LIMESTONE PARTICLE SIZE IN LAYER DIETS

by

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Dissertation submitted in accordance with academic requirements for the degree

MAGISTER SCIENTIAE AGRICULTURE

to the

Faculty of Natural and Agricultural Sciences
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Bloemfontein
30 November 2007

DECLARATION

I declare that the dissertation hereby submitted by me for the **MAGISTER SCIENTIAE AGRICULTURE (ANIMAL SCIENCE)** degree at the University of Free State is my own independent work and has not previously been submitted by me at another University/Faculty. I further more cede copyright of the dissertation in favour of the University of the Free State.

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ACKNOWLEDGEMENTS

I would like to express the most sincere gratitude to my supervisor Prof. H.J. van der Merwe for encouragement, guidance, invaluable advice and constructive criticisms and for allowing me this great opportunity to further my education.

I am grateful to my co-supervisor Mr. F. De Witt for all his support, guidance, encouragement and assistance in preparing this dissertation.

Special thanks to Mr. M. Fair for assistance with the statistical analysis of the data.

Thanks to the Government of Lesotho for awarding the scholarship and the National University of Lesotho for granting the study leave to undertake this study.

The following organization and or individual contributed to this study: Mr. G. Maritz and Viljoen from Agri-Lime for providing limestone, Mr. A. de Vries from Senwesko Feeds for the formulation of the diet, Mr. P. Venter from Nutrifeed for mixing the basal diet, Mr. J.du Plessis from the Pioneer Group for organizing the hens and Paardefontein Farm of Nulaid for donating the experimental birds.

I am deeply indebted to Mrs. K. Habai, Mr. B. Ledimo, Mrs. M. Phehlane, Ms. K. Mokhutsoane Mr. T. Nkofu, Mr. M. and T. Kuleile for their assistance in the laborious eggshell thickness measurements and excreta sample preparations.

Special gratitude to Prof. I. Okello-Uma for authorizing my release in order to embark on my study leave during a very critical time of my life and for his encouragement during the writing of this dissertation.

Special thanks to my Head of Department Dr. G. Adoko for giving me the opportunity to write the dissertation at work place and to my colleague Mr. M. Ntakatsane for his assistance with my duties during the critical stages of the writing of this dissertation.

Sincere acknowledgement is also expressed to Mr. Combrink (laboratory technician) and all the farm personnel at the Experimental farm of the University of the Free State for providing a warm, friendly and comfortable environment to enjoy working with, and not forgetting Mrs. F. Mohapi, Mrs. T. Lepheana and Mrs. M. Malitse for typing this dissertation.

A sincere thanks to all my colleagues and friends, for the support and encouragement through my MSc. studies namely B. Kuenene, K. Ncheche, L. Lebesa, M. Yinka, and T. Phirinyane.

Special thanks to my wife, my baby girl, parents, and parents-in-law for their constant encouragement, love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACRONYMS AND ABBREVIATIONS

%	Percentage
$1,25(OH)_2D_3$	1,25-Dihydroxycholecalciferol
ADF	Acid detergent fibre
Al	Aluminium
As	Arsenic
BE	Blunt end
Ca	Calcium
Ca:P	Calcium to phosphorus ratio
$Ca_3(PO_4)_2$	Calcium phosphate
$CaCO_3$	Calcium carbonate
Cd	Cadmium
cm^2	Centimeter squared
CO_2	Carbon dioxide
Cu	Copper
CV	Coefficient of variation
D_2	Ergocalciferol
D_3	Cholecalciferol
DM	Dry matter
EQ	Equator
EW	Egg weight
F	Fluorine
FCR	Feed conversion ratio
Fe	Iron
g	Gram
g/h/d	Gram per hen per day
GIT	Gastrointestinal tract
GLM	General linear model
ICU	International chick unit
ILC	Iowa Limestone Corporation
HCO_3	Bicarbonate
kg	Kilogram
ME	Metabolisable energy
mg	milligram
MJ	Mega joule

mm	Millimeter
Mn	Manganese
N/m^2	Newton per meter squared
NDF	Neutral detergent fibre
NRC	National research council
^oC	Degrees Celsius
P	Phosphorus
Pb	Lead
PCO_2	Partial pressure of carbon dioxide
pH	Hydrogen ion concentration
PTH	Parathyroid hormone
s.e.	Standard error
SAS	Statistical analysis system
SE	Sharp end
Si	Silicon
ST	Shell thickness
SW	Shell weight
SWUSA	Shell weight per unit surface area
U.S.	United States
V	Vanadium
Zn	Zinc

CHAPTER 1

GENERAL INTRODUCTION

As a result of the high cost of meat, people are constantly looking for a cheaper protein source. Eggs provide a valuable yet affordable source of high quality protein and vitamins that is required for normal growth, especially in children, when meat is too expensive or unavailable. The rapid increase in the demand for eggs, as well as the human population growth, caused a dramatic increase in the total demand for eggs. It is estimated that the total demand for eggs will increase by almost a factor of 5 between 1990 and 2050, and by almost a factor of 8 between 1990 and 2100 (Bouwman, 1997). The region with the highest increase in egg consumption is Sub-Saharan Africa with an estimated factor of more than 10 in the period between 1990 and 2050. The total number of eggs reaching their final market, or the consumer, is largely dependent on their shell quality.

