60% to 100%. Lastly, the PS of limestone will not influence the porosity characteristics of the limestone (Guinotte & Nys, 1991). The *in vitro* testing of the solubility of limestone has provided a tool to estimate the efficacy of limestone for dissolution in the gizzard and proventriculus; however, there is no set methodology between labs. Zhang and Coon (1997) found that 99.9% of fine limestone dissolved within one minute in HCL (0.1 N), and only 62% of limestone grit dissolved after one hour in solution. Further laboratory methods for determining limestone solubility are briefly discussed (Table 2.9.2.1).

Table 2.9.2.1 Methods for determining limestone solubility *in vitro* (Cheng & Coon, 1990a)

Method	Brief description of the method
Percentage weight loss method (WLM)	2 g of limestone is dissolved in 0.1 M HCL for 10 minutes, and the change in weight of the limestone after filtering the solution sample is recorded.
pH change method (pHC)	2 g of limestone is dissolved as above (WLM), and pH measurements are taken at the beginning and end of each 10 minutes.
Proton consumption method (PC)	The pH readings from the (pHC) method are transformed into H^+ ion concentrations. The moles of proton consumption (PC) is calculated as the difference between the H^+ readings.
pH plateau time	2 g of limestone is dissolved in 0.1 M HCL, a pH probe is left in the solution, and measurements are taken hourly until a pH plateau is reached. Solubility is expressed as the time taken to reach a pH plateau.
Percentage weight loss method (Auburn WLA)	Samples are treated with HCL in a set ratio of 1:100 wt/vol (pH 1.5) and allowed to react for 15 minutes in a beaker. Samples are then entirely dried and weighed. Half of the sample is used to repeat the method, and the other half is reacted again after that from the second reaction. Solubility is expressed as the mean percentage of limestone weight loss per 15 minutes.

2.10 Commercial strategies to improve calcium and phosphorus digestibility

The understanding of calcium (Ca) and phosphorus (P) nutrition is vital in a poultry production system, although it is practical measures that the producer can implement that will make the largest difference to production figures. Several commercial strategies are currently in use to improve Ca and P supply to the hen during increased Ca demand periods. Midnight feeding uses a brief period of light (45 minutes to an hour) during the dark phase to stimulate feed intake, usually around midnight. Midnight feeding has been found to increase the eggshell quality of hens that lay their eggs in the morning (Harms *et al*., 1996) due to the midnight feeding regime providing a source of Ca and P during evening eggshell formation (Scott *et al*., 1971). Midnight feeding has been shown to significantly increase Ca digestibility ($P<0.05$) in laying hens (Lichovnikova, 2007). An alternative to midnight feeding is the use of split feeding programs. When implementing split feeding systems, the hens are fed two different diets, the first diet soon after the beginning of the light period, and the second diet is fed a few hours before the dark period. During the early hours of the light period (three-and-a-half to four hours post-oviposition), the albumin of the egg is formed (Nys *et al*., 2011; Kaspers, 2016) and demands a higher energy and amino acid requirement from the hen. Split feeding recognizes the increased nutrient demand, and the initial diet meets these requirements. Eggshell calcification occurs during the later phase of the day, with peak eggshell formation occurring at roughly around midnight (Hurwitz and Barr 1965). During this period, there is a large demand for Ca and P. Split feeding uses a diet rich in Ca grit and P to supply the hen with her requirements in the second diet ration. It has been shown that during the first five to six hours of egg development where ovulation and albumin formation occurs, only 40% of Ca is absorbed from the diet; however, during shell formation, up to 80% of the Ca is absorbed from the diet (Hurwitz, 1965; 1973). Therefore, it can be seen that by feeding a diet with a constant level of nutrients, they will not be utilized optimally as different nutrients are required at different times. Birds that are fed a diet where they are allowed to select their nutrients will eat more energy and protein in the early hours and increase their Ca intake towards the end of the day. Benefits of split feeding include increased profit per egg, improved eggshell quality, decreased number of cracked eggs, and improved performance (De Los Mozos & Sanchez, 2014). Other benefits to split feeding

systems are a decreased incidence of osteoporosis in older hens as the bone is diminished to a lesser extent than birds who experience a Ca shortage in the later parts of the day (Molnar *et al.*, 2018).

2.11 Conclusion

From the above literature, it is evident that there is limited research that focuses on laying hen Ca and P digestibility and blood Ca status. The modern commercial hen differs vastly from the hen of more than 30 years ago due to improved genetic selection. The advances in genetic selection have led to differences in body composition, egg composition, and nutrient requirements. There is a vast research scope in the area of understanding the mechanics of Ca and P digestion and their regulation in the commercial laying hen and this is supported by Adedokun & Adeola (2013). Updating laying hen research on Ca and P digestibility and blood ionized calcium (iCa) would benefit the sustainability, welfare, and the efficacy of extended production cycle hens.

CHAPTER 3 Materials and methods

3.1 Facilities and husbandry

The use of animals was approved by the Animal Ethics Committee (AEC) of the University of Pretoria (Ethics clearance number: EC052-18). The study was conducted on the experimental farm at the University of Pretoria (Hatfield, Pretoria) in metabolic cages. Strict biosecurity measures were followed, such as footbaths and controlled access, and only authorized personnel were allowed to enter the trial facility and interact with the hens. Personnel entering the trial facility were expected not to have had contact with live poultry for 14 days prior to entry. Prior to placement of hens into cages, the metabolic housing facility was disinfected thoroughly and allowed to stand for 14 days. The aim of this trial was to provide an understanding of the Ca and P digestibility dynamics in the laying hen at different periods of the day, depending on the egg production cycle.

A total of 160 Dekalb Amberlink hens at 30 weeks of age were weighed and randomly allocated to metabolic cages assigned to four treatment groups. Each treatment group had 40 hens and each hen served as a replicate unit. The house was divided into four blocks, and there was an equal number of treatments per block. The facility consisted of 102 metabolic cages that were 84 cm x 69 cm x 52 cm in size. Each cage was divided into two to yield 204 individual cages with a stocking density of 1449 cm^2 per hen caged. This is a lower stocking density to the 450 cm^2 that is commercially used (Cook & Nettleton, 2006; South African Poultry Association Code of Practice 2012). The hens were individually allocated into cages to allow the recording of individual oviposition times. The individual cages were clearly labelled to ensure that the correct treatments were given to the correct hens. The hens were exposed to 16 hours of only artificial lighting and eight hours of darkness each day, with the lights turning on at 05h00 and turning off at 21h00. Feed and water were offered *ad libitum* throughout the trial period. Each day, at 07h00 and 13h00, approximately 65 g of feed, was provided to each hen such that the total feed provided in a day was 130 g. During the first two weeks of the trial, the hens were allowed to adapt to the new cages and feeding system.

The hens were handled with care to ensure that they become accustomed to human contact and presence; this reduced the stress of the hens during the blood sampling week and during ileal sampling. Three checks on the hens and housing conditions were conducted daily for the duration of the trial and the trial took place over 21 consecutive days.

Feed intake (FI) was determined as follows;

Feed intake (g) = (Bl_W+130 g) - (Bl_W+ RF)

Where:

Bl_W = Weight of the individual feed bowl.

RF = Feed remaining in the bowl (g).

3.2 Preparation of the basal diet

The basal diet was formulated using Format NC software, Format International U.K.; using the Kuipers Group nutrient specifications. Ingredients that were used in the basal diets were analysed for Dry matter (DM), ash, crude protein (CP), ether extract (EE), calcium (Ca), and phosphorus (P). The diet was then adjusted for the analysed individual ingredient proximate values. A single batch of basal diet was mixed that did not contain a premix, limestone, mono-dicalcium phosphate (MDCP), soya oil, or phytase. This basal diet was used as a base for the adaptation diet and the dietary treatments in the trial, respectively. The basal diet inclusion levels are shown in Table 3.2.1.

Table 3.2.1 Basal diet mixing proportions

Ingredient	Level (%)
Soya Oil Cake Meal	12.838
Soya Full Fat	9.054
Sunflower Oilcake Meal	9.054
Maize	67.987
Salt	0.345
Biolysine (51%)	0.115
Sodium Bicarbonate	0.132
Synthetic Methionine	0.189
Red Pigment (2%)	0.054
Yellow Pigment (10%)	0.030
Sal CURB® S Liquid	0.203

3.3 Preparation of the adaptation diet

The adaptation diet (Table 3.3.1) was fed for two weeks prior to the experimental diets. The feed was split into two 65 g meals to ensure that the hens did not waste their feed. The basal feed was used as a base for the adaptation diet, to which the layer hen premix, limestone, mono-dicalcium phosphate (MDCP), soya oil, and phytase were added to reach the desired formulated values for the hens. The adaptation diet was mixed at a facility designed to accurately mix experimental diets. Prior to mixing the feed, the ingredients were isolated their composition was analysed for proximate values. The proximate analysis tested for dry matter (DM), crude protein (CP), ether extract (EE), and ash. Further mineral analyses (calcium Ca and phosphorus P) were conducted on the basal ingredients using the AOAC method 935.13 (AOAC, 1998).

Table 3.3.1 Adaptation diet mixing proportions

Ingredient	Level (%)
Basal Diet	89.13
MDCP	0.47
Limestone Grit	6.18
Fine Limestone	2.65
Layer Premix (No Selenium or phytase)	0.25
Phytase (600 FTU) + Carrier	0.25
Soya Oil	1.07

3.4 Treatments

The experimental diets were set up in a 2x2 factorial design. The treatments consisted of two levels of phytase (600 phytase units [FTU] or 0 FTU) and two particle sizes of limestone (1.5 mm and 0.25 mm) to yield 4 dietary treatments. The treatments are described below, and their composition is indicated in Table 3.4.1. Each hen served as a replicate unit to yield 40 replicates per treatment. These treatments were randomly assigned to 160 cages, with all treatments appearing in each of the blocks at least 9 times

Treatment 1- Fine Limestone (0.25 mm) and 600 FTU phytase
Treatment 2- Fine Limestone (0.25 mm) and 0 FTU phytase
Treatment 3- Limestone Grit (1.5 mm) and 600 FTU phytase
Treatment 4- Limestone Grit (1.5 mm) and 0 FTU phytase

Table 3.4.1 Dietary treatment composition and analysed values

Ingredient, %	T1	T2	T3	T4
Basal Feed	88.09	87.94	87.89	87.73
Fine Limestone	9.31	9.31	-	-
Limestone Grit	-	-	9.11	9.11
Premix[1] (No Se* or Phytase)	0.25	0.25	0.25	0.25
Phytase + Carrier	0.25	-	0.25	-
Celite Marker	1.00	1.00	1.00	1.00
Chromium Marker	0.4	0.4	0.4	0.4
Organic Selenium	0.030	0.030	0.030	0.030
Analysed nutrient composition				
Crude protein, %	15.47 (15.40)	15.96 (15.40)	15.26 (15.40)	15.50 (15.40)
Moisture, %	9.23 (10.61)	9.24 (10.61)	9.37 (10.61)	9.10 (10.61)
Crude fibre, %	4.60 (4.50)	4.43 (4.50)	4.14 (4.50)	4.39 (4.50)
NFE, %	52.81	61.86	62.19	61.65
Ash, %	12.99 (11.89)	12.82 (11.89)	13.65 (11.89)	13.56 (11.89)
Fat (EE), %	4.91 (4.93)	4.94 (4.93)	4.77 (4.93)	4.92 (4.93)
Ca, %	3.58 (3.70)	3.53 (3.70)	3.80 (3.70)	3.80 (3.70)
P, %	0.34 (0.36)	0.28 (0.36)	0.30 (0.36)	0.33 (0.36)
Phytase, FTU	823.35 (600)	113.32 (600)	1021.94 600)	105.84 (600)

Data in brackets are formulated values. *Se, selenium

[1]Premix composition: Vitamin A, Vitamin D_3, Vitamin E, Vitamin K3, Vitamin B1, Vitamin B2, Vitamin B3, Cal Panthonate (B5), Vitamin B6, Folic Acid, Vitamin B12, Biotin, Antioxidant, Manganese, Iron, Zinc, Copper, Cobalt, Iodine, Selenium, Choline.

Table 3.4.2 Phytase levels and limestone particle size (PS) in dietary treatments

Treatment	Phytase Level (FTU)[1]	Limestone Particle Size
1	600	(0.25 mm) GMD Fine Limestone
2	0	(0.25 mm) GMD Fine Limestone
3	600	(1.5 mm) GMD Limestone Grit
4	0	(1.5 mm) GMD Limestone Grit

[1] Phytase derived from *Buttiauxella sp*. And expressed in *T.reesei*.

Table 3.4.3 Mineral composition of trial limestone

Mineral	Fine Limestone	Limestone Grit
Calcium (Ca), %	36.97	35.91
Copper (Cu), mg/kg	1.17	0.79
Iron (Fe), mg/kg	530.77	452.76
Magnesium (Mg), %	0.56	0.79
Manganese (Mn), mg/kg	42.72	22.29
Phosphorus (P), %	0.01	0.01
Potassium (K), %	0.01	0.01
Sodium (Na), %	0.01	0.01
Zinc (Zn), %	4.93	5.22

3.5 Calcium and phosphorus analysis

The feed and ileal samples were analysed for Ca and P according to the AOAC method 935.13 (AOAC, 199). The samples were initially digested as described below, and the digested samples were then placed into a spectrophotometer.

Digestion of samples for calcium and phosphorus:

A heating block was set to 240 °C, and 0.500 g of feed sample was transferred into a digestion-tube. The mass of the digestion-tube, and sample was then recorded and 25 ml of nitric acid (HNO_3) was added to the tube. The digestion-tube containing the acid and the sample was boiled for 15 minutes on the pre-heated heating block. Following this, the sample was allowed to cool for five minutes. 10 ml of $HCLO_4$ was then added to the cooled tube and the tube and its contents were boiled for a further 35 minutes. The tubes were then removed from the heat and placed into a fume cupboard until no further fumes were released. 30 ml of deionized water was then added into the tube. The addition of the deionized water generated heat; therefore, the samples were allowed to cool once more. The solution was then transferred into a volumetric flask and well mixed.

Spectrophotometry calcium analysis:

Different strength Ca solutions were placed into the spectrophotometer (one part per million (ppm), three ppm, and five ppm) along with 1.0 ml of 1000 ppm certified Ca solution. These solutions were used to calibrate the spectrophotometer. The diluted sample solution from the acid digestion described above was then placed into the spectrophotometer, and the concentration of Ca was determined. The reading on the spectrophotometer was used to calculate the Ca concentration of the original feed sample, keeping the dilution factors in mind.

The P content was analysed according to the AOAC method 965.17 (AOAC, 1998). To analyse for P, 1.0 ml of the sample solution from the mineral digestion described above was diluted with 7.0 ml of deionized water. Following this 2.0 ml of the colour solution (preparation described below, in steps) was then added to the diluted sample solution. The colour of the colour solution combined with the diluted sample solution was allowed to develop for 30 minutes. The developed solution was analysed at 400 nm using a spectrophotometer and, this value was used to determine the P content of the feed.

Colour solution preparation:

- Four grams of Ammonium molybdate tetrahydrate was dissolved in 50 ml of deionized water. This was heated at 60 °C until dissolved.

- 0.2 g of ammonium vanadate was then dissolved in 50 ml of deionized water and heated until dissolved, to this 45 ml of perchloric acid was added.
- The ammonium molybdate solution was then added to the ammonium vanadate solution.
- The mixture of these two solutions was then allowed to cool and then used as the "colour solution."

3.6 Phytase analysis

Phytase activity was analysed according to the method of Dilger *et al.*(2004) by incubating phytase with sodium phytate (phytic acid dodecasodium). From the incubation, the inorganic P was released, and the inorganic sodium reacted with molybdate vanadate to produce a yellow colour complex. The colour of the complex was measured using a spectrophotometer set at 450 nm; the measure of colour is directly related to the activity of the phytase enzyme and the level of P it released.

Phytase activity was measured using phytase units (FTU), with an FTU being defined as the amount of enzyme that releases one µmol of inorganic orthophosphate from a sodium phytate substrate per min at pH 5.5 and 37 °C (Dilger *et al.*, 2004).

To prepare the feed sample for phytase analysis, 200 g of the feed was ground down to pass through a 0.75 mm sieve. The feed was mixed thoroughly, and individual 10 g sub-samples were weighed out. The feed sub-samples were added to 100ml of buffer solution (FX 10) and stirred for 10 minutes using a magnetic stirrer. Following this, the feed and solution were filtered through a glass filter. From the filtrate, 250 µl were placed into test tubes, and 750 µl of acetate buffer (F4X) was added. The water bath was then heated to 37 °C, and the filtrate samples, as well as the sodium phytate, were heated for five minutes. The sodium phytate was then added to the sample filtrate at a ratio of 2ml to every sample, in five second intervals. The water bath was then allowed to reach an equilibrium of 37 °C for an hour. The chemical reaction was stopped by adding 2ml of the stop solution, in the same sequence as when the sodium phytate was added.

The reacted samples were then mixed using a vortex mixer below 200 rpm and centrifuged at 3500 rpm for 10 minutes. From each sample, 2ml of the solution was transferred into plastic cuvettes and then into the spectrophotometer along with a blank sample and measured at 415nm. For each sample of feed analysed, there was a control sample that was analysed using the same methods, as well as a zero-time period sample. The zero-time period used the F4X, and the filtrate dilution (1.0 ml) in four test tubes, two of which were not incubated, but instead had the stop solution added immediately. The time zero samples were then centrifuged and measured as per the procedures described above.

3.7 Ash

The ash percentage of each feed sample was determined using the method 942.05 from AOAC (AOAC, 1942). The method used two grams of sample feed, which was subsampled from a representative feed sample. The 2.0 g sample was placed into a crucible and heated to 600 ℃ for two hours. After heating, the crucible and sample were placed into a desiccator and weighed once cool. The percent ash was calculated using the following formula:

$$\text{Ash \%} = \frac{Weight\ of\ test\ portion\ (g) - weight\ loss\ on\ ashing\ (g)}{Weight\ of\ test\ portion\ (g)} * 100$$

3.8 Ether extract

The ether content of the feed samples was determined using the official AOAC method 920.39 (AOAC, 1998). Initially, the moisture of the samples was determined using the AOAC method 934.01 (AOAC, 1998), this dry sample was weighed, and the difference in weight was reported as moisture content.

Solid sodium hydroxide was added to a water-ether mix. Once the water has been abstracted, the mixture was decanted into a dry bottle and pure metallic sodium pieces were added to

remove H^+ ions from the mixture. Two grams of the dry feed sample from method (934.01) was then added to the prepared ether and extracted using a thimble that allowed a rapid passage of ether. The sample was allowed to extract for five hours and dried at 100 °C for 30 minutes. Once the sample had cooled, it was weighed. The difference in weight between the original two grams of dry feed sample and the weight of the ether-extracted feed sample was recorded as the ether portion of the sample. This weight loss was divided by the original sample weight multiplied by 100 and reported as the ether extract (%).

3.9 Crude fibre

The crude fibre (CF) of the feed samples was determined using the filter bag technique in the *official methods* of the AOCS, procedure Ba 6a-05 (AOAC, 1998). The crude fibre was determined after digesting the feed using 0.255 N H_2SO_4 and 0.313 N NaOH. This method was used to remove compounds such as proteins, sugar, starch, lipids, and structural carbohydrates and lignin. The full procedure is described as follows below.

The empty filter bags were labelled and weighed (W1), after which the balance was zeroed with the filter bag on it. Following this, 1.0 g of feed sample (W2) was added into the filter bags. The filter bags were sealed using a heat sealer. A blank bag was also weighed to account for a blank bag correction factor (C1). The sealed bags were then placed into a 250 ml beaker, and petroleum ether was added. The addition of the petroleum ether for 10 minutes removed the fat from the sample. After the ether extraction, the bags were allowed to air dry and suspended in the ANKOM digester, and 1500 ml of ambient temperature acid (0.255N H_2SO_4) was added to the machine. The ANKOM digester was sealed and the heat and agitate setting was activated for 40 minutes.

Following acid digestion, the heat and agitate function was turned off, and the machine was drained using the drain valve. 1900 ml of water was then added to the ANKOM and the samples were agitated for a further five minutes to rinse the samples. The rinsing process was repeated three times; and the samples were then removed and squeezed to remove excess water. The sample bags were then placed into 250mL beakers and covered with acetone for

five minutes. After soaking in acetone, the bags were placed on a wire screen and dried in the oven at 102°C for four hours. The dry bags were placed into a desiccator until cooled and then weighed.

After weighing, the bags containing the samples were ashed in a pre-weighed crucible at 600 °C for two hours and then weighed (W3). The crude fibre percentage was calculated as follows:

$$\text{Crude fibre \%} = 100 * \frac{(W3-(W1-C1))}{W2}$$

Where:

W1= Bag tare weight
W2= Sample weight
W3= Weight of organic matter (weight loss on ignition of bag and fibre)
C1= Ash corrected blank bag factor (Loss of weight on ignition of the blank bag)

3.10 Dry matter

To determine the dry matter content of the feed samples, the moisture loss is calculated and then subtracted from 100. The moisture loss was calculated using the *official method of* AOAC 934.0 (1998). A feed sample of two grams was dried at 95-100 °C at a pressure of ≤100 mm Hg for five hours. The moisture loss on drying (LOD) was reported as the moisture content of the feed sample and was calculated as described below.

$$\text{\% Moisture (LOD)} = 100 * \frac{wt\ loss\ on\ drying\ (g)}{wt\ of\ test\ portion\ (g)}$$

% Dry matter = 100- (LOD)

3.11 Crude protein

The crude protein (CP) of the feed samples was determined using the Dumas method, according to the official method of the AOAC procedure 4.2.04 (AOAC, 1998). The feed samples were milled and passed through a 0.2 mm screen. The furnaces were then run until reaching a thermal equilibrium of between 850 ℃ and 900 ℃. The combustion-tube was prepared by placing a stainless-steel screen into the tube, followed by a six mm plug of glass wool. Using a glass rod, the glass wool was pushed towards the steel screen.

Keeping the combustion-tube vertical, a copper oxide and platinum (CuO-Pt) catalyst was added into the tube. An empty sample boat was then weighed (A_1). A 200 mg milled sample of feed was added into a pre-weighed boat (A_1). The sample boat was then placed into the open end of the combustion-tube and the tube was tapped to move the sample boat into place. A 200 mg volume of cobalt oxide (Co_3O_4) and copper oxide (CuO) was then added to the tube. The prepared combustion tube was installed into the nitrogen (N_2) analyser.

The N_2 analyser contains a potassium hydroxide (KOH) level that is used to calculate the N_2 volume emitted from a sample. On the N_2 analyser the 45 % KOH solution meniscus was adjusted to the calibration mark. This reading was recorded and denoted as R_1. The syringe temperature was also recorded as per the scale thermometer on the N_2 analyser (t_1). Following this the analyser was set to run a combustion cycle.

Following the combustion cycle, the second level of the KOH meniscus was recorded (R_2), and the second syringe temperature was also recorded (t_2). A blank value for the instrument was also prepared using the same methods above; however, the sample was omitted from the combustion tube. The observed N_2 volume was recorded using the formula ($V_0 = R_2-R_1$) Where:

V_0=Observed N_2 volume (µL)

R_1= initial KOH meniscus reading

R_2=final KOH meniscus reading

The observed N_2 volume was corrected for temperature and pressure using the Table 968.06A (AOAC, 1998). The corrected N_2 was then converted to a % N_2; and multiplied by 6.25 to calculate the % CP in the feed sample.

3.12 Limestone particle size calculation

For the calculation of limestone particle size, 15 individual sieves were placed into a shaker for ten minutes. The differences in the weights of the sieves after shaking were used to calculate the geometric mean diameter of the limestone samples. The 14 sieves and the base pan were arranged from the coarsest sieve on top to the finest sieve at the bottom, the bottom-most sieve being the collection pan. The sieves were then weighed individually and recorded and then stacked back in the same order. Exactly 100 g of the limestone samples were weighed out and placed into the top sieve (coarsest) of the stack. The lid of the stack was then placed onto the stack, and the stack was placed into the shaker. The shaker was run for ten minutes. After ten minutes, the stack of sieves was removed from the shaker and each sieve containing the retained limestone was weighed. The particle size is determined in duplicate by repeating the above process.

The method for calculating the geometric mean diameter (GMD) of limestone particles by mass (d_{gw}) is described by Wilcox *et al*. (1962) and Baker & Herrman (2002) and was calculated as follows:

$$d_{gw} = log^{-1}\left[\frac{\sum_{i=1}^{n} W_i \, log \, \bar{d}_\iota}{\sum_{i=1}^{n} W_i}\right]$$

Where:

$\bar{d}\iota$ is the diameter of the *i*th sieve: which is calculated using the equation $\bar{d}_\iota = (d_i \times d_{i+1})^{0.5}$

di = Nominal sieve aperture size in ith sieve.

di+1 = Nominal sieve aperture size in next larger than *i*th sieve (just above in a set)

Wi = Mass on ith sieve, g

n = Number of sieves +1 (pan)

Standard deviation (S_{gw})

$$S_{gw} \approx \frac{1}{2} d_{gw} \left[log^{-1} S_{log} - (log^{-1} S_{log})^{-1}\right]$$

$$S_{log} = \left[\frac{\sum W_i (\log \bar{d}_i - log d_{gw})^2}{\sum W_i}\right]^{0.5}$$

Sgw = Geometric standard deviation of particle diameter by mass (mm).

S_{log} = Geometric standard deviation of log-normal distribution by mass in ten-based logarithm, dimensionless.

The results of the limestone particle size calculation were as follows for the limestone used in the trial. The fine limestone had a GMD of 0.25 mm and the limestone grit was calculated to have a GMD of 1.5 mm. The particle size distribution range for each limestone PS is depicted in Figure 3.12.1 and in Figure 3.12.2.

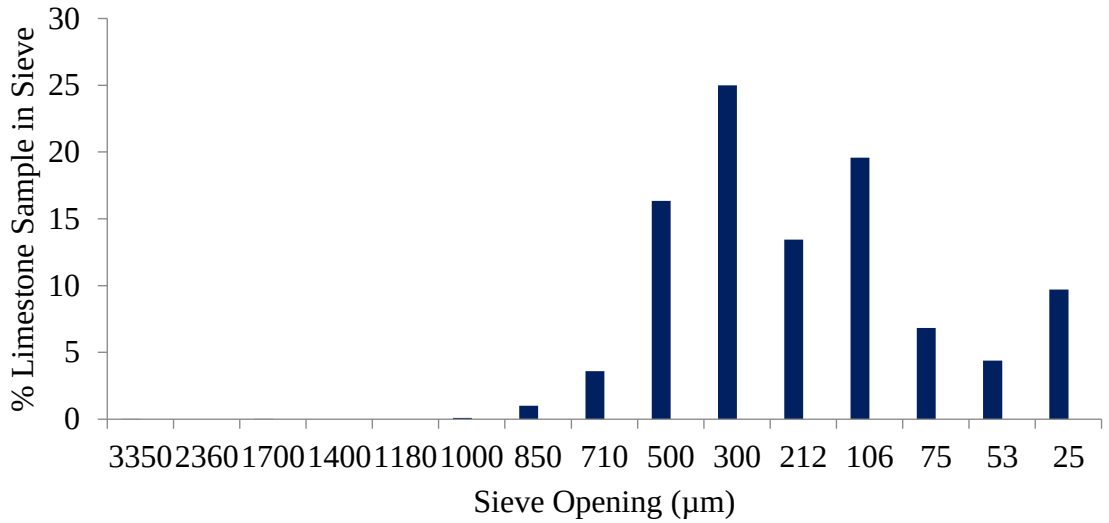


Figure 3.12.1 Particle size distribution of the trial fine limestone

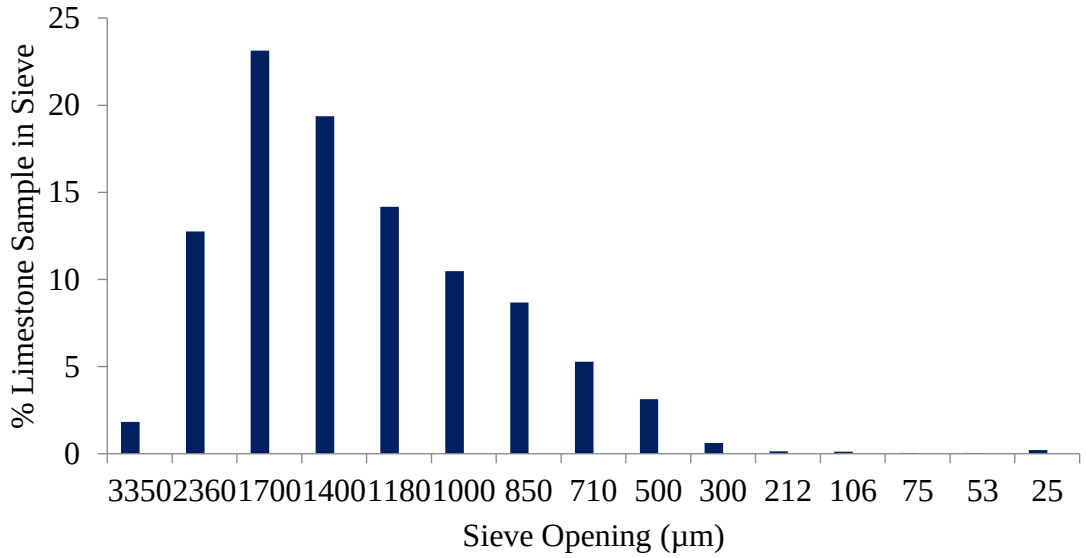


Figure 3.12.2 Particle size distribution of the trial limestone grit

3.13 Limestone solubility assay

Limestone solubility tests were conducted according to the methods described by Kim *et al.* (2018). The process followed is described below.

Prior to testing the solubility of the limestone, the acid solution was prepared, and all Erlenmeyer flasks (EF) and aluminium pans were labelled. Exactly 1.0 g of sample was weighed into the aluminium trays to three decimal points. A buffered pH 3 hydrochloric acid (HCL) was used. The buffered acid solution was prepared as follows.