According to Hunton (2005) the eggshell is an important structure due to two reasons. Firstly, it forms an embryonic chamber for the developing chick, thereby providing mechanical protection and a controlled gas exchange medium. Secondly, it serves as a container for the marketed egg, providing protection of the contents in a unique package for a valuable food. The main factor contributing to shell quality and strength is shell thickness and it is affected by genetic (breed) and non-genetic factors (diets) (Mohammed *et al.,* 2005).

Worldwide, the poultry industry suffers enormous economic losses from breakages due to poor shell quality. Although strain of hen and nutritional regime are the major factors affecting quality, age of hen also has an important influence. Other factors that have an influence on eggshell quality include: rate of calcium (Ca) deposition, flock health, general management practices, and environmental conditions (Butcher & Miles, 2003; Koelkebeck, 2006). Brooks (1971) reported that total egg breakage was 2.7% during the 1st month and 13.5% in the 15th month of lay. As a hen ages, she will have more difficulty mobilizing Ca from her bones and her ability to absorption dietary Ca from the digestive system also decreases with age (Mckillop & Rathgeber, 2006). Economic losses due to poor shell quality are worldwide estimated at approximately 500 million United States (U.S.) dollars per year (Etches, 1996). Macleod (2002) reported that the annual financial loss due to eggshell breakages in the United Kingdom egg industry is more than 8 million British pounds per annum.

The discovery of Ca and bone related disorders that affected both production and welfare of hens have stimulated interest in the bone biology of the laying hen (Whitehead, 2004). Osteoporosis is a condition in which the structural components of bones become abnormally thin (Webster, 2002). The condition arises from bone weakness in high producing hens leading to fractures of thoracic vertebrae (Webster, 2002). Osteoporosis can result in excessive bone breakage when spent cage layer flocks are caught and processed. Fractures in egg laying birds due to handling during depopulation and transportation have been reported in 29% of the birds that reach the processing facilities (Gregory & Wilkins, 1989) and in 98% of the carcasses at the end of the processing line (Belyavin, 1995). According to Schreiweis *et al*. (2004) these practices create bone splinters in the processed meat products, causing food safety concerns for the consumers. Therefore, food companies are reluctant to use spent cage laying hens for processed meat products and have turned to the broiler industry for meat supply (Wilson & Harner, 1988; Whitehead & Wilson, 1992; Bhat, 1993; Brown, 1993). A lack of market for spent hen meat imposes additional economic and environmental burdens on producers (Roland & Rao, 1992; McCoy *et al.*, 1996; Newberry *et al*., 1999). Steeves (2006) reported that the estimated U.S. annual economic losses due to spent layers that are not used in processed meat products are 18 million U.S. dollars.

Calcium is one of the key elements required by laying hens for maintenance and production (Turner, 1999). It is the most abundant mineral element in the body and fulfills two key functions in laying hens, namely the formation of eggshells and the development of skeletal bones (Gurr, 1999). More than 99% of the Ca is located in the bones, where it plays an important role in their structure and strength (Gurr, 1999). It is primarily present in bone tissue as the hydroxyapatite form of Ca phosphate (De Groote *et al*., 2002). A very small proportion of body Ca fulfills a vital role in regulating critical functions such as nerve impulses, muscle contractions and the activities of enzymes (De Groote *et al*., 2002). In laying hens Ca has an additional function of being the main mineral component of eggshells which are almost entirely calcium carbonate ($CaCO_3$) of which 40% is Ca (Roudybush & Grau, 1987).

The eggshell contains on average about 2.0 to 2.5 g Ca (Larbier & Leclercq, 1994). Buss & Guyer (1984) stated that this mineral must ultimately be absorbed from the gut if the hen is to maintain Ca balance during egg formation days. Some researchers found that the need for Ca during shell formation could be met by an increased intestinal absorption thereof (Hurwitz & Bar, 1969). However, other researchers showed that only 60-75% of the Ca needed for eggshell formation came directly from the feed while the remainder is withdrawn from body stores (Drigger & Comar, 1949; Mueller *et al*., 1964). A number of studies have been conducted to

investigate the Ca requirements for laying hens, resulting in a variability of results ranging from 3.25 to 5.57 g/hen/day (Roush *et al*., 1986; Frost & Roland, 1991; Keshavarz & Nakajima, 1993; Roland & Bryant, 1994; NRC, 1994; Roland *et al*., 1996; Ahmad *et al*., 2003). Wu *et al*. (2005) reported that newer strains of commercial laying hens had higher egg yields than older strains, resulting in an increased Ca requirement.

Medullary bone consists of nonstructural woven bone matrix, rendering it as the responsive type of bone regarding Ca turnover due to the large vascular surface area for mineral exchange and the large number of osteoclasts (Dacke *et al*., 1993). Medullary bone maintains the blood Ca level during shell formation when the removal of Ca at the uterus is greater than the absorption from the gut (Simkiss, 1967). Presumably, medullary bone's role in shell formation occurs during the dark period (lights off) when the hen consumes little or no food (Clunies *et al*., 1992). Inadequate dietary Ca create a conflict between structural bone maintenance and eggshell formation, resulting in the majority of Ca being transferred from structural and medullary bones to the uterus where it is used for eggshell formation (Webster, 2002).

There is considerable literature reporting on the benefits of feeding large particle $CaCO_3$ to layers. Larger Ca particles improve shell quality, bone ash and bone strength of layers (Guinotte & Nys, 1991; Fleming *et al*., 1998; Lichovnikova, 2007; Manangi & Coon, 2007). Whitehead (2004) reported that particulate Ca sources remain in the digestive system for a longer period of time during the night and provide a greater dietary source of Ca during the period of shell formation, thereby making the birds less dependent upon bone mobilization to provide Ca for eggshells. Nys (1999) indicated that factors favoring the supply of Ca during eggshell formation are of the utmost importance in the improvement of eggshell quality.

$CaCO_3$ in the form of limestone or oyster shell are probably the most common concentrated sources of Ca fed to laying hens (Roland, 1986). Since oyster shell and limestone are the two principal sources of Ca used in laying hen diets, most experiments have been based on them. Limestone and oyster shell sources contain relatively the same concentration of Ca, however, oyster shell is much more expensive than limestone (Saunders-Blades & Anderson, 2003). Roland (1986) reported that it was not until 1970 that the controversy concerning oyster shell and limestone really gained momentum. The researcher added that from 1972 to 1985, at least 12 papers were published in which large particles of oyster shell were compared to similar sized limestone particles. From these studies 10 reported an equal response when the same size particles were compared, and two reported that oyster shell gave better results than limestone. Dale (1999) reported that the closure of major oyster shell supplying companies resulted in

many poultry producers questioning whether it might be possible to satisfactorily substitute oyster shell with large particle size limestone and still maintain the same degree of shell quality. Roland & Bryant (1999) confirmed that the size of the Ca source, rather than the source itself (limestone versus oyster shell) was responsible for the improvement in shell quality.

In South Africa, calcitic limestone is the most common source of Ca used in layer diets (Chrystal, 2000). Other sources of Ca include oyster shell, snail shell and dried eggshell although these are often not readily available. Also, the cost of oyster shell in relation to limestone, as mentioned earlier, limited the use of this Ca source in South Africa. The largest limestone supplier to the feed manufacturing sector in South Africa is situated between the towns of Rustenburg and Thabazimbi in the North West Province. This particular calcitic limestone source contains about 36% Ca and is mainly supplied in three different particle sizes namely; Al 1000 (0-1.0 mm), Al 2000 (1.0-2.0 mm) and Grit (2.0-3.8 mm).

De Witt (2006) studied the effects of different particle sizes as well as particle size distribution ratios of this particular limestone source on bone and eggshell quality during the early laying period between 24 and 32 weeks of age. According to De Witt (2006) limestone particle size and particle size distribution ratio had generally no significant effect on bone as well as eggshell quality during early stages of production. Several researchers (Brister *et al*., 1981; Fleming *et al.*, 1998) also confirmed that Ca particle size had no significant influence on bone and eggshell quality during the early laying period. Contrary to these results (Rennie *et al*., 1997; Guinotte & Nys, 1991; Maff, 2000; Pavloski *et al*., 2003; Lichovnikova, 2007) reported that limestone particle size had a significant effect on bone and eggshell quality during the late laying period.

Due to the lack of statistical significance regarding the effect of specific calcitic limestone particle sizes on bone and eggshell quality during the early laying period and the significant effect of limestone particle size during the late laying period, as recorded in literature, the need arises to study the effects of this specific calcitic limestone on bone and eggshell quality characteristics during the late laying period. This is very important because the information obtained can be compared to the results of the early stage of production and enable better conclusions regarding the overall effect of this specific Ca source on bone and eggshell quality. The necessity of this study also originated from the various variables like particle size, Ca-content of diets, genotype and age of hens that hampers meaningful comparisons and conclusions among different studies regarding the desirable limestone particle size in diets of layer hens. Therefore, the aim of this study was to investigate the influence of a particular

calcitic limestone source differing in particle size or distribution ratios of particle sizes on egg shell and bone quality during the later stages of the laying period.

This dissertation is presented in the form of two separate articles, supported by a general introduction, literature review and conclusion in an effort to create a single unit. Although great care has been taken to avoid unnecessary repetition, some repetition has been inevitable.

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CHAPTER 2

LITERATURE REVIEW

Bone strength in layer hens is of utmost importance to prevent bone related disorders and especially bone fragility that normally occurs with age. Accordingly, eggshell strength should enjoy special attention, to avoid egg breakage and economic losses. In this literature review the factors affecting bone strength and eggshell quality are investigated with special reference to the role of calcium (Ca).

2.1 Functions of calcium

Ca is the most abundant mineral element in the body. Approximately 99% of the Ca in the animal body is found in the bones and teeth, with the remaining one percent widely distributed in various soft tissues. Ca also has a very close interrelationship with phosphorus (P) and vitamin D (Hegsted, 1973). Hunton (2005) indicated that the Ca content of blood at any given time is not more than 30 mg/ml, whereas the eggshell contains 80 times more Ca than blood. Elaroussi *et al.* (1994) stated that Ca is one of the key elements required for maintenance and egg production in laying hens. Quantitatively, the participation of Ca in the formation of bone is the most important function of the mineral. Bone acts not only as a supportive or structural component of the body, but also as a vital physiological tissue serving to provide a steadily available source of Ca for maintenance of Ca homeostasis in the laying hen.

The one percent of the body's Ca outside the bone, functioned in a number of essential processes and is found in extra cellular fluid, soft tissue and as a component of various membrane structures (Bronner, 1964). It occurs as the free ion form (50-60%), bound to serum protein or complexed to organic and inorganic acids. De Groote *et al*. (2002) indicated that the ionized form is extremely important in cellular metabolism, blood clotting enzyme activation and neuromuscular action. In poultry, Ca has the unique function of protecting the egg content through the deposition of an eggshell which has a high concentration of $CaCO_3$. Eastin & Spaziani (1978) reported that in the domestic hen, the shell gland extracts 2.0-2.5 g of Ca from blood and transfers the element without accumulation to the egg over a period of 15-20 hours. Soares (1987) stated that eggshell deposition dominates the Ca metabolism in the laying hen.