Table 3.13.1 Volumes of the ingredients needed to mix a pH 3 HCL buffered glycine (Gly) solution

Litres prepared	Distilled water (ml)	Weight of Gly (g)	Weight of HCL (ml)	Distilled water (ml)
1L	500	225.21	50	250
2L	1000	450.42	100	500
3L	1500	675.63	150	750
4L	2000	900.84	200	1000

Initially, 1126.05 g of glycine (Gly) was added to a plastic jug. In a 5.0 L EF, 2500 ml of deionized distilled water was added. The Gly from the plastic jug was then added to the EF using a funnel. The solution in the EF was mixed using a magnetic stir plate and heated to temperatures that did not exceed 40 °C. To the EF containing the glycine and water, 250 ml of HCL was slowly added and while the solution was stirring a further 1250 ml of distilled water was added. The solution continued to mix on the stir plate until the Gly had completely dissolved.

The pH meter was calibrated at a pH of 4.0 and at a pH of 7.0 and then used to measure the pH of the solution in the EF, which was approximately at a pH of 3.4. HCL at 37 % dilution was then added to the solution using a Pasteur pipette to drop the pH of the solution further

to pH 3.0. The solution was then transferred to a 5.0 L volumetric flask, and the volume was corrected to exactly five litres using distilled water. After the correction, the solution was added back to the 5.0 L EF. The 5.0 L EF was then decanted into several 2.0 L flasks for ease of use during the assay. Each of the flasks was sealed using foil and kept in the water bath until the next day. The Ca solubility assay then proceeded as followed.

All flasks, filter papers, and aluminium pans were labelled and had their individual weights recorded. The buffered Gly solution, described above, was added to six small EF flasks at 140 ml per flask. These small EF flasks with solution were placed into a water bath containing shaking apparatus. Distilled water was added to the small EF ensure that the level of Gly solutions was similar to the level of the water in the water bath. The water bath was heated to 42 °C and set to shake at 135 shakes per minute. Once shaking, the weighed 1.0 g of limestone samples from the day before were added to the flasks.

The fine limestone samples were agitated in the water bath at 5 minutes, 15 minutes and 30 minutes. The limestone grit samples were agitated until 30 minutes, 90 minutes and 150 minutes. As the timer for each set of samples was complete, the flask was removed from the water bath, and immediately, 100 ml of cold distilled water was added to the flask solution. The purpose of this was to stop the reaction of the limestone and the solution. The water and solution were swilled in the flasks to ensure that it was sufficiently mixed. The limestone and solution were then poured onto a filter paper. The filtration system ran for 2 minutes to ensure that all of the liquid was removed from the limestone sample. The filter paper was then removed and placed onto pre-weighed labelled aluminium pans that were dried in an oven at 100 °C for 24 hours. After the samples dried, they were weighed (pan, filter paper, and limestone sample) and the Ca solubility was then calculated as follows:

Solubility of limestone (%) = ((WDs-(Wfp * DMfp/100) – (Wp * DMp/100)) / (Ws * DMs/100)

Where:

S = Solubility of limestone.

Ws = Weight of sample.

DMs = Dry matter of sample.

WD s= Weight of the dry sample.

Wfp = Weight of the filter paper.

DMfp = Dry matter of the filer paper.

Wp = Weight of the aluminium pan.

DMp = Dry matter of the aluminium pan.

The limestone solubility (%) was then plotted onto a graph to illustrate how the limestone dissolved over the three time points. The graph allowed us to understand how the differences in limestone solubility affected both Ca and P digestibility *in vivo*.

The limestone solubility of three different South African limestones at different particle sizes (PS) (<1 mm and >1 mm) is shown in the figures below (Figure 3.13.1 and Figure 3.13.2). The solubility of the limestone source used in this trial (Limestone 1) was the lowest of all the South African limestone sources tested in the Chemunique' (Pty) Ltd. Laboratory (2019).

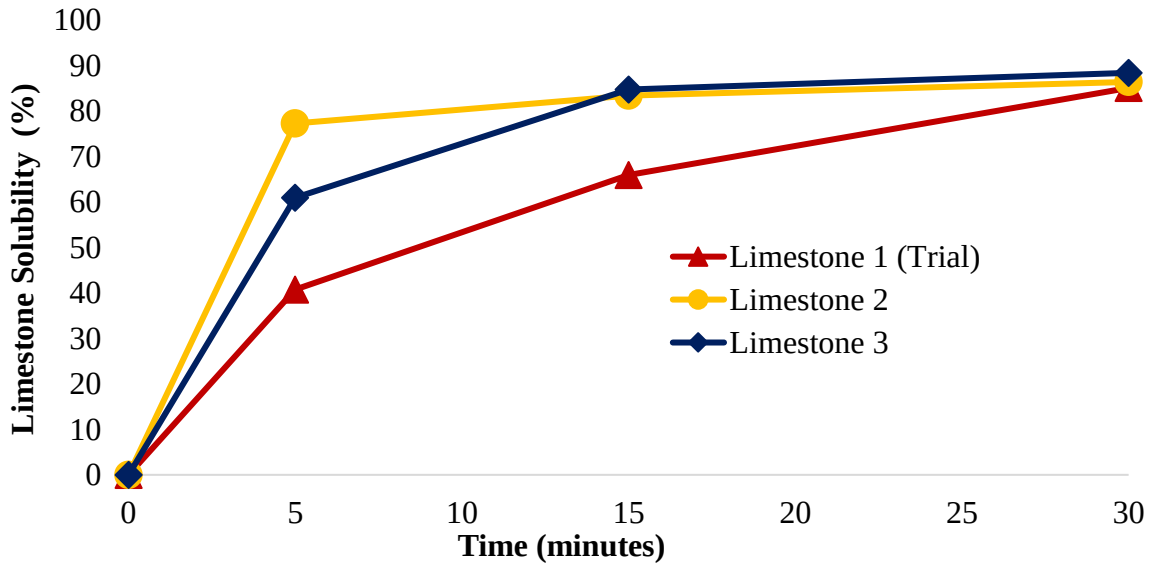


Figure 3.13.1 *In-vitro* solubility of fine limestone (<1 mm) sources from South Africa

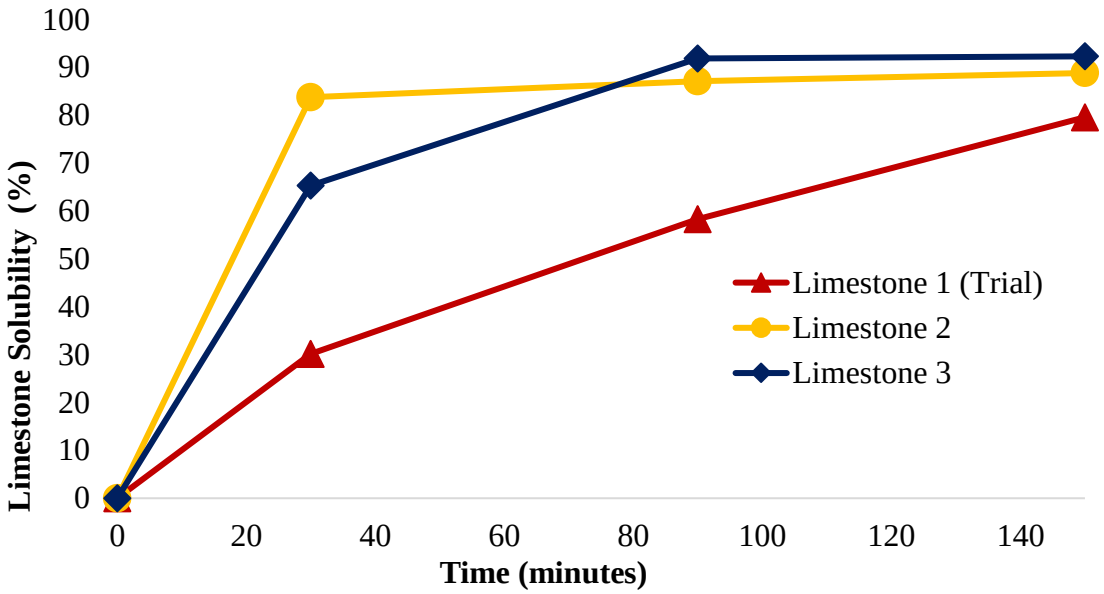


Figure 3.13.2 *In-vitro* solubility of limestone grit (>1 mm) sources from South Africa

3.14 Ileal digestibility

A total of 160 Amberlink hens were selected based on their oviposition times to have their ileal contents analysed for Ca and P. Ileal digesta was extracted over three days at 3 h and 11 h POP. In total, 20 hens from each treatment at each time period were euthanized. At the corresponding time period (3 h or 11 h) POP, the selected hen was removed from its cage and weighed. The hen was then placed in a dark box and a gas mixture that consisted of 35 % CO_2, 30 % O_2 and 35 % N_2 was applied into the box. The gas mixture acted as an anesthetizer to render the hen unconscious. Following the gas mixture, pure CO_2 was administered with to euthanize the hen (Vaughn, 2012). This step was vital as it prevented the hen from experiencing excess smooth muscle contraction associated with flapping during slaughter. The gas mixture was applied for three minutes, and then the second gas consisting of pure CO_2 was applied for two minutes.

Ileal digestibility required the identification of the Merkel's diverticulum and the ileocecal junction. Once these landmarks were identified, the segment was divided in half. The half that was proximal to the ileocecal junction was removed and flushed of its digestive contents. The ileal contents were removed using a syringe and medical-grade distilled water (Ravindran *et al.*, 1999). The syringe contained 2 ml of distilled and 8 ml of air. The end of the dissected ileum was placed over the tip of the syringe with the distilled water, and the air and water were injected into the intestinal portion. The distilled water in the syringe acted as an airtight seal that aided in removing the intestinal contents with minimal physical force used on the gut, the air within the syringe forced the watertight seal on the digestive contents. This method reduced the number of intestinal cells that may contaminate the ileal digesta sample. The ileal digesta contents were flushed into a sterilized container and frozen immediately after sampling.

The frozen digesta samples were lyophilized for seven days to remove any moisture from the digesta sample. The dried digesta was then finely ground to pass through a 0.25 mm sieve to increase the homogeneity of the sample and to remove any visible contaminants. The ground sample was analysed for calcium (Ca), phosphorus (P), and chromium (Cr).

3.15 Blood ionized calcium analysis

Blood analyses from laying hens are able to provide an indication physiological factors taking place within the body of the hen. During the present study, 64 hens had their blood ionized calcium (iCa) measured every three hours from oviposition over four days. Each day 16 hens (four per treatment) had blood collected at (0, 3, 6, 9, 12, 15, 18, and 21) hours post oviposition (POP). Each day yielded 128 individual blood samples, and over the four days, a total of 512 blood samples were analysed. The blood samples were analysed using an Abbott point of care i-STAT device. The Abbott point of care i-STAT device is designed to give accurate and precise blood measurements within minutes of blood collection. The rapid analysis of the sample removes the requirement for blood collection tubes and cold chain storage of the sample. For this experiment, the i-STAT EG7+ cartridge was utilized for the i-STAT device.

To collect blood samples, a detailed record of each hen's oviposition time was logged. At the time of oviposition, the hen was removed and restrained using two trained personnel on a sterile worktop. The collection site for the blood was the medial metatarsal vein located on the leg of the hen. While the hen was restrained, a third trained person located the collection point and inserted a 30-gauge insulin needle at an angle parallel to the vein (Owen, 2011) A total volume of 0.15 ml of blood was drawn from the medial metatarsal vein from each hen per time period. Care was taken to ensure that the hen did not move during this procedure as this would have damaged the vein. The collected blood sample was inserted directly into the i-STAT cartridge; the cartridge was then sealed and placed into the i-STAT device for analysis. The i-STAT device reported the blood results within four minutes of cartridge insertion. The above process was repeated every three hours over each 24-hour period to yield eight sampling periods per day. The blood sampling was repeated over four days.

3.16 Dry eggshell percentage

Eggs were collected over a period of five consecutive days. Each egg was labelled with the corresponding date and hen number. The whole eggs were individually weighed on a calibrated scale and their weight recorded. After weighing the whole eggs, they were opened to remove the internal albumin and yolk contents, and care was taken to ensure that no eggshell pieces were lost during this process. The inner and outer membranes within the shell were removed on all of the eggs to ensure that only the eggshell remained. The eggshells were placed into labelled tins and dried for 24 hours at 60 °C. After the shells were dried, they were weighed. The percent eggshell was determined by the following formula

Eggshell Percentage (%) = (Weight of Dry Shell / Weight of whole egg) x 100

3.17 Statistical analysis

A randomized block design was used. The house was divided into 4 blocks based on their position in the metabolic house. Within each of the 4 blocks, 4 treatments were randomly assigned to 48 hens per block, with an extra block containing 3 replications of each treatment

for extra hens that may have been required. The layout of the housing is shown below in table 13.18.1

Digestibility data of Ca and P digestibility were analysed statistically with the Proc Mixed model (Statistical Analysis System, 2019) for the average effects. Means and standard error were calculated, and a significant difference of (P<0.05) between means was determined by Fischer's test (Samuels, 1989). Time post oviposition (POP) (hours) were used as repeated measures.

The linear mixed model used is described by the following equation:

$$Y_{ijklmn} = \mu + P_i + F_j + H_k + B_l + L_m + S(B)_n + PF_{ij} + PH_{ik} + FH_{jk} + PFH_{ijk} + e_{ijklmn}$$

Where Y_{ijklmn} = Ca and P digestibility.

μ = Overall mean of the population.

P_i = Effect of the i^{th} phytase level.

F_j = Effect of the j^{th} limestone particle size (PS).

H_k = Effect of the k^{th} hour.

L_m = Effect of the m^{th} location in tier.

$S(B)_n$ = Effect of the n^{th} stack in block

PF_{ij} = Effect of the ij^{th} interaction between phytase and limestone PS.

PH_{ik} = Effect of the ik^{th} interaction between phytase and hours POP.

FH_{ik} = Effect of the jk^{th} interaction between limestone PS and hour POP.

PFH_{ijk} = Effect of the ijk^{th} interaction between phytase, limestone PS, and hour POP.

Blood iCa data were also analysed with the Proc Mixed model (Statistical Analysis System, 2019) for the average effects. Means and standard error were calculated, and a significant difference of (P<0.05) between means was determined by Fischer's test (Samuels, 1989). Time post POP (hours) were used as repeated measures.The linear mixmodel used is described by the following equation:

$$Y_{ijklmn} = \mu + P_i + F_j + H_k + B_l + L_m + PF_{ij} + PH_{ik} + FH_{jk} + PFH_{ijk} + e_{ijklmn}$$

Where Y_{ijklmn} = Blood iCa

μ = overall mean of the population

P_i = effect of the i^{th} phytase.

F_j = effect of the j^{th} limestone PS.

H_k = effect of the k^{th} hours POP .

L_m = effect of the m^{th} location in tier

PF_{ij} = effect of the ij^{th} interaction between phytase and limestone PS.

PH_{ik} = effect of the ik^{th} interaction between phytase and hours POP.

FH_{ik} = effect of the jk^{th} interaction between limestone PS and hour POP

PFH_{ijk} = effect of the ijk^{th} interaction between phytase, limestone PS, and hour POP.