2.2 Calcium metabolism

Because of the vital role of Ca in cellular communication, it is essential that the concentration of ionized (Ca^{2+}) dissolved in the blood is regulated within narrow limits (Gurr, 1999). The control of Ca metabolism in birds have developed into a highly efficient homeostatic system, able to respond quickly to increased Ca demands for both egg production and growth rate (Aslam *et al*., 1998; Bentley, 1998). Parathyroid hormone (PTH), metabolites of vitamin D_3 and calcitonin regulates the concentration of Ca, by acting on the main target organs such as the liver, kidneys, gastrointestinal tract and bones (Taylor & Dacke, 1984). Estrogen and prostaglandins also appear to have an important role in Ca regulation in the bird (Aslam *et al*., 1998; Bentley, 1998).

The most distinct differences between mammalian and avian systemic regulation of Ca are the rate of skeletal metabolism at the specific time of Ca demand. Koch *et al*. (1984) reported that the domestic chicken would respond to hypocalcemic challenges within minutes compared with response to similar challenges in mammals that can take approximately 24 hours. This is best demonstrated by an egg laying bird where 10% of the body Ca reserves can be required for egg production in a 24 hour period (Klasing, 1998). The Ca required for eggshell production is mainly obtained from increased intestinal absorption and a highly labile reservoir found in the medullary bone, normally visible radiographically in female birds.

A hen lays approximately 250 eggs per year which corresponds to 20 times the quantity of Ca in her bones at any one time (Elaroussi *et al*., 1994). This amount of Ca is the total quantity of Ca in a normal hen's circulating system at any given point in time. Koelkebeck (1999) indicated that eggshell consists of about 94 to 97% $CaCO_3$ that is equivalent to 2.0 g Ca. Stout & Buss (1980) indicated that a normal laying hen mobilizes about 2.4 g of Ca in 20 hr to produce a thick shell for a 60 g egg and about 1.6 g for similar sized thin shelled egg. Georgievskii (1982) added that the total Ca content of the eggshell and shell membranes is 1.76 g Ca while the albumen and yolk contains 30-40 mg Ca per egg. It can be calculated that during the 15-20 hours required for eggshell formation, 25 mg of Ca must be deposited on the egg every 15 minutes (Butcher & Miles, 2003). This mineral must ultimately come from the gut if the hen is to maintain a Ca balance.

2.3 Vitamin D_3 (Cholecalciferol)

The vitamin D_3 metabolism in birds has been extensively reviewed by authors (Taylor & Dacke, 1984; Aslam *et al*., 1998). Vitamin D_3 is a necessity for normal Ca and P metabolism and absorption (Hayes & Saunders, 2002). The main role of vitamin D_3 is the control of bone

metabolism by strictly regulating mineral absorption, but more recently, it was found to have a profound effect on the immune system as well as skin and cancer cells (Stanford, 2006). In laying hens a deficiency of vitamin D_3 results in poor eggshells and weakening of bone structure (Hayes & Saunders, 2002).

The laying hen's minimum requirements for vitamin D_3 is stated as 500 international chick unit (I.C.U.) per kg of feed (Garlich & Wyatt, 1971). Edwards *et al.* (1994) indicated that due to the importance of this vitamin in bone development and the requirement for ultraviolet (UV) light in the metabolic conversion of provitamin D to vitamin D_3 the commercial availability of dietary vitamin D_3 is essential to allow the indoor production of poultry. Klasing (1998) stated that Vitamin D occurs naturally in plants as ergocalciferol (vitamin D_2) that will fulfill in the needs of mammals, however birds do not respond well to dietary vitamin D_2. This is due to increased renal excretion of vitamin D_2 rather than lack of intestinal absorption.

Cholecalciferol is initially metabolized to 25-hydroxycholecalciferol ($25(OH)D_3$) in the liver, where after 25-hydroxycholecalciferol is transported to the kidneys via carrier proteins and converted to either 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) / 25-dihydroxycholecalciferol ($25(OH)_2D_3$), the active metabolites of cholecalciferol in the domestic fowl (Stanford, 2006). Hurwitz (1989) and Stanford (2003) reported that the most significant active metabolite of vitamin D_3 in domestic chicken is ($1,25(OH)_2D_3$) which displays a hypercalcemic action.

PTH tightly regulates the synthesis of $1,25(OH)_2D_3$ depending on the Ca status of the bird. The metabolite regulates Ca absorption across the intestinal wall by inducing the formation of the carrier, calcium binding protein (calbindin-D28K). The presence of this protein reflects the gut's ability to absorb Ca (Bronner, 1998). Taylor *et al.* (1982) indicated that calbindin-D28K is also found in the oviduct wall and that its concentration increases during egg laying, although this process is not directly related to the actions of $1,25(OH)_2D_3$. Bone formation is stimulated by 1, $25(OH)_2D_3$ which induce osteoclastin production from osteoblasts. In egg laying birds, 30 to 40% of the Ca required for eggshell formation is acquired from medullary bone. The control of this labile pool of Ca involves both 1, $25(OH)_2D_3$ and estrogen activity. The function of vitamin D_3 is reliant on the presence of normal vitamin D_3 receptors. The receptors have been found in bone, skin, skeletal muscle, gonads pancreas, thymus, lymphocytes and the pituitary gland.