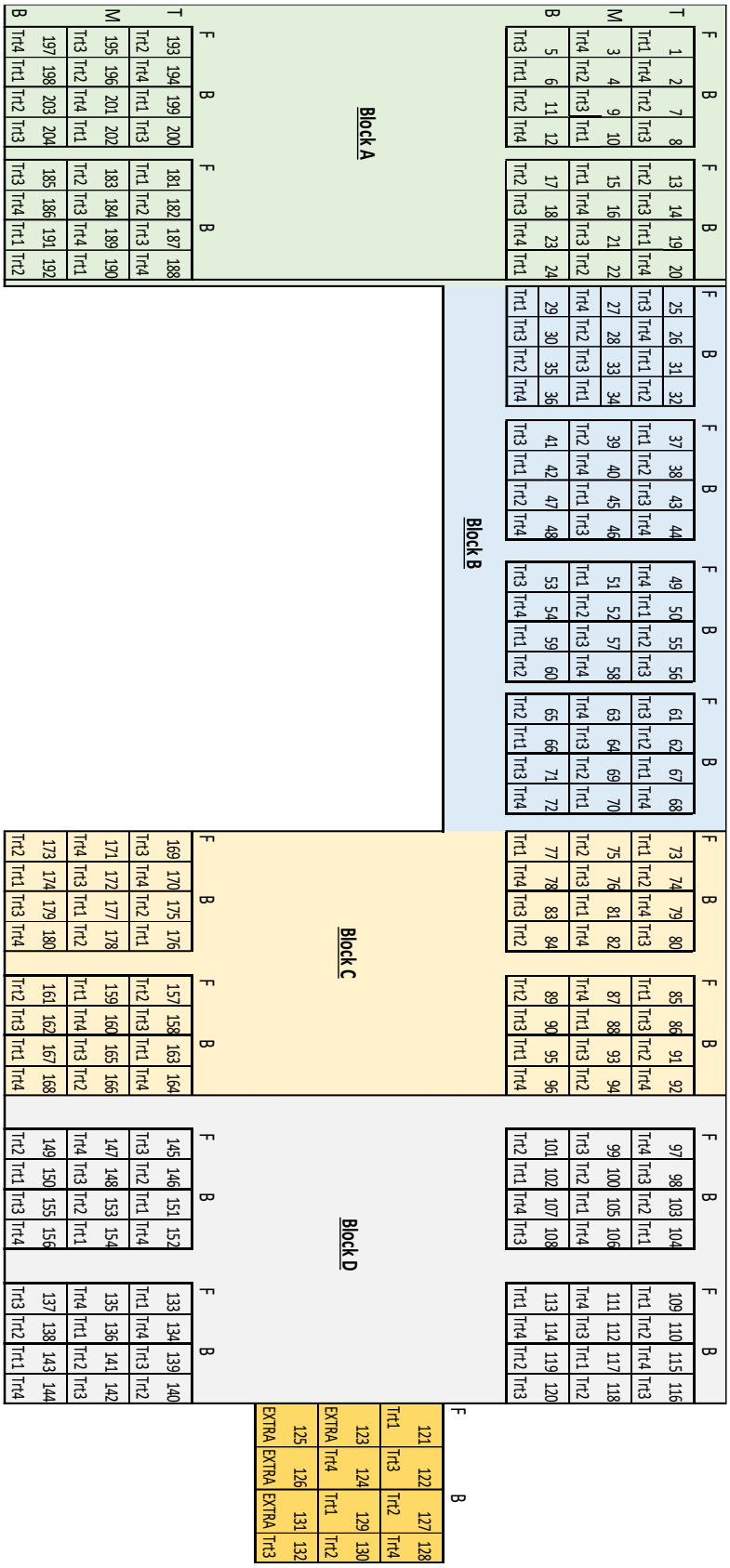


Table 3.17.1 Housing block design

CHAPTER 4 Results

4.1 Calcium and phosphorus digestibility

The largest significant effect on Ca digestibility was the influence of time POP (P<0.01) The Ca digestibility at 11 h POP was significantly greater than the Ca digestibility at 3 h POP (P<0.01) (Table 4.1.4). Ca digestibility was also significantly influenced by a two-way interaction between phytase inclusion and limestone particle size (P<0.01), with Treatment 1 (fine limestone and 600 FTU phytase) having the best Ca digestibility of the four treatments (Table 4.1.4). Limestone particle size had no significant effect on Ca digestibility, and nor did phytase inclusion have an influence on Ca digestibility, as shown in Table 4.1.1. There was no significant interaction between the time POP and the limestone particle size. The Ca digestibility of fine limestone appeared higher at both 3 h and 11 h POP; however, these values were not significantly different from the Ca digestibility of the limestone grit at the respective time periods, as shown in Table 4.1.2. There was no significant interaction between time POP and phytase inclusion level on Ca digestibility (Table 4.1.3).

As shown in Table 4.1.1, the addition of phytase enzymes to the diet had a significant positive effect on P digestibility (P<0.01), while the limestone particle size, however, did not have an effect on P digestibility. Time POP had a significant effect on the P digestibility values, with the P digestibility value being greater 3 h POP than 11 h POP (P<0.01) (Table 4.1.5). A significant two-way interaction was noted between the level of phytase inclusion and the time POP (P<0.05), with the highest P digestibility occurring at 3 h POP with 600 FTU phytase (Table 4.1.3). There was no significant interaction between the limestone particle size and the phytase inclusion level. However, the highest P digestibility was noted with Treatment 1 and this treatment was significant different to the other treatments (P<0.05) (Table 4.1.5). There was no significant interaction between particle size and time POP as shown in Table 4.1.2.

Table 4.1.1 Influence of limestone particle size (PS) and phytase on calcium (Ca) and phosphorus (P) digestibility

Digestibility (%)	PS (mm)		Phytase (FTU)	
	0.25	1.5	0	600
Ca	62.34^{A}	56.99^{A}	60.00^{A}	59.37^{A}
P	36.79^{A}	31.78^{A}	24.00^{B}	44.56^{A}

^{ABC} Row means with the same superscript do not differ significantly from each other (P<0.05).

Table 4.1.2 Interactions of limestone particle size (PS) and time post-oviposition (POP) on calcium (Ca) and phosphorus (P) digestibility

Interactions		Digestibility (%)	
PS (mm)	Time POP (hours)	Ca	P
0.25	3	57.39^{cd}	43.00^{ab}
0.25	11	67.38^{ab}	30.57^{cd}
1.5	3	51.83^{d}	40.20^{b}
1.5	11	62.13^{bc}	23.34^{d}

^{abcd} Column means with the same superscript do not differ significantly from each other (P<0.05).

Table 4.1.3 Interactions of time post-oviposition (POP) and phytase on calcium (Ca) and phosphorus (P) digestibility

Interactions		Digestibility (%)	
Phytase (FTU)	Time POP (hours)	Ca	P
600	3	54.21^{cd}	55.33^{a}
600	11	64.52^{abd}	33.78^{bc}
0	3	55.00^{bc}	27.88^{cd}
0	11	65.01^{a}	20.13^{d}

^{abcd} Column means with the same superscript do not differ significantly from each other (P<0.05).

Table 4.1.4 The influence of limestone particle size (PS), phytase and time post-oviposition (POP) on calcium digestibility

PS (mm)	Phytase (FTU)	Time POP (hours)		
		3	11	$\bar{x}^1$
0.25	600	63.78^{ab}	69.51	66.63^{ab}
0.25	0	51.02^{cdB}	65.26^{A}	58.14^{abc}
1.5	600	44.66^{dB}	59.53^{A}	52.10^{c}
1.5	0	58.98^{bc}	64.78	61.88^{b}
$\bar{x}^2$		54.61^{A}	64.77^{B}	

^{abcd} Column means with the same superscript do not differ significantly from each other (P<0.05).

^{ABC} Row means with the same superscript do not differ significantly from each other.

^{1} Mean values across treatments.

^{2} Mean values across time periods.

Table 4.1.5 The influence of limestone particle size (PS), phytase, and time post-oviposition (POP) on phosphorus (P) digestibility

PS (mm)	Phytase (FTU)	Time POP (hours)		
		3	11	$\bar{x}^1$
0.25	600	58.75^{abA}	40.22^{aB}	49.49^{a}
0.25	0	27.26^{d}	20.93^{c}	24.10^{cd}
1.5	600	51.92^{bcA}	27.36^{bcB}	39.64^{b}
1.5	0	28.50^{cd}	19.33^{cd}	23.92^{d}
$\bar{x}^2$		41.61^{A}	26.96^{B}	

[abcd] Column means with the same superscript do not differ significantly from each other (P<0.05).

[ABC] Row means with the same superscript do not differ significantly from each other (P<0.05).

[1] Mean values across treatments.

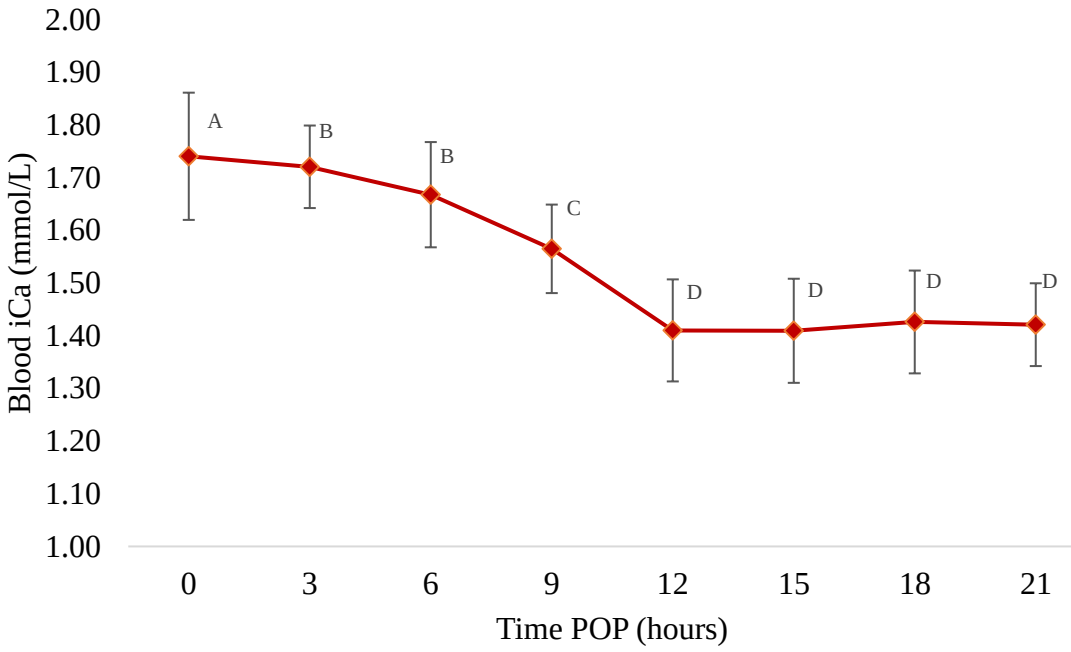
[2] Mean values across time periods

Table 4.1.6 Main and interaction effects on calcium (Ca) and phosphorus (P) digestibility

Ca digestibility		P digestibility	
Source of variance	*P*-value	Source of variation	*P*-value
PS	0.10	PS	0.12
Phytase	0.84	Phytase	<0.01
Time POP[1]	<0.01	Time POP[1]	<0.01
PS x Phytase	<0.01	PS x Phytase	0.13
PS x Time POP	0.96	PS x Time POP	0.51
Phytase x Time POP	0.97	Phytase x Time POP	<0.05

4.2 Blood ionized calcium

Blood iCa was significantly affected by time POP (P<0.001); the blood iCa remained high from 0 h to 6 h POP and decreased rapidly from 9 h to 21 h POP (Figure 4.2.1). There were two-way interactions between limestone particle size (PS) and phytase (P<0.05) (Table 4.2.1), and between time POP and POP (P<0.05) (Table 4.2.2). There was a significant improvement to the blood iCa at 15 h POP with the limestone grit versus the fine limestone (P<0.05). There were no significant effects of limestone PS or phytase on the overall blood iCa level (P>0.05).



[ABC] Data points with the same superscript do not differ significantly from each other (P<0.05).

Figure 4.2.1 The effect of time post-oviposition (POP) on blood ionized calcium (iCa)

Table 4.2.1 Blood ionized calcium (iCa) as affected by the time post-oviposition (POP) and experimental treatment (T)

T	Time POP (hours)								
	0	3	6	9	12	15	18	21	$\bar{x}^1$
1	1.717^{A}	1.702^{AB}	1.683^{AB}	1.594^{B}	1.421^{C}	1.387^{Ca}	1.437^{C}	1.410^{C}	1.544^{a}
2	1.689^{A}	1.654^{A}	1.668^{AB}	1.570^{B}	1.340^{C}	1.333^{Cb}	1.413^{C}	1.407^{C}	1.509^{b}
3	1.722^{A}	1.703^{A}	1.630^{AB}	1.535^{BC}	1.404^{D}	1.408^{CDa}	1.404^{CD}	1.363^{D}	1.521^{a}
4	1.740^{A}	1.719^{A}	1.656^{A}	1.592^{B}	1.440^{C}	1.437^{Ca}	1.439^{C}	1.432^{C}	1.557^{a}

^{abcd} Column means with the same superscript do not differ significantly from each other (P<0.05).

^{ABC} Row means with the same superscript do not differ significantly from each other.

Treatments: T1 (fine limestone, 600 FTU phytase), T2 (fine limestone, 0 FTU phytase, T3 (limestone grit, 600 FTU phytase), T4 (limestone grit, 0 FTU phytase).

[1] Mean values for all time periods over each treatment.

Table 4.2.2 Blood ionized Ca (iCa) as affected by limestone particle size (PS) and time post oviposition (POP)

PS (mm)	Time POP (hours)							
	0	3	6	9	12	15	18	21
0.25	1.70^{A}	1.68^{A}	1.68^{A}	1.58^{B}	1.38^{C}	1.36^{Cb}	1.42^{C}	1.41^{C}
1.5	1.73^{A}	1.71^{AB}	1.64^{B}	1.56^{C}	1.42^{D}	1.42^{Da}	1.42^{D}	1.40^{D}

^{abcd} Column means with the same superscript do not differ significantly from each other (P<0.05)

^{ABC} Row means with the same superscript do not differ significantly from each other. [1]Limestone particle size

Table 4.2.3 Main and interaction effects on blood ionized calcium (iCa)

Source of Variance	F>*P* Value
Limestone PS	0.45
Phytase inclusion	0.97
Limestone PS x phytase	<0.05
Time POP	<0.01
Limestone PS x Time POP	<0.05
Phytase x Time POP	0.72

4.3 Dry eggshell percentage

Dry eggshell percentage, or the PE, is an indication of the Ca deposited versus the total weight of the egg. There was a significant interaction of phytase and limestone particle size (PS) (P<0.01) as shown in Table 4.3.1, with the highest shell percentage appearing in Treatment 3 (limestone grit, 600 FTU phytase). Limestone grit maintained an increased PE (9.71%) in comparison to the fine limestone (9.46%) (P<0.01), as shown in Table 4.3.2.

Table 4.3.1 Effect of treatment on dry eggshell percentage (PE)

PS (mm)	Phytase (FTU)	PE (%)
0.25	600	9.43^{c}
0.25	0	9.49^{c}
1.5	600	9.79^{a}
1.5	0	9.62^{b}

^{abcd} Column means with the same superscript do not differ significantly from each other (P<0.05).

Table 4.3.2 Effect of limestone particle size (PS) on dry eggshell percentage (PE)

PS (mm)	PE (%)
0.25	9.46[b]
1.5	9.71[a]

[abcd] Column means with the same superscript do not differ significantly from each other (P<0.05).

Table 4.3.3 Main and interaction effects on dry eggshell percentage or percentage eggshell (PE)

Source of Variance	F>P Value
Limestone PS	<0.01
Phytase	0.46
Limestone PS x Phytase	<0.01

CHAPTER 5 Discussion

5.1 Calcium digestibility

In this trial, the time POP had a significant effect on Ca digestibility ($P<0.01$). Results showed that the overall Ca digestibility during the period of shell gland inactivity (3 h POP), was 54.61%; however, during the period of eggshell calcification, the hen was able to increase her Ca digestibility to 64.77% (Table 4.1.4). These differences in Ca digestibility confirm previous findings in literature (Hurwitz & Bar 1965; Hurwitz & Bar 1973) and support the theory of a diurnal circadian rhythm in laying hen (Hughes, 1972; Etches, 1987; Lin *et al*., 2018). These differences in digestibility are also supported by increased circulating levels of PTH as previously reported (Van de Velde *et al*., 1984; Singh *et al*.,1986; Johnston *et al*., 2002) and the corresponding increase in 25-$(OH)_2$-D_3 hydroxylation during eggshell formation (Bar & Norman, 1980; Van de Velde *et al*., 1984). The higher Ca digestibility during the scotophase found in this study is supported by the mechanism described by Mongin (1976). Mongin (1976) found that the hen was able to increase the solubility of limestone during the time of eggshell formation and thereby increase the digestibility of Ca from the gut. The hen does this by increasing the amount of HCL secreted by the proventriculus during eggshell formation. The hen increases her Ca absorption from the gut through the above mechanisms in order to supply the large demand for Ca required during eggshell formation.

There was a two-way interaction between limestone PS and phytase on Ca digestibility ($P<0.01$), potentially due to the interactions of Ca ions with the phytate molecule found within the grain in the hens' diet (Selle & Ravindran, 2007). Within a diet containing no phytase, fine limestone in the gizzard releases its Ca ions at a rapid rate (Zhang & Coon, 1997), and these Ca ions bind to the phytate molecule (Graf, 1983). The binding of Ca ions to phytate causes the molecule to precipitate, and the hen's endogenous phytase enzymes are less effective at breaking down the phytate molecule (Tamim *et al*., 2004). The net result is that the P and Ca digestibility of poultry diets containing fine limestone, without phytase, is

lower than diets containing phytase. This was numerically illustrated in the present study (Tables 4.1.4 and 4.1.5).

The addition of exogenous phytase to poultry diet cleaves P ions from the phytate molecules (Driver, 2004) prior to the Ca ions being able to bind to the phytate. The decreased binding of Ca reduces the extent of chelation of the phytate molecule and the result is increased availability of Ca and P to the hen. This result is noted in the present study with Treatment 1 (fine limestone and 600 FTU phytase) having the numerically highest Ca digestibility (66.63%) of the four treatments (Table 4.1.4). The effect of phytase on limestone grit, however, is less prominent in this study, potentially due to the slowly solubilizing limestone grit that was used (Table 3.13.2). The slow release of Ca ions from grit decreases the level of Ca binding and phytate chelation in the digestive tract of the hen. When Ca ions are slowly solubilized from the limestone, the hen may be able to utilize her endogenous phytase enzymes located in the brush border of the epithelial cells more efficiently. This is likely the reason that phytase was more effective at increasing the Ca digestibility of the fine limestone fraction than in the limestone grit fraction. A further factor that may explain the increased Ca digestibility noted between Treatment 1(fine limestone, 600 FTU phytase) and Treatment 3 (limestone grit, 600 FTU phytase) is the overall analysed Ca value of each diet. Treatment 1 had an analysed Ca value of 3.58%, while Treatment 3 had an analysed Ca value of 3.80% (Table 3.4.1). It has been shown in the literature (Lim *et al*., 2003) that increased levels of Ca in the diet will decrease the efficacy of phytase enzymes and decrease the level of Ca available to the hen for digestion.

The influence of phytase alone, however, on Ca digestibility was not significant. This result differs from findings in the literature (Mroz *et al*., 1994; Um & Paik, 1999; Jalal & Scheideler, 2001; Bradbury *et al*., 2016), which showed phytase to increase Ca digestibility. In contrast, and in support of results of the current study, studies by Sohail & Roland (2000); Araujo *et al*. (2011), Englmaierová *et al*. (2017) and Sommerfeld & Schollenberger (2018) showed no effect of phytase on Ca digestibility. The lack of significant effects of phytase in the present study may be due to a high variation in Ca digestibility between hens that resulted in an inability to detect small differences in Ca digestibility from supplemental phytase. The

extent to which phytase will influence the level of Ca available to the hen will depend on the amount of phytate that is not hydrolysed by phytase, as well as the rate at which limestone solubilizes and releases free Ca ions that are able to bind phytate (Sebastian *et al*., 1998). A slowly solubilizing limestone, such as the source used in this trial, may bind fewer Ca ions to the phytate molecule due to the slow release of Ca^{2+}. Consequently, this may have prevented a significant increase in Ca digestibility from phytase in this study.

The influence of limestone PS on overall Ca digestibility in the present study, together with the increased variation in Ca digestibility observed, may be due a high bird-to-bird variation in Ca intake due to selection by individual birds. Brister *et al*. (1981) and Araujo *et al*. (2011) found that a coarser limestone did not increase Ca digestibility in broilers and layers, respectively. In contrast, Anwar *et al*. (2017) showed Ca digestibility in broilers to increase when fed a coarser limestone PS. However, in that study, broilers were fed semi-synthetic diets that did not contain phytate levels typically found in poultry diets. From literature, it would be expected that the limestone grit would provide a higher Ca digestibility value (Anwar *et al*., 2016; Zang *et al*., 2017) due to its slow release of Ca to the hen.

There was no significant interaction between the time POP and limestone PS on Ca digestibility (Table 4.1.2). Similar to the effects of limestone PS, there was also no significant interaction between time POP and phytase on Ca digestibility (Table 4.1.3).

It can be seen from these results that Ca digestibility follows a diurnal circadian rhythm to provide Ca to the hen when she needs it most for eggshell calcification. While neither limestone PS nor phytase had a significant effect on Ca digestibility, the interaction of limestone PS and phytase yielded a numerically higher Ca digestibility value for Treatment 1.

5.2 Phosphorus digestibility

The digestibility of P in the diets supplemented with 600 FTU of phytase were significantly higher ($P<0.01$). Table 4.1.1 shows that the addition of phytase to the diet almost doubles

the P digestibility (24.00% to 44.56%). This is in support of previous research in broilers (Qian *et al*., 1997; Driver, 2004; Rutherford *et al*., 2004; Bradbury *et al*., 2016) and laying hens (Gordon & Roland, 1997; Gordon & Roland, 1998; Keshavarz & Austic, 2004). Present results confirm the efficacy of phytase in providing an improved P supply to the hen to improve bone and eggshell formation.

The current study also investigated the effect of time POP on P digestibility. It was found that P digestibility during active eggshell formation (11 h POP) was significantly lower (P<0.01) than during the period of eggshell gland inactivity (3 h POP) (Table 4.1.1). It is also important to note that during the period of eggshell gland inactivity (3 h POP), the hen is actively adding minerals into the organic matrix of the medullary bone (Dacke *et al*., 1993). The addition of minerals into the bone requires P to form hydroxyapatite crystals needed for bone formation (Whitehead, 2004; Bonjour, 2011). During the period of eggshell calcification, the hen is in a state of hyperphosphatemia due to elevated bone breakdown (Nys *et al*., 1986; Manangi *et al*., 2018), and, therefore, does not require elevated levels of P digestibility from the gut. These results pose an interesting hypothesis that the hen may have a circadian rhythm for P as well as Ca, and that P has a higher digestibility during the early photophase of medullary bone formation and that Ca has a higher digestibility during the later scotophase of eggshell formation.

No significant effect of limestone PS on P digestibility was observed (Table 4.1.1). These results are in support of the laying hen study by Araujo *et al*. (2011), but are not in agreement with observations reported by the broiler studies of Anderson *et al*. (1984), Atia *et al*. (2000) and Manangi & Coon (2007). From relevant literature in broilers, one would expect a strong interaction between limestone PS and phytase. Fine limestone has a greater surface area than limestone grit, meaning that HCL from the proventriculus has a greater area to react upon. Limestone grit has a relatively lower surface area than fine limestone, and the Ca ion release from limestone grit would this be relatively slower. Fine limestone releases its Ca ions very rapidly, depending on the source of the limestone and its geographic composition. The rate of release of the Ca ions will influence how readily they bind to phytate in the crop and gizzard (Manangi & Coon, 2007). As discussed above, the bonding of Ca ions to the phytate

molecule will negatively impact the P digestibility of the hen as she is unable to break down the precipitated phytate molecule using endogenous phytase enzymes (Maenz & Classen, 1997). The limestone source utilized in the present study is one of the slowest solubilizing limestone sources in South Africa (Chemuniqué lab work, 2019), which may explain the lack of significant interaction observed between limestone PS and phytase. The slow release of Ca from this source may have a beneficial effect on P digestibility (Manangi & Coon, 2007). In future experiments, the influence of different limestone sources and PS should be investigated in order to quantify the differences in P digestibility from different South African limestone sources.

A significant two-way interaction occurred between the level of phytase and the time POP (P<0.05)(Table 4.1.3), with the highest P digestibility occurring at 3 h POP with 600 FTU phytase (58.75%). The effect of the combination was significantly different to all the other effect combinations in this table. The interaction noted was likely due to the efficacy of the phytase enzyme to increase P digestibility coupled with the increased digestibility of P during medullary bone remodelling. The result is that the hen is more efficient at absorbing P during medullary bone formation with the addition of phytase to the diet. The practical relevance of this result is to ensure that the hen is consuming sufficient feed during medullary bone formation in order to ensure that she is consuming enough phytase to sustain medullary bone mineralization.

Contrary to what would be expected according to literature (Englmaierová *et al*., 2017), there were no significant interactions between limestone PS and phytase on P digestibility in the present study (Table 4.1.6). Lastly, it was noted that there was no significant interaction between limestone PS and time POP (Table 4.1.6), which illustrated that the increase seen in P digestibility was related more to time than by an interaction between time and PS.

This trial demonstrated that phytase enzymes are highly efficient in increasing P digestibility (P<0.01) in laying hens, as well as that the hen has an increased capacity to digest P during medullary bone formation (P<0.01). There is a significant benefit in providing phytase during the medullary bone formation period (3 h POP) (P<0.05). The investigations of

phytase level and limestone PS have shown beneficial effects in increasing the P available to the laying hen for bone and eggshell formation.

5.3 Blood ionized calcium

Blood iCa is the Ca that is physiologically available for the hen to utilize for functions such as eggshell formation and bone remodelling (Parsons & Combs, 1980). It is an indicator of the Ca status of the hen. Both a deficiency and an excess of Ca will have a negative impact on performance, and therefore the blood iCa is maintained in a strict homeostatic balance (Parsons & Combs, 1980).

The pattern of the blood iCa followed a sinusoidal curve with the initial nine hours post-oviposition (POP) being significantly greater ($P<0.05$) than the remaining 12 hours POP (Figure 4.2.1). The time POP had the greatest influence on blood iCa ($P<0.001$), because the metabolic events of bone remodelling and eggshell formation form at specific periods POP (Parsons & Combs, 1980; Van de Velde & Vermeiden, 1984). The elevated blood iCa from 0 h to 7 h POP (Figure 4.2.1) coincides with the mineralization of medullary bone (Van de Velde & Vermeiden, 1984). During this period, the hen is consuming sufficient soluble Ca to maintain an elevated blood iCa level. The process of egg calcification, however, occurs from six hours to 21 hours POP (Van de Velde *et al*., 1984). During this period, there is a large demand for Ca and the hen is unable to meet the demand through her diet, blood, and bone reserves (Nys *et al*., 1986), which is why a steep drop in blood iCa is noted that directly coincides with the beginning of eggshell formation (Figure 4.2.1).

The Ca status of the hen is directly linked to blood iCa levels. A decrease in blood iCa causes an increase in the release of parathyroid hormone (PTH) (Nys *et al*., 1986). The role of PTH in the body is to increase the blood iCa to maintain a homeostatic balance and prevent hypocalcaemia (Van de Velde *et al*., 1984). PTH increases the rate of bone resorption, and repeated elevated levels of PTH have a negative effect on skeletal integrity (Dacke *et al*., 1993). Factors that limit the decrease in blood iCa during eggshell formation have a positive effect through decreasing the circulating levels of PTH and, therefore bone breakdown. In

the present study, the factors investigated that would improve the Ca status of the hen were the addition of different PS of limestone and the addition of phytase to the diet. Whilst neither PS nor phytase alone had significant effects on the blood iCa level, a result which is supported by Manangi *et al*. (2018), there was a significant interaction between limestone PS and phytase (Table 4.2.1) ($P<0.05$). Table 4.1.2 indicates that Treatment 2 (fine limestone, 0 FTU phytase) had significantly lower blood iCa than Treatment 1 (fine limestone, 600 FTU phytase). The phytase enzymes likely reduced the binding of Ca ions to the phytate molecule which may have played a role in increasing the available Ca to the hen (Bradbury *et al*., 2016).

The interaction between time POP and limestone PS observed is supported by the study done by Manangi *et al*. (2018). The limestone grit significantly improved the blood Ca status of the hen ($P<0.05$) at 15 h POP versus the fine limestone due to the retention of limestone grit in the gizzard during the scotophase. During the scotophase, the hen is no longer consuming feed, and she must rely upon the large particles retained in the gizzard (Lichovnikova, 2007). The grit, therefore, provides a slow release of Ca from the gizzard during eggshell calcification. This result was significant as this is the period of peak eggshell calcification (Sauveur & Mongin, 1983; Van De Velde *et al*., 1984).