2.4 Dietary calcium level

The inclusion of Ca in the layer diets at levels beyond the requirements may reduce the rate of egg production (March & Amin, 1981). Ca level should be adjusted according to the anticipated

level of feed intake. A high level of dietary Ca decreases intestinal absorption of the mineral (Larbier & Leclercq, 1994). Shafey *et al.* (1991) indicated that excess dietary Ca inhibits the absorption of micro elements (zinc, manganese, iron and copper) and interferes with the assimilation of phytate P. Leeson (2001) stated that high Ca concentration in the form of $CaCO_3$ effectively dilute other feed nutrients. The true Ca absorption is an inverse function of Ca intake, decreasing from approximately 70% at very low feed intake levels to about 35% at high intake levels.

2.5 Calcium absorption

During shell formation, Ca contained in the digestive tract is dissolved by abundant secretion of hydrochloric acid. Ca absorption occurs mostly in the duodenum and jejunum of broilers and layers (Van der Klis *et al*., 1990). However, in laying hens some Ca absorption has also been observed in the lower gastrointestinal tract. The secretion and absorption of Ca by the different intestinal segments of layers are dependent on the stage of eggshell formation (Nys & Mongin, 1980; Waddington *et al*., 1989).

Ca is transported across the intestinal membranes by both a saturable, active (transcellular) process and a non-saturable (paracellular) process (Bronner, 1998). There is evidence that the active transport of Ca is regulated to meet the Ca needs of the body and is most active when dietary needs are great, such as during egg production and eggshell formation (Wills, 1973). The quantity of mineral absorbed by the paracellular route is determined by the quantity solubilized in the intestinal lumen that depends on mineral solubility, paracellular permeability and retention time of the chyle in the gut (Bronner, 1998). The researcher added that in the case of Ca, solubility is function of the chemical form of the Ca salt and that of the pH at a given intestinal region. Hendrix Genetics (2006) indicated that Ca absorption varies from approximately 30% to over 70% between periods without calcification and periods of shell formation. For this reason, an increase in the quantity of available Ca at the end of the night resulted in an improvement in shell quality (Hendrix Genetics, 2006). The researchers added that limestone of a large particle size (> 2 mm) is retained in the digestive tract and dissolve slowly during shell formation, providing a more regular release of Ca. Gurr (1999) stated that the efficiency of Ca absorption declines with age, reflecting either a reduced ability to produce 1,25 $(OH)_2 D_3$ or more probably, a lower activity of the vitamin D receptor.

2.5.1 Factors affecting calcium absorption

Many factors influence the availability of Ca for absorption and the absorptive mechanism itself. Factors which may affect gastrointestinal absorption of Ca include dietary mineral levels,

physical and chemical form of the source, mineral interactions, passage rate, viscosity of digesta, chelating agents, site of absorption, calcium to phosphorus ratio (Ca:P), dietary fat, and gastrointestinal tract pH (Van der Klis, 1993). However, the discussion in this literature review will be limited to the last four factors.

2.5.1.1 Site of absorption

The secretion and absorption of Ca by different intestinal segments is dependent on the stage of eggshell formation (Hurwitz & Bar, 1965). Since Ca is absorbed in its ionic (Ca^{2+}) form, the quantity absorbed depends largely on the activity of the agents that reduce $CaCO_3$ to ionized Ca in the intestines. Georgievskii (1982) reported that Ca^{2+} transfer mechanism is more active in the vicinity of the stomach, where the contents of the duodenum are still acidic. Guinotte *et al.* (1995) stated that it is generally accepted that gastric acid secretion is a prerequisite for $CaCO_3$ solubilization, before its intestinal absorption in the ionic form. Guinotte *et al*. (1995) also indicated that dietary Ca is usually supplied in a coarse particle form in laying hens to order in improve eggshell quality, and therefore, the acidic condition of the duodenum plays a vital role in Ca solubilization.

2.5.1.2 Calcium: phosphorus ratio (Ca:P)

There has been considerable interest in the importance of dietary (Ca:P) ratio in both bone development and eggshell formation. However, the effects of changes in the ratio are variable and dependent upon the absolute levels of the two minerals. Previous studies established that phosphorus is an essential nutrient for laying hens due to its role in eggshell formation and metabolism (Said *et al*., 1984; Rao *et al*., 1992). Miles *et al.* (1983) stated that excess dietary P is detrimental for eggshell quality. Kaplan (2005) added that too much dietary P forms an insoluble Ca phosphate, which renders the Ca unavailable for absorption, while too much Ca result in a P deficiency and impaired metabolic function.

A Ca:P ratio of 2:1 (weight/weight) basis is appropriate for most poultry diets, with the exception of egg laying diets (NRC, 1994). When poultry are laying eggs, a much higher level of Ca is needed for eggshell formation, and a ratio as high as 12 Ca to 1 nonphytate P (weight/weight) may be correct (NRC, 1994). Highfill (1998) indicated that ratios of 1:1 are required to support adequate growth, 1.5:1 for maintenance of adequate serum Ca while 2:1 are for the achievement of maximum bone density. However, high levels of $CaCO_3$ (limestone) and Ca phosphate may tend to make the diet unpalatable and dilute the other nutrients. A high P intake causes an increased concentration of serum P, which secondarily results in a decrease of serum Ca. This decrease stimulates the parathyroid gland to increase serum Ca by resorption of

bone and increase renal phosphate excretion (Wideman, 1984). Thus, a pronounced bone loss in adult animal can occur by feeding excess dietary P or insufficient dietary Ca.