5.4 Dry eggshell percentage

Although dry eggshell percentage, or PE, is not the most accurate indication of breaking strength, it is an indication of the hen's Ca status and ability to deposit Ca into the eggshell. The PE decreases when Ca is being utilized from mainly the hen's bone reserves (Sauveur & Mongin, 1983) due to insufficient Ca originating from the diet. In the present study, limestone particle size (PS) had a significant effect on PE (Table 4.3.3). The diets containing limestone grit had an increased PE (9.71%) compared to the diets containing fine limestone (9.46%) ($P<0.01$) (Table 4.3.2) There was also an interaction between limestone PS and phytase ($P<0.01$). This interaction can be seen in the eggshells from Treatment 3 (limestone grit, 600 FTU phytase), where the PE was significantly higher than the other treatments. This

indicates that the addition to phytase, along with a slowly solubilizing limestone grit, has the greatest effect on improving PE. Other findings from this trial indicate that limestone grit maintains a higher blood iCa during eggshell formation (Figure 4.2.1), and this, in turn, will decrease the level of bone breakdown. The decreased utilization of bone, coupled with a more sustained Ca supply from the limestone grit during eggshell formation, may provide evidence as to why the PE of limestone grit is greater than that of fine limestone. The PE results from the present study are supported by Koreleski & Swiatkiewicz (2004), Lichovnikova (2007) and Englmaierová *et al*. (2017), although they are in conflict to the findings by Witt *et al*. (2009), *Araujo et al*. (2010) and Cufadar *et al.* (2011). These results illustrate that the choice of limestone PS and phytase inclusion have an influence on the hen's ability to deposit Ca into the eggshell and that PE be influenced by the Ca status of the hen.

CHAPTER 6 Conclusion

Results from the present study indicated that Ca` and P digestibility, as well as iCa, followed a circadian rhythm that coincided with eggshell formation and bone remodelling.

Ca digestibility was significantly higher during eggshell calcification than during the inactive phase of the eggshell gland. Ca digestibility was also improved by an interaction between phytase and limestone particle size (PS), with Treatment 1 (fine limestone, 600 FTU phytase) having the greatest Ca digestibility.

P digestibility was almost doubled (41.16%) during the phase of medullary bone mineralization (3 h post-oviposition, POP) as compared to a digestibility of 26.96% during the active stage of eggshell calcification (11 h POP). The addition of exogenous phytase vastly improved P digestibility and had its most beneficial effect during the medullary bone mineralization phase due to an interaction between time POP and phytase. Treatment 1 (fine limestone, 600 FTU phytase) yielded the highest P digestibility value (49.49%), which was also significantly greater than the other treatments ($P<0.05$)

Blood iCa was improved during peak eggshell formation by the addition of limestone grit. Blood iCa remained level during the inactive phase of eggshell calcification (between 0 h and 6 h POP) and decreased significantly during active eggshell calcification (between 9 h and 21 h POP). Treatment 1 (fine limestone, 600 FTU phytase) had a significantly lower blood iCa, which will have a long-term negative impact on skeletal integrity. A diet containing sufficiently large particle size limestone grit and 600 FTU phytase is recommended to maintain an elevated blood iCa status.

Dry eggshell percentage (PE) is an indication of the Ca deposited into the eggshell. PE was improved significantly by the treatments containing limestone grit, with the greatest PE occurring in Treatment 3 (limestone grit, 600 FTU phytase). The retention of the limestone grit by the gizzard benefited the deposition of increased Ca into the eggshell and increased PE.

Limestone grit had a beneficial effect on blood iCa and PE. Fine limestone had a positive influence on Ca and P digestibility. The interaction between phytase and limestone PS had a beneficial effect on all parameters measured.

To conclude, it is recommended to provide a laying hen diet that contains a combination of fine limestone and limestone grit that is also supplemented with exogenous phytase enzymes.

CHAPTER 7 Critical review

This study provided several significant findings that could have been improved by reducing the variation.

Recommendations to reduce variation in future studies are as follows:

1. Decrease the level of feed being fed to the hens. The hens in this trial were fed two meals of 65 g per day, which was above the breed recommendation of 120 g per day, resulting in feed remaining in the feeders.
2. Modify the feeders. In the present study, the hens were able to flick their feed from their feeders, resulting in inaccurate feed intake measurements and feed wastage.
3. Pellet the feed. The feed provided in this trial was in mash form. The result was that the hens were selectively feeding, and this varied from hen to hen.

Future study opportunities:

1. Investigate the effects of different limestone sources on Ca and P digestibility in commercial laying hens. This study investigated the effects of limestone particle size from a single source; however, recent literature (Safaa *et al*., 2008) has shown that there are large differences in Ca utilization from different sources of Ca with different solubilities.
2. Investigate the effects of fine and limestone grit ratios and the influence of Ca and P utilization when different ratios are applied to the diet.
3. Investigate the Ca and P utilization in older hens (80-100 weeks). The current industry practice is to extend laying hen production cycles from 80 to 100 weeks. However, there is limited information on the Ca and P requirements in hens past 60 weeks of age.

REFERENCES

Adedokun, S. A., Adeola, O., 2013. Calcium and phosphorus digestibility: Metabolic limits. *Journal of Applied Poultry Research*, 22(3), 600–608.

Afify, A. E. M. R., El-beltagi, H. S., El-salam, S. M. A, Omran, A. A., 2011. Bioavailability of Iron, Zinc, Phytate and Phytase Activity during Soaking and Germination of White Sorghum Varieties. *Plos One*, 10(6), 1–7.

al-Batshan, H. A., Scheideler, S. E., Black, B. L., Garlich, J. D., & Anderson, K. E., 1994. Duodenal calcium uptake, femur ash, and eggshell quality decline with age and increase following molt. *Poultry Science* 73(10), 1590–1596.

Anderson, J. O., Dobson, D. C., & Jack, O. K., 1984. Effect of Particle Size of the Calcium Source on Performance of Broiler Chicks Fed Diets with Different Calcium and Phosphorus Levels. *Poultry Science*, 63, 311–316.

Angel, C. R., 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. *Poultry Science*, 83, 1358–1367.

Anwar, M. N., Ravindran, V., Morel, P. C. H., Ravindran, G., & Cowieson, A. J., 2016. Effect of limestone particle size and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. *British Poultry Science*, 5(57), 707–713.

Anwar, M. N., Ravindran, V., Morel, P. C. H., Ravindran, G., & Cowieson, A. J., 2017. Effect of calcium source and particle size on the true ileal digestibility and total tract retention of calcium in broiler chickens. *Animal Feed Science and Technology*, 224, 39–45.

AOAC. 1942. Official Methods of Analysis. Association of Official Analytical Chemists, Washington, DC.

AOAC. 1998. Official Methods of Analysis. Association of Official Analytical Chemists, Washington, DC.

Applegate, T. J, Angel, R., 2014. Nutrient requirements of poultry publication: History and need for an update. *Applied Poultry Research*, 23(3), 567–565.

Araujo, J. A. De, Humberto, J., Guilherme, F., Costa, P., Maria, J., Sousa, B. De, Sakomura, N. K., 2011. Effect of the levels of calcium and particle size of limestone on laying hens. *Revista Brasileira de Zootecnia*, 997–1005.

Armbrecht, H. J., Hodam, T. L., Boltz, M. A, Kumar, V. B., 1999. Capacity of a low calcium diet to induce the renal vitamin D 1a-hydroxylase is decreased in adult rats. *Biochemical and Biophysical Research Communications*, 255(3), 731–734.

Atia, F. A., Waibel, P. E., Hermes, I., Carlson, C. W., & Walser, M. M., 2000. Effect of Dietary Phosphorus, Calcium, and Phytase on Performance of Growing Turkeys. *Poultry Science*, 79, 231–239.

Atteh, J. O, Leeson, S., 1983. Influence of increasing the calcium and magnesium content of the drinking water on performance and bone and plasma minerals of broiler chickens. *Poultry Science*, 62(5), 869–874.

Bain, M. M., Nys, Y, Dunn, I. C., 2016. Increasing persistency in lay and stabilising egg quality in longer laying cycles. What are the challenges? *British Poultry Science*, 57(3), 330-338.

Baker, S., Herrman, T., 2002. Evaluating Particle Size. *Evaluating Particle Size*, 1–6.

Bannister, D. W, Candlish, J. K., 1973. The collagenolytic activity of avian medullary bone: Effect of laying status and parathyroid extract. *British Poultry Science*, 14(1), 121–125.

Bar, A, Hurwitz, S., 1987. Vitamin D metabolism and calbindin (calcium-binding protein) in aged laying hens. *Journal of Nutrition*, 117(10), 1775–1779.

Bar, A., & Norman, A. W., 1980. Studies on the Mode of Action of Calciferol. Relationship of the Distribution of 25-Hydroxyvitamin D 3 Metabolites to Gonadal Activity and Egg Shell Formation in the Quail. *Endocrinology*, 109, 950–955.

Bar, A., 2009. Calcium transport in strongly calcifying laying birds: Mechanisms and regulation. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, 152(4), 447–469

Bar, A., Razaphkovsky, V, Vax, E., 2002. Re-evaluation of calcium and phosphorus requirements in aged laying hens. *British Poultry Science*, 43(2), 261–269.

Bar, A., Striem, S., Rosenberg, J., & Hurwitz, S., 1988. Egg shell quality and cholecalciferol metabolism in aged laying hens. *Journal of Nutrition*, 118(8), 1018–1023.

Bar, A., Vax, E, Striem, S., 1998. Effects of age at onset of production, light regime and dietary calcium on performance, eggshell traits, duodenal calbindin and cholecalciferol metabolism. *British Poultry Science*, 39(2), 282–290.

Bikle, D. D., 2014.Vitamin D metabolism, mechanism of action, and clinical applications. *Chemistry and Biology* 21(3), 319–329.

Bonjour, J. P., 2011. Calcium and phosphate: A duet of ions playing for bone health. *Journal of the American College of Nutrition*, 30,438–448.

Bradbury, E. J., Wilkinson, S. J., Cronin, G. M., Walk, C. L., & Cowieson, A. J., 2016. Effects of phytase, calcium source, calcium concentration and particle size on broiler performance, nutrient digestibility and skeletal integrity. *Animal Production Science*, 58(2), 271-283.

Brister, R. D., Linton, S. S., & Creger, C. R., 1981. Effects of Dietary Calcium Sources and Particle Size on Laying Hen Performance. *Poultry Science*, 60(12), 2648–2654.

Buss, E. G, Guyer, R. B., 1984. Bone parameters of thick and thin eggshell lines of chickens. *Comparative Biochemistry and Physiology - Part A: Physiology*, 78(3), 449–452.

Candlish, J. K., 1971. The formation of mineral and organic matrix of fowl cortical and medullary bone during shell calcification. *British Poultry Science*. 12(1), 119–127.

Carlos, A. B, Edwards, H. M., 1998. The Effects of 1,25-Dihydroxycholecalciferol and Phytase on the Natural Phytate Phosphorus Utilization by Laying Hens. *Poultry Science*, 77(6), 850–858.

Castillo, C., Cuca, M., Pro, A., González, M, Morales, E, 2004. Biological and economic optimum level of calcium in white leghorn laying hens. *Poultry Science*, *83*(6), 868–872.

Castillo, L., Tanaka, Y., Wineland, M. J., Jowsey, J. O, Deluca, H. F., 1979. Production of 1, 25-dihydroxyvitamin D3 and formation of medullary bone in the egg-laying hen. *Endocrinology* 104(6), 1598–1601.

Chemunique (Pty) Ltd, (2019), *Limestone analysis*, (unpublished).

Cheng, T. K, Coon, C. N., 1990. Comparison of Various In Vitro Methods for the Determination of Limestone Solubility. *Poultry Science*, 69(12), 2204–2208.

Cheng, T. K, Coon, C. N., 1990. Effect of calcium source, particle size, limestone solubility in vitro, and calcium intake level on layer bone status and performance. *Poultry Science*, 69(12), 2214–2219.

Cornish, A., Ashton, B., Raubenheimer, D, McGreevy, P., 2019. Australian Consumers' Knowledge and Concern for Animal Welfare in Food Production: Influences on Purchasing Intention. *Society & Animals*, 1–28.

Costa, F. G. P., Goulart, C. C., Figueiredo, D. F., Oliveira, C. F. S, Silva, J. H. V., 2008. Economic and environmental impact of using exogenous enzymes on poultry feeding. *International Journal of Poultry Science*, 7(4), 311–314.

Cufadar Y., Olgun, O., & Yildiz, A. Ö., 2011. The effect of dietary calcium concentration and particle size on performance, eggshell quality, bone mechanical properties and tibia mineral contents in moulted laying hens. *British Poultry Science*, 52(6), 761–768.

Dacke, C. G., Arkle, S., Cook, D. J., Wormstone, I. M., Jones, S., Zaidi, M., & Bascal, Z., 1993. Medullary Bone and Avian Calcium Regulation. *Journal of Experimental Biology*, 88, 63–88.

Dacke, C. G., Boelkins, J. N., Smith, W. K, Kenny, A. D., 1972. Plasma calcitonin levels in birds during the ovulation cycle. *The Journal of Endocrinology*, 54(2), 369–370.

Davidson, J, Boyne, A. W., 1970. The calcium and phosphorus requirements of laying hens. *British Poultry Science*, 11(2), 231–240.

De Los Mozos, J., & Sanchez, F., 2014. Split feeding meets layers' specific needs. Retrieved from https://www.poultryworld.net/Nutrition/Articles/2014/6/Split-feeding-meets-layers-specific-needs-1474191W/

de Matos, R., 2008. Calcium Metabolism in Birds. Veterinary Clinics of North America - *Exotic Animal Practice*, 11(1), 59–82.

Dilger, R. N., Onyango, E. M., Sands, J. S, Adeola, O., 2004. Evaluation of Microbial Phytase in Broiler Diets. *Poultry Science.*, 83, 962–970.

Driver, J. P., 2004. *Performance and bone quality of the modern broiler chicken as affected by dietary calcium, phosphorus, phytase and 1-alpha-hydroxycholecalciferol*. PhD thesis. Univ. of Georgia, Athens, Georgia

Elaroussi, M. A., Forte, L. R., Eber, S. L, Biellier, H. V., 1994. Calcium Homeostasis in the laying hen. Age and dietary calcium effects. *Poultry Science.*, 73, 1581–1589.

Englmaierová, M., Skřivan, M., Skřivanová, E., Čermák, L., & Englmaierov, M. (2017). Limestone particle size and Aspergillus niger phytase in the diet of older hens. *Italian Journal of Animal Science*.

Etches, R. J., 1987. Calcium logistics in the laying hen. *Journal of Nutrition*. 117, 619–628.

Eusebio-Balcazar, P. E., Purdum, S., Hanford, K., & Beck, M. M., 2018. Limestone particle size fed to pullets influences subsequent bone integrity of hens. *Poultry Science*, 97(5), 1471–1483.

Fisher, L. W, Schraer, H., 1982. Keratan sulfate proteoglycan isolated from the estrogen-induced medullary bone in Japanese quail. *Comparative Biochemistry and Physiology* 72(2), 227–232.

Fleming, R. H., 2008. Nutritional factors affecting poultry bone health. *Proceedings of the Nutrition Society*, 67(2), 177–183.

Fleming, R. H., Mccormack, H. A., & Whitehead, C. C., 1998. Bone structure and strength at different ages in laying hens and effects of dietary particulate limestone, vitamin K and ascorbic acid Bone structure and strength at different ages in laying hens and effects of dietary particulate limestone and vitamin K. *British Poultry Science*, 39, 434–440.

Fraser, D, Kodicek, E., 1973. Regulation of 25-hydroxycholecalciferol-1-hydroxylase activity in the kidney by parathyroid hormone. *Nature: New Biology*, 241(110), 163-166

Goodland, R., 1997. Environmental sustainability in agriculture: diet matters. *Ecological Economics*, 23, 189–200.