2.5.1.3 Dietary fat

Fats have been reported to reduce mineral absorption with the formation of insoluble soaps when cations come in contact with free fatty acids that are released during digestion (Whitehead *et al*., 1971; Kaplan, 1995; Highfill, 1998). Atteh *et al*. (1983) reported that a variation occurred between different sources of fat and that corn oil (poly-unsaturated fatty acid) have a more detrimental effect on mineral metabolism than animal (saturated fatty acids) or a blend of vegetable fat. The dietary soaps are dissociated at a low pH in the stomach and cannot reform until they reach the ileum, where Ca absorption is limited (Gueguen, 2000). Supplementation of palmitic and stearic acid resulted in soap formations, which lead to a decreased Ca retention as evident by the decrease in bone ash and blood plasma content (Waibel & Mraz, 1964; Whitehead *et al*., 1971; Dewar *et al*., 1975; Hakansson, 1975; Gardiner & Whitehead, 1976).

2.5.1.4 Gastrointestinal pH

Laying hens are characterized during the period of shell formation by a large quantity of soluble Ca in the duodenum and jejunum despite high intestinal pH values (Mongin, 1976). Bronner (1987) stated that different intestinal segments have different pH levels, therefore affecting Ca absorption differently. McDonald *et al*. (2002) indicated that the low alimentary pH favours Ca absorption because it ensures ionic bonding which is necessary for intestinal uptake. High dietary Ca levels increase gizzard pH due to the buffering action of carbonates (Guinotte *et al*., 1995). At a pH higher than 6.5, manganese (Mn) and zinc (Zn) forms an insoluble complex, rendering them unavailable to the young chick. Whiting (2006) stated that if the stomach produces too little hydrochloric acid, Ca remains insoluble and cannot be ionized. The lower pH helps ionic bonding which is necessary for intestinal uptake of Ca^{2+}.

2.6 Bone characteristics

Bone is a complex tissue composed of inorganic and organic matrixes that provide support and mechanical strength (Bristol, 2004). The inorganic matrix, primarily hydroxyapatite, provides compressional strength, and the organic matrix that are predominantly collagen provide tensile strength and structural scaffolds to the inorganic matrix (Einhorn, 1996). Although bone is one of the hardest structures in the body, it maintains a degree of elasticity owing to its structure and composition. The principal functions of the skeleton are mechanical support, maintenance of Ca homeostasis and haematopoiesis in the bone marrow.

2.6.1 Types of bone

The skeleton of the domestic fowl is composed of three different types of bone tissue, namely compact cortical bone found in the diaphysis of the long bones, secondly, cancellous bone found in the vertebrae and epiphyses of long bones providing structural integrity and thirdly, a non-structural medullary bone formed at sexual maturity in the marrow cavities of certain bones (Newman & Leeson, 1997).

2.6.1.1 Cortical bone

Cortical bone is sometimes referred to as lamella bone. This is due to the layered manner in which it is formed (Newman & Leeson, 1997). Cortical bone consists of a number of irregularly spaced and over-lapping cylindrical units termed haversian systems (Courtney & Keaveny, 1994). Each haversian system consists of a central haversian canal surrounded by the concentric lamella of bony tissue. The haversian canals carry blood vessels that provide nutrients to the bone tissue (Newman & Leeson, 1997). Vaughn (1975) reported that the size of the canal might increase as the bone ages, giving rise to the characteristic pores from which the term osteoporosis, or "porous bone" originated.

2.6.1.2 Cancellous bone

Cancellous bone is spongy in appearance and it has a lower Ca content than cortical. It is primarily composed of fine sheets of mineralized bone, interlaced with marrow spaces where red blood cells are produced and contains a few haversian canals (Hodges, 1974). Cancellous bone has a higher turnover rate compared to cortical bone and is more vulnerable to bone loss. As a result, the regions in the skeleton that are constituted of cancellous bone are more susceptible to fracture later in life.

2.6.1.3 Medullary bone

Medullary bone consists of a woven bone that provides a labile source of Ca for eggshell formation (Whitehead & Fleming, 2000) and is characterized by the haphazard organization of collagen fibres in its matrix making it mechanically weaker than structural bone types. The highest concentration of medullary bone is usually found in leg bones (Whitehead & Fleming, 2000). Clunies *et al.* (1992b) indicated that this specialized bone tissue is formed in the long bones, pelvic girdle and ribs of pullets when sexual maturity approaches. Medullary bone has a larger surface area, is more vascularized and better mineralized and can be metabolized at a rate of 10 to 15 times faster than cortical bone (Hurwitz, 1965; Simkiss, 1967). Medullary bone is

therefore capable of supplying the hen with Ca needed for eggshell formation, when dietary Ca supplementation is inadequate. However, during a prolonged period of Ca deficiency, the laying hen responds by increasing the size of the medullary bone reservoir at the expense of cortical bone.

2.6.2 Bone strength

Since bone status is commonly used as an indicator of mineral adequacy in poultry diets, several bone measurements to evaluate bone status exists. Both invasive and non-invasive methods have been used to evaluate bone mineralization in poultry (Rao *et al.,* 1993). Invasive methods include bone ash, bone breaking strength and bone weight (Rao *et al.,* 1993). Among the different bone measurements, bone breaking strength is one of the most accurate parameters to evaluate direct bone fracture resistance (Kim *et al*., 2004). Rath *et al.* (2000) define bone strength as the toughness or ability of the bone to endure stress. The major minerals forming the inorganic matrix of bone are Ca and P (Reichmann & Connor, 1977; Ali, 1992; Watkins, 1992; Rath *et al*., 1999). The extent of bone mineralization affects bone strength (Reichmann & Connor, 1977), and poor mineralization has been associated with increased risk of fractures (Blake & Fogelman, 2002).

One of the common traits of bone breaking strength is bending moment, which measures the amount of force withstood by the bone (Crenshaw *et al*., 1981). Although these procedures have been used with a certain degree of accuracy, considerable variation occur in reported values for bone strength because of inadequate standardized test procedures (Orban *et al*., 1993). Such variation might be partly attributed to the type of instrument used to determine the physical state of bone, the procedures used to prepare the bones for testing and/or the lack of consideration for physical and mechanical properties of bones.

Differences exist in the physical and mechanical properties of wet and dry bones (Crenshaw *et al.,* 1981). Crenshaw *et al*. (1981) indicated that wet bones bend more than dry bones and even a short exposure period to air can change the mechanical properties of wet bones. Lott *et al*. (1980) reported that the breaking strength of dry tibia was significantly lower than that of fresh tibia. Park *et al.* (2003) indicated that breaking strength was significantly higher in refrigerated tibias than in frozen bones.

2.6.2.1 Factors affecting bone strength

(a) Age and growth

Growth rate is one of the most important determinants of bone strength due to the fact that bone mass increase with growth and bone strength is proportional to its mass (Frost, 1997; Seeman, 1999). However, there are very limited data on the age-related changes in bone parameters of poultry. Rath *et al*. (2000) reported that tibia weight, length, diameter and the pyridinium cross link content of broiler breeder hens reached a maximum at 25 weeks of age, whereas the mineral content, density and breaking strength of bone did not reach a maximum until 35 weeks of age. Fleming *et al*. (1998) indicated that the small increase in tibia breaking strength between 15 and 25 weeks of age could indicate some accumulation of medullary bone and relatively little loss of structural bone while the major decrease in bone strength between 25 and 50 weeks of age implies a considerable loss of structural bone.

(b) Nutrition

The role of nutritional factors is probably the most relevant to poultry bone strength. Nutritional management of birds is important in maximizing the mineralization of the skeleton, and ultimately minimizing the severity of osteoporosis that will occur as the laying cycle progresses (Newman & Leeson, 1997). Ca and P are primary inorganic nutrients, forming 95% of the mineral matrixes. However, several other inorganic elements such Cu, Zn, Mn and Mg are also present in the bone and may be important for bone strength (Rath *et al.,* 2000).

Low level of serum Ca stimulate the secretion of PTH and vitamin D synthesis, which in turn activate the release of bone minerals (Rath *et al*., 2000), causing a decrease in the amount of bone and an increase in the osteoid (Zambonin-Zallone & Teti, 1981). The structural bone loss in laying hens could cause high incidences of fractures at various parts of the skeleton (Whitehead & Fleming, 2000), and the relation of bone loss to osteoporosis can enhance skeletal fragility and contribute to the high fractures incidence in end-of-lay hens (Gregory & Wilkins, 1989). Therefore, accurate measurements of bone status in laying hens are critical to develop nutritional strategies that can reduce and/or prevent structural bone loss.

Adequate dietary Ca is necessary to decrease bone turnover, but due to the interactions, between Ca, P and vitamin D_3, it is often difficult to relate bone problems to dietary levels of a specific nutrient (Newman & Leeson, 1997). Leeson & Summers (1997) indicated that excess Ca in the diet leads to an imbalance in the ratio of Ca to P that will be excreted as $Ca_3(PO_4)_2$ causing a metabolic deficiency of P. Insufficient levels of 1, $25(OH)_2D_3$ the active metabolite of vitamin D, will lead to reduced absorption and retention of Ca and P (Elaroussi *et al.,* 1994) and

ultimately, osteopenia. These examples illustrate the difficulties involved in determining the exact dietary composition for maximal skeletal integrity.

Dietary factors such as physical form, digestibility and intrinsic factors have an influence on the availability of supplemental minerals (Cheng & Coon, 1990b; Guinotte & Nys, 1991). Rennie *et al*. (1997) and Fleming *et al*. (1998) stated that the dietary inclusion of a particulate source of Ca, such as large particles limestone or oystershell, before onset of egg production can reduce loss of structural bone early in lay and increase the accumulation of medullary bone. Dietary Ca in particulate form appears to promote better sustained mineralization of medullary bone, resulting in less resorption of structural bone (Webster, 2003). Several researchers (Miller & Sunde, 1975b; Cheng & Coon, 1990b; Guinotte & Nys, 1991; Rennie *et al*., 1997; Fleming *et al*., 1998; Schreiweis *et al*., 2003) reported that an increased Ca particle size and dietary Ca levels resulted in an increase in bone breaking strength. Particulate Ca sources have a prolonged retention time in the digestive system, thereby providing a Ca source during the period of shell formation and making birds less dependent upon bone mobilization to provide Ca for eggshell (Whitehead, 2004). Provision of Ca in particulate form can increase the amount of medullary bone which in turn will prevent Ca being withdrawn from more valuable bone structures and bone cortex (ILC, 2000).