Gordon, R. W., & Roland, D. A., 1997. Performance of Commercial Laying Hens Fed Various Phosphorus Levels, with and Without Supplemental Phytase. *Poultry Science*, 76(8), 1172–1177.

Gordon, R. W., & Roland, D. A.,1998. Influence of Supplemental Phytase on Calcium and Phosphorus Utilization in Laying Hens. *Poultry Science*, 77(2), 290–294.

Graf, E., 1983. Calcium Binding to Phytic Acid. *Journal of Agricultural and Food Chemistry*, 31(4), 851–855.

Guinotte, F, Nys, Y., 1991. Effects of Particle Size and Origin of Calcium Sources on Eggshell Quality and Bone Mineralization in Egg Laying Hens. *Poultry Science*. 70(3), 583–592.

Guinotte, F., Gautron, J., Nys, Y, Soumarmon, A., 1995. Calcium solubilization and retention in the gastrointestinal tract in chicks (Gallus domesticus) as a function of gastric acid secretion inhibition and of calcium carbonate particle size. British Journal of Nutrition, 73(01),125-139.

Hamdi, M., Solà-Oriol, D., Davin, R., & Perez, J. F., 2015. Calcium sources and their interaction with the different levels of non-phytate phosphorus affect performance and bone mineralization in broiler chickens. *Poultry Science*, 94(9), 2136–2143.

Harms, R. H, Waldroup, P. W., 1982. The effects of high dietary calcium on the performance of laying hens. *Poultry Science*, 50(3), 967.969.

Harms, R. H., Douglas, C. R., & Sloan, D. R., 1996. Midnight feeding of commercial laying hens can improve eggshell quality. *Journal of Applied Poultry Research*, 5(1), 1–5.

Hein, L, Leemans, R., 2012. The impact of first-generation biofuels on the depletion of the global phosphorus reserve. *Ambio*, 41(4), 341–349.

Heuser, G. F, Norris, L. C., 1945. Oyster Shells, Calcite Grit, Ground Limestone, and Granite Grit in Rations for Hens. *Poultry Science*, 25(2), 173–179.

Hoenderop, J. G. J., Nilius, B, Bindels, R. J. M., 2003. Epithelial calcium channels: From identification to function and regulation. *Pflugers Archiv European Journal of Physiology*, 446(3), 304–308.

Holt, P. S., Davies, R. H., Dewulf, J., Gast, R. K., Huwe, J. K., Jones, D. R, Willian, K. R., 2011. The impact of different housing systems on egg safety and quality. *Poultry Science*, 90(1), 251–262.

Horikawa, H., Sugahara, M., Kubota, M., Masumura, T., Abe, E, Suda, T., 1982. Disorders of Cholecalciferol Metabolism in Old Egg-Laying Hens. *The Journal of Nutrition*, 112(3), 436–446.

Hudson, H. A., Britton, W. M., Rowland, G. N, Buhr, R. J., 1993. Histomorphometric bone properties of sexually immature and mature White Leghorn hens with evaluation of fluorochrome injection on egg production traits. *Poultry Science*. 72(8), 1537–1547.

Hughes, B. O., 1972. A circadian rhythm of calcium intake in the domestic fowl. *British Poultry Science*, 13(5), 485–493.

Humer, E., Schwarz, C, Schedle, K., 2012. Phytate in pig and poultry nutrition. *Journal of Animal Physiology and Animal Nutrition*, 99(4), 605–625.

Hurwitz, S, Bar, A., 1965. Absorption of calcium and phosphorus along the gastrointestinal tract of the laying fowl as influenced by dietary calcium and eggshell formation. *Journal of Nutrition*, 86(840), 433–438.

Hurwitz, S, Bar, A., 1969. Calcium Reserves in Bones of Laying Hens: Their Presence and Utilization. *Poultry Science*. 48(4), 1391–1396.

Hurwitz, S, Bar, A., Cohen, I., 1973. Regulation of calcium absorption by fowl intestine. *American Journal of Physiology*, 225(1), 150-154.

Hurwitz, S, Griminger, P., 1962. Estimation of calcium and phosphorus requirements in laying hens by balance techniques. *Journal of Science for Food and Agriculture*, 13, 185–191.

Hurwitz, S., Wax, E., Nisenbaum, Y., Ben-Moshe, M, Plavnik, I., 1998. The Response of Laying Hens to Induced Molt as Affected by Strain and Age. *Poultry Science*, 77(1), 22–31.

Hy-line International, 2014.*Performance Standards*. Retrieved from https://www.hyline.com/userdocs/pages/PERF_STDS_2-12-15.pdf

Hy-line International, 2018. *Hy-line International Management Guide*. Retrieved from https://www.hyline.com/UserDocs/Pages/BRN_COM_ENG.pdf

Izat, A. L., Gardner, F. A, Mellor, D. B., 1985. Effects of Age of Bird and Season of the Year on Egg Quality 1. Shell Quality. *Poultry Science*, 64(10), 1900–1906.

Jalal, M. A, Scheideler, S. E., 2001. Effect of supplementation of two different sources of phytase on egg production parameters in laying hens and nutrient digestibility. *Poultry Science*, 80, 1463–1471.

Jing, M., Zhao, S., Rogiewicz, A., Slominski, B. A, House, J. D., 2018. Assessment of the minimal available phosphorus needs of pullets during the pre-laying period. *Poultry Science*. 97(2), 557–567.

Johnston, M. S., Ivey, E. S., & Abvp, D., 2002. Parathyroid and Ultimobranchial Glands: Calcium Metabolism in Birds. *Seminars in Avian and Exotic Pet Medicine*

Juanpere, J, Brufau, J., 2004. Effect of microbial phytase on broilers fed barley-based diets in the presence or not of endogenous phytase. *Animal Feed Science and Technology*, 115, 265–279.

Kaspers, B., 2016. An egg a day – the physiology of egg formation. *Lohmann Information*. 13–15.

Kavanagh, S., Lynch, P. B., Mara, F. O., & Caffrey, P. J., 2001. A comparison of total collection and marker technique for the measurement of apparent digestibility of diets for growing pigs. *Animal Feed Science and Technology*, 89, 49–58.

Kebreab, E., France, J., Kwakkel, R. P., Leeson, S., Darmani Kuhi, H, Dijkstra, J., 2009. Development and evaluation of a dynamic model of calcium and phosphorus flows in layers. *Poultry Science*, 88(3), 680–689.

Kenny, A. D., 1971. Determination of calcitonin in plasma by bioassay. *Endocrinology*, 89(4), 1005–1013.

Kenny, A. D., 1986. Parathyroid and Ultimobranchial Glands. *Avian Physiology* 466–478.

Kerschnitzki, M., Zander, T., Zaslansky, P., Fratzl, P., Shahar, R, Wagermaier, W., 2014. Rapid alterations of avian medullary bone material during the daily egg-laying cycle. *Bone*, 69, 109–117.

Keshavarz, K, Nakajima, S., 1993. Re-Evaluation of Calcium and Phosphorus Requirements of Laying Hens for Optimum Performance and Eggshell Quality. *Poultry Science.*, 72, 144–153.

Keshavarz, K., & Austic, R. E., 2004. The use of low-protein, low-phosphorus, amino acid- and phytase-supplemented diets on laying hen performance and nitrogen and phosphorus excretion. *Poultry Science*, 83(1), 75–83.

Keshavarz, K., 2003. A Comparison Between Cholecalciferol and 25-OH-Cholecalciferol on Performance and Eggshell Quality of Hens Fed Different Levels of Calcium and Phosphorus 1. *Poultry Science*, 82, 1415–1422

Kim, S. W., Li, W., Angel, R., & Proszkowiec-Weglarz, M., 2018. Effects of limestone particle size and dietary Ca concentration on apparent P and Ca digestibility in the presence or absence of phytase. *Poultry Science*, 97(12), 4306–4314.

Kim, W. K., Bloomfield, S. A., Sugiyama, T, Ricke, S. C., 2012. Concepts and methods for understanding bone metabolism in laying hens. *World's Poultry Science Journal*, 68(1), 71–82.

Kim, W. K., Donalson, L. M., Bloomfield, S. A., Hogan, H. A., Kubena, L. F., Nisbet, D. J, Ricke, S. C., 2007. Molt performance and bone density of cortical, medullary, and cancellous bone in laying hens during feed restriction or alfalfa-based feed molt. *Poultry Science*, 86(9), 1821–1830.

Klandorf, H., Boyce, C. S., Killefer, J., McGowan, J. A., Peterson, R. A., Valent, M, Deaver, D. R., 1997. The effect of photoperiod and food intake on daily changes in plasma calcitonin in broiler breeder hens. *General and Comparative Endocrinology*, 107(3), 327–340.

Koreleski, J, Świątkiewicz, S., 2004. Calcium from limestone meal and grit in laying hen diets - effect on performance, eggshell and bone quality. *Journal of Animal Feed Science* 13(4), 635–645.

Krehbiel, C., 2013. The role of new technologies in global food security: Improving animal production efficiency and minimizing impacts. *Animal Frontiers*, 3(3), 4–7.

Lambert, W., Van Krimpen & M., Star, L., 2014. Phosphorus Requirement in Laying Hens *Report November 2014*.

Lay, D. C., Fulton, R. M., Hester, P. Y., Karcher, D. M., Kjaer, J. B., Mench, J. A, Porter, R. E., 2011. Hen welfare in different housing systems. *Poultry Science*, 90(1), 278–294.

Li, X., Zhang, D, Bryden, W. L., 2017. Calcium and phosphorus metabolism and nutrition of poultry: Are current diets formulated in excess? *Animal Production Science* 57(11), 2304–2310.

Lichovnikova, M., 2007. The effect of dietary calcium source, concentration and particle size on calcium retention, eggshell quality and overall calcium requirement in laying hens. *British Poultry Science*, 48(1), 71–75.

Lim, H. S., Namkung, H., & Paik, I. K., 2003. Effects of phytase supplementation on the performance, egg quality, and phosphorous excretion of laying hens fed different levels of dietary calcium and nonphytate phosphorous. *Poultry Science*, 82(1), 92–99.

Lin, X., Liu, Y., Xie, C., Wu, X, Yin, Y., 2018. Circadian rhythms and dynamic dietary calcium feeding affect laying performance, calcium and phosphorus levels in laying hens. *Biological Rhythm Research*, 49(2), 227–236.

Lohmann Tierzucht, 2018. *Hy-line International Management Guide*. Retrieved from http://www.ltz.de/en/e-guide/new_e-guide/HTML/

Luck, M. R., Sommerville, B. A., & Scanes, C. C.,1980. The effect of egg-shell calcification on the response of plasma calcium activity to parathyroid hormone and calcitonin in the domestic fowl (Gallus domesticus). *Comparative Biochemistry and Physiology -- Part A: Physiology*, 65, 151–154.

Lutz, W., Sanderson, W, Scherbov, S., 2014. Doubling of world population unlikely. *Nature*, 387(6635), 803-805.

Maddaiah, V., Kurnick, A, Reid, B., 1964. Phytic Acid Studies. *Proceedings of the Society of Experimental Biology and Medicine* 115, 391–393.

Maenz, D. D., & Classen, H. L., 1997. Phytase Activity in the Small Intestinal Brush Border Membrane of the Chicken Preparation of Chicken Intestinal BBV Calculation of Total Brush Border Phytase Activity in Sections of Small Intestine Assay of Phytate Hydrolysis by BBV Assay of Nonspecific P. *Poultry Science.*, 77(4), 557–563.

Manangi, M. K., & Coon, C. N., 2007. The effect of calcium carbonate particle size and solubility on the utilization of phosphorus from phytase for broilers. *International Journal of Poultry Science*, 6(2), 85–90.

Manangi, M. K., Maharjan, P., & Coon, C. N., 2018. Calcium particle size effects on plasma, excreta, and urinary Ca and P changes in broiler breeder hens. *Poultry Science*, 97(8), 2798–2806.

Martin, M. P., Wineland, A. C. M, Barnesa, H. J., 2010. Selected Blood Chemistry and Gas Reference Ranges for Broiler Breeders Using the i-STAT ® Handheld Clinical Analyzer. *Avian Diseases*, 54(3), 1016–1020.

Menezes-Blackburn, D., Gabler, S., & Greiner, R., 2015. Performance of Seven Commercial Phytases in an in Vitro Simulation of Poultry Digestive Tract. *Journal of Agricultural and Food Chemistry*, 63(27), 6142–6149.

Herrman, T., & Harner, J.P., 1995. *Evaluating Particle Size*. Kansas State University Agricultural Experiment Station and Cooperative Extension Service. Manhattan, KS.

Miles, R. D., Costa, P. T., & Harms, R. H., 1983. The influence of dietary phosphorus level on laying hen performance, eggshell quality, and various blood parameters. *Poultry Science*, 62(6), 1033–1037.

Milhaud, G., Rankin, J. C., Bolis, L., & Benson, A. A., 1977. Calcitonin: Its hormonal action on the gill. *Proceedings of the National Academy of Sciences of the United States of America*, 74(10), 4693–4696.

Miller, S. C., 1978. Rapid activation of the medullary bone osteoclast cell surface by parathyroid hormone. *Journal of Cell Biology*, 76(3), 615–618.

Mitchell, R. D, Edwards, H. M., 1995. Effects of 1, 25-Dihydroxycholecalciferol and Phytase on Phytate Phosphorus Utilization and Related Parameters in Broiler Chickens. *Poultry Science*, 75(1), 95-110.

Mohammed, A., Gibney, M. J., & Taylor, T. G., 1991. The effects of dietary levels of inorganic phosphorus, calcium and cholecalciferol on the digestibility of phytate-P by the chick. *British Journal of Nutrition*, 66(2), 251–259.

Molnár, A., Kempen, I., Sleeckx, N., Zoons, J., Maertens, L., Ampe, B., Buyse, J, Delezie, E., 2018. Effects of split feeding on performance, egg quality, and bone strength in brown laying hens in aviary system. *Journal of Applied Poultry Research*, 27(3), 1–15.

Mongin, P., 1976. Composition of crop and gizzard contents in the laying hen, *British Poultry Science*,17(5), *37*–41.

Moore, E. W., 1970. Ionized Calcium in Normal Serum, Ultrafiltrates, and Whole Blood Determined by Ion-Exchange Electrodes. *The Journal of Clinical Investigation*, (49), 318-334.

Mroz, Z., Jongbloed, A. W., & Kemme, P. A., 1994. Apparent digestibility and retention of nutrients bound to phytate complexes as influenced by microbial phytase and feeding regimen in pigs. *Journal of Animal Science*, 72(1), 126–132.

Nash, K. L., Jensen, M. P, Schmidt, M. A., 1998. Actinide immobilization in the subsurface environment by in-situ treatment with a hydrolytically unstable organophosphorus complexant: Uranyl uptake by calcium phytate. *Journal of Alloys and Compounds*, 273, 257–261.

Neijat, M., House, J. D., Guenter, W., & Kebreab, E., 2011. Calcium and phosphorus dynamics in commercial laying hens housed in conventional or enriched cage systems. *Poultry Science*, 90(10), 2383–2396.

Nicol, C. J., Brown, S. N., Glen, E., Pope, S. J., Short, F. J., Warriss, P. D., Wilkins, L. J., 2006. Effects of stocking density, flock size and management on the welfare of laying hens in single-tier aviaries. *British Poultry Science*, 47(2), 135–146.

NRC. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

NRC. 1997. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Nys, Y., & Guyot, N., 2011. *Egg formation and chemistry*. *Improving the Safety and Quality of Eggs and Egg Products: Egg Chemistry, Production and Consumption*. Woodhead Publishing Limited.

Nys, Y., N'Guyen, T. M., Williams, J., & Etches, R. J., 1986. Blood levels of ionized calcium, inorganic phosphorus, 1,25-dihydroxycholecalciferol and gonadal hormones in hens laying hard-shelled or shell-less eggs. *Journal of Endocrinology*, 111(1), 151–157.

Odgaard, A., 1997. Three-Dimensional Methods for Quantification of Cancellous Bone Architecture. *Bone*, 20(4), 315–328.

Owen, J. C., 2011. Collecting, processing, and storing avian blood: A review. *Journal of Field Ornithology*, 82(4), 339–354.

Parsons, A. H, Combs, G. F., 1980. Blood Ionized Calcium Cycles in the Chicken. *Poultry Science*, 60(7), 1520–1524.

Pelicia, K., Garcia, G., Faitarone, A. B. G., Silva, A. P., Berto, D. A., Molino, A. B., & Vercese, F., 2009. Calcium and available phosphorus levels for laying hens in second production cycle. *Revista Brasileira de Ciencia Avicola*, 11(1), 39–49.

Pines, M., & Polin, D., 1981. Urinary Cyclic AMP Excretion in Birds: Dependence Hormone Activity on Parathyroid. *General and Comparative Endocrinology*, (40), 90–96.

Proszkowiec-Weglarz, M, Angel, R., 2013. Calcium and phosphorus metabolism in broilers: Effect of homeostatic mechanism on calcium and phosphorus digestibility. *Journal of Applied Poultry Research*. 22(3), 609–627.

Qian, H., Kornegay, E. T., & Denbow, D. M., 1997. Utilization of Phytate Phosphorus and Calcium as Influenced by Microbial Phytase, Cholecalciferol, and the Calcium: Total Phosphorus Ratio in Broiler Diets. *Poultry Science*, 76(1), 37–46.

Rao, K. S, Roland, D. A., 1989. Influence of dietary calcium level and particle size of calcium source on in vivo calcium solubilization by commercial Leghorns. *Poultry Science*. 68(11), 1499–1505.

Rao, K. S., & Roland, D. A., 1990. In vivo limestone solubilization in commercial Leghorns: role of dietary calcium level, limestone particle size, in vitro limestone solubility rate, and the calcium status of the hen. *Poultry Science*, 69(12), 2170–2176.

Ravindran, V., Hew, L. I., Ravindran, G., & Bryden, W. L., 1999. A comparison of ileal digesta and excreta analysis for the determination of amino acid digestibility in food ingredients for poultry. *British Poultry Science*, *40*(2), 266–274.

Roland, D. a, Sloan, D. R, Harms, R. H., 1971. Calcium Metabolism in the Laying Hen. *Poultry Science*. 51(4467), 598–601.

Roland, D. A., Sloan, D. R., & Harms, R. H., 1973. Calcium Metabolism in the Laying Hen: 1. Calcium Retention in the Digestive Tract of the Laying Hen. *Poultry Science*, 52(1), 351–354.

Roland, S., 1986. Eggshell Quality IV: Oyster shell versus limestone and the importance of particle size or solubility of calcium source. *World Poultry Science Journal* 42(2), 166–171.

Rutherfurd, S. M., Chung, T. K., Morel, P. C. H., & Moughan, P. J., 2004. Effect of Microbial Phytase on Ileal Digestibility of Phytate Phosphorus, Total Phosphorus, and Amino Acids in a Low-Phosphorus Diet for Broilers. *Poultry Science*, 83, 61–68.

Safaa, H. M., Serrano, M. P., Valencia, D. G., Frikha, M., Jiménez-Moreno, E., & Mateos, G. G., 2008. Productive performance and egg quality of Brown egg-laying hens in the

late phase of production as influenced by level and source of calcium in the diet. *Poultry Science*, 87(10), 2043–2051.

Sales, J., & Janssens, G.P.J., 2005. The use of markers to determine energy metabolizability and nutrient digestibility in avian species. *World Poultry Science Journal*, 59(03), 314–327.

Samuals, M.L., 1989. Statistics for the life sciences. Collier MacMillan Publishers, London.

Sauveur, B, Mongin, P., 1983. Plasma inorganic phosphorus concentration during eggshell formation. II. - Inverse relationships with intestinal calcium content and eggshell weight. *Reproduction Nutrition Development*, 23(4), 755–764.

Schaal, T. P., Arango, J., Wolc, A., Brady, J. V., Fulton, J. E., Rubinoff, I., O'sullivan, N. P., 2016. Commercial Hy-Line W-36 pullet and laying hen venous blood gas and chemistry profiles utilizing the portable i-STAT®1 analyzer. *Poultry Science*, 95(2), 466–471.

Scott, M. L., Hull, S. J, Mullenhoff, P. A., 1971. The calcium requirements of laying hens and effects of dietary oyster shell upon eggshell quality. *Poultry Science*. 50(4), 1055–1063.

Sebastian, S., Touchburn, S. P., & Chavez, E. R., 1998. Implications of phytic acid and supplemental microbial phytase in poultry nutrition: a review. *World's Poultry Science Journal*, 54, 28–47.

Selle, P. H., & Ravindran, V., 2007. Microbial phytase in poultry nutrition, *Journal of animal feed science*, 135, 1–41.

Selle, P. H., Cowieson, A. J, Ravindran, V., 2009. Consequences of calcium interactions with phytate and phytase for poultry and pigs. *Livestock Science*, 124(1–3), 126–141.

Shafey, T. M., Mcdonald, M. W, Dingle, J. G., 1991. Effects of dietary calcium and available phosphorus concentration on digesta PH and on the availability of calcium, iron,

magnesium and zinc from the intestinal contents of meat chickens. *British Poultry Science*. 32(1), 185–194.

Simkiss, K., 1961. Calcium metabolism and avian reproduction. *Biological Reviews*, 36(3), 321–359.

Singh, R., Joyner, C. J., Peddie, M. J., & Taylor, T. G., 1986. Changes in the concentrations of parathyroid hormone and ionic calcium in the plasma of laying hens during the egg cycle in relation to dietary deficiencies of calcium and vitamin D. *General and Comparative Endocrinology*, 61(1), 20–28.

Siqueira, J., Sakomura, N., Dourado, L., Marcato, S., Silva, J., Fernandes, J., & Pinheiro, S., 2010. Poultry feed metabolizable energy determination using total or partial excreta collection methods. *Revista Brasileira de Ciência Avícola*, 12(2), 129–132.

Snow, J., Douglas, M, Parsons, C., 2003. Phytase effects on amino acid digestibility in molted laying hens. *Poultry Science*. 82, 474–477.

Sohail, S. S., & Roland, D. A., 2000. Influence of phytase on calcium utilization in commercial layers. *Journal of Applied Poultry Research*, 9(1), 81–87.

Sommerfeld, V., & Schollenberger, M., 2018. Interactive effects of phosphorus, calcium, and phytase supplements on products of phytate degradation in the digestive tract of broiler chickens. *Poultry Science.*, 1, 1–12.

South African Poultry Association, 2018. *Egg Industry Production Report for January 2018*. Available at: http://www.sapoultry.co.za/pdf-statistics/egg-industry.pdf.

Split feeding system for more sustainable egg production 2014. Available at: http://www.thepoultrysite.com/articles/3091/split-feeding-system-for-more-sustainable-egg-production/.

Statistical Analysis Systems, 2019. SAS User's Guide : Statistics Version 9.4 SAS Institute Inc. Cary, NC, USA.

Steinmetz, H. W., Vogt, R., Kästner, S., Riond, B, Hatt, J. M., 2007. Evaluation of the i-STAT portable clinical analyser in chickens (Gallus gallus). *Journal of Veterinary Diagnostic Investigation,* 19(4), 382–388.

Sturkie, P., 1998. *Avian physiology*. Fakulta¨fur Psychologie Ruhr-Universitat Bochum 44780, Academic Press.

Świątkiewicz, S., Arczewska-Włosek, A., Krawczyk, J., Puchała, M., & Józefiak, D., 2015. Effects on performance and eggshell quality of particle size of calcium sources in laying hens' diets with different Ca concentrations. *Archiv Tierzucht*, 58(2), 301–307.

Tamim, N. M., Angel, R., & Christman, M., 2004. Influence of Dietary Calcium and Phytase on Phytate Phosphorus Hydrolysis in Broiler Chickens. *Poultry Science*, 83, 1358–1367.

Taylor, P., Cransberg, P. H., Parkinson, G. B., Wilson, S., & Thorp, B. H., 2010. Sequential studies of skeletal calcium reserves and structural bone volume in a commercial layer flock Sequential studies of skeletal calcium reserves and structural bone volume in a commercial layer flock, *British Poultry Science*, 42(2), 260-265.

Tiggelaar, R. M., Verdoold, V., Eghbali, H., Desmet, G, Gardeniers, J. G. E., 2009. Characterization of porous silicon integrated in liquid chromatography chips. *Royal Society of Chemistry,* (3)1–5.

Um, J. S., & Paik, I. K., 1999. Effects of microbial phytase supplementation on egg production, eggshell quality, and mineral retention of laying hens fed different levels of phosphorus. *Poultry Science*, 78(1), 75–79.

Vaccari, D. A., 2009. Phosphorus: A looming crisis. *Scientific American*, 300(6), 54–59.

Van De Velde, J. P., & Vermeiden, J. F. W., 1984. Changes in Activity of Chicken Medullary Bone Cell Populations in Relation to the Egg-laying Cycle. *Metabolic Bone Disorders and Related Research*, 193, 191–193.

Van De Velde, J. P., Loveridge, N., & Vermeiden, J. P. W., 1984. Parathyroid hormone responses to calcium stress during eggshell calcification. *Endocrinology*, 115(5), 1901–1904.

Van Der Klis, J. D., Versteegh, H. A. J., Simons, P. C. M, Kies, A. K., 1997. The Efficacy of Phytase in Corn-Soybean Meal-Based Diets for Laying Hens. *Poultry Science*. 76(11), 1535–1542.

Van Vuuren, D. P., Bouwman, A. F., & Beusen, A. H. W., 2009. Phosphorus demand for the 1970-2100 period: A scenario analysis of resource depletion. *Global Environmental Change*, 20(3), 428–439.

Vaughn, S., 2012. FASS Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching. *Journal of the American Association for Laboratory Animal Science* . 51. 298-300.

Veum, T.L., 2010. Phosphorus and Calcium Nutrition and Metabolism. *Phosphorus and Calcium Utilization and Requirements in Farm Animals*, 94-111.

Wang, T., Ragland, D., & Adeola, O., 2016. Investigations of marker and fiber effects on energy and nutrient utilization in growing pigs. *Journal of Animal Science*, 94, 473.

Whitehead, C. C., 2004. Overview of bone biology in the egg-laying hen. *Poultry Science*. 83(2), 193–199.

Wilcox, R., Deyoe, C., & PFost, H., 1962. A Method for Determining and Expressing the Size of Feed Particles by Sieving. *Poultry Science.*, 9–13.

Wilson, S., Solomon, S. E., & Thorp, B. H., 1998. Bisphosphonates: A potential role in the prevention of osteoporosis in laying hens. *Research in Veterinary Science*, 64(1), 37–40.

Witt, F. H. De, Kuleile, N. P., Merwe, H. J. Van Der, & Fair, M. D., 2009. Effect of Limestone Particle Size on Bone Quality of Layers. *South African Journal of Animal Sciences*, 39, 41-44

Zhang, B., & Coon, C. N., 1997. The Relationship of Calcium Intake, Source, Size, Solubility In Vitro and In Vivo, and Gizzard Limestone Retention in Laying Hens. *Poultry Science.* 76(12), 1702–1706.

Zhang, F., & Adeola, O., 2017. Techniques for evaluating digestibility of energy, amino acids, phosphorus, and calcium in feed ingredients for pigs. *Animal Nutrition*, 3(4), 344–352.

CHAPTER 8 Annexures

Images of setup and apparatus

A)

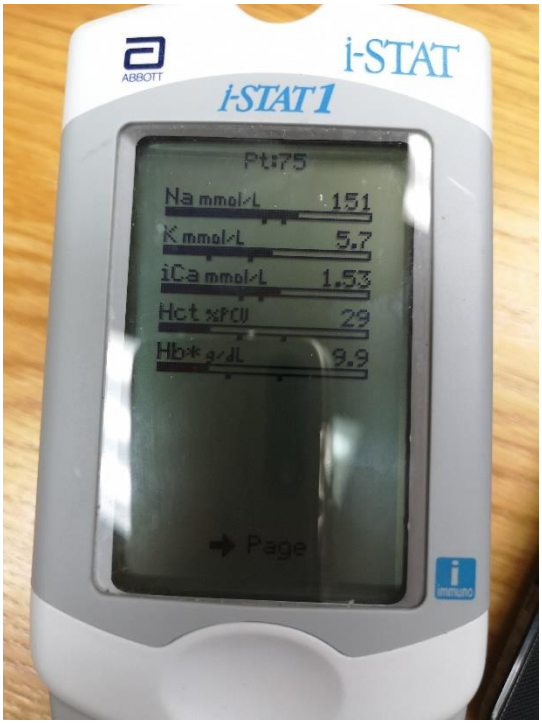
B)

C)

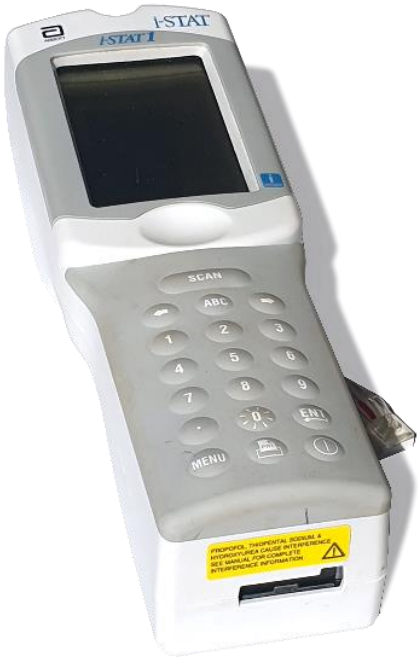
A) Cage system prior to the hen's arrival

B) Cage system with hens

C) Hens were able to exhibit individual behaviour in their separate cages



D)



E)

F)

D) I-STAT blood reading

E) I-STAT point of care device

F) Blood sampling team during the evening shift

G)

H)

I)

G) Dr P.W Plumstead and Prof R. Angel assisting on the sampling day

H) Student dissecting one of the hens to remove the ileal contents

I) Dr P.W. Plumstead assisting with dissection to remove ileal contents

J)

K)

L)

J) Ileal contents being sieved through a 0.25 mm sieve

K) Pestle and mortar used to grind the ileal contents after freeze drying

L) Example of feed label on feed bag used in the trial

M)

N)

M) Uncalcified egg, (3 hours post oviposition)

N) The primary stages of eggshell calcification (11 hours post oviposition)