(c) Physical activity

Cage layer osteoporosis severely reduces bone strength due to the high bone turnover rate related to eggshell formation and inadequate physical activity (Rath *et al*., 2000). Lanyon (1993) reported that physical activity is essential for the maintenance of cortical bone mass. Several studies illustrated a clear relationship between the physical exercise of hens and their bone strength (Whitehead, 1996; Newman & Leeson, 1997). Knowles & Broom (1990) and Norgaard-Nielsen (1990) found a significant increase in movement as well as stronger bones in the least restrictive systems compared to battery cages. The humerus strength of caged hens was only about 54% from that of perchery hens (Knowles & Broom, 1990) and 57% from that of hens kept in a deep litter system with perches (Norgaard-Nielsen, 1990). Newman & Leeson (1998) reported that tibial strength increased within 20 days after transferring hens from cages to an aviary suggested that the mechanism may involve stimulation of structural bone formation, rather than inhibition of resportion. Welch (2004) indicated that the impact exercise improved bone strength irrespective of the dietary Ca content. However, there is little literature information on the exact mechanism by which exercise improves bone characteristics in the hen.

(d) Genetics

Susceptibility to osteoporosis is significantly heritable, and it is possible to select hens for stronger bones (HSUS, 2007). Older strains such as Roslin J-Line Brown Leghorns are relatively resistant to osteoporosis, even in battery cages (HSUS, 2007). Newer strains have shown a six-fold decrease in humeral fractures after four generations and two-fold increase in humeral strength after seven generations of selection for these traits (Rennie *et al*., 1997; Bishop *et al*., 2000). Moreover, studies suggest that breeders can select for increased bone strength without necessarily sacrificing egg production (Bishop *et al*., 2000; Whitehead, 2004).

Whitehead & Wilson (1992) compared the histology of vertebrae from different strains of birds and observed the lowest cancellous bone volume in high performance strains. Within the same strain of birds, it appears that some high producing birds have better skeletal structures than others, resulting in the potential of genetic selection for improved bone strength (Whitehead, 1994). Bone strength characteristics in end-of-lay hens have been found to be moderately to strongly inherited and respond readily to selection. The implication of these findings is that selection for enhanced bone strength can be used as a long-term measure to help alleviate osteoporosis.

(e) Hormones and cytokines

Hormones and cytokines have a profound effect on bone metabolism, growth, remodeling and subsequent bone strength. Knowledge of cytokines which influence osteoclast formation and activity as well as their capacity to modulate bone resorption should provide critical insights into normal Ca homeostasis, as well as bone disorders such as osteoporosis (Roodman, 1993). Rath *et al.* (1996) showed that testosterone implantation caused a significant increase in bone strength of young chickens, while synthetic corticosteroid, decreased bone strength of turkey.

(f) Diseases or disorders

Metabolic bone disease (MBD) is an umbrella term that covers a number of disorders related to the weakening of bones or impaired system function caused by an imbalance in vitamin D_3, Ca and P (Kaplan, 2003). This imbalance might be caused by either a lack or an excess of any of these three essential nutrients and/or a failure to provide these nutrients in a bioavailable form. A number of MBD's in poultry are associated with Ca deficiency including cage paralysis, osteomalacia, osteoporosis, nutrition hyperparathyroidism and tibial dyschondroplasia (Kaplan, 1995; Pesek, 2001). Osteoporosis is the most important MBD in laying hens, due to its severity especially towards end-of-lay. The increased Ca demand for egg formation compounds the

inability of actively laying hens to deposit new structural bone. Alberdi (1996) stated that each year approximately 65 million laying hens suffer from bone fractures in the United Kingdom as a result of bone fragility caused by osteoporosis, clearly illustrating the severe welfare implications of this MBD.

Osteoporosis is defined as a progressive decrease in the amount of mineralized structural bone, leading to bone fragility and susceptibility to fractures (Whitehead, 2004). This condition is precipitated by inadequate intakes of Ca and/or P, thereby causing an increased mobilization of medullary bone in order to maintain shell production (Newman & Leeson, 1997). The onset of this disease in individual hens decreases bird welfare, eggshell quality and egg production. It also increases the incidences of broken bones and lameness (Korver, 2004). The major economical concern associated with decreased skeletal strength is the reduction in meat quality of the carcasses from birds that have suffered breakages (Whitehead & Wilson, 1992). The presence of bone splinters in the meat of carcasses that have suffered breakages during processing, create a product which is unsafe for the consumer. As a result, carcasses from spent hens are worth little, and processing plants may refuse to process these birds. If the problem is not dealt with, spent hens may become the liability of the producer.

2.7 Eggshell characteristics

Eggshell is a highly ordered mineral structure deposited in a cellular milieu, secreted by the distal parts of the oviduct, the isthmus and the uterus (Nys *et al*., 1999). The calcification of eggshell is the results of the precipitation phenomenon occurring onto the eggshell membrane, which takes place during passage of the egg through distinct regions of the oviduct (Hincke *et al*., 2000). Calcification of the avian eggshell is one of the fastest processes, where 2.0-2.5 g of mineral is deposited in less than 15 to 20 hours during the daily egg formation cycle (Westmoreland, 2003).

The eggshell consists of the inner and outer shell membrane, the true shell and the cuticle (Tullett, 1987). In total it is approximately one third of millimeter thick and more than 90% of it consists of $CaCO_3$ (Maff, 2007). There are also small quantities of proteins and other minerals. Several thousands tiny pores permeate the shell with the majority of these pores situated at the broad end of the egg (Kermanshahi & Hadavi, 2006). The pores allow gases to move between the inner contents of the egg and the outer surroundings (Hincke, 2006). Cuticle is the outermost part of the shell which gives the egg its characteristic bloom or shine. The cuticle normally covers the pores and this provides extra microbiological protection especially when the egg is fresh (Maff, 2007).

2.7.1 Eggshell quality

The eggshell is the natural packing material for the egg contents, and it is important to obtain proper shell strength, to resist all impacts an egg is subjected to during the production chain (Mertens *et al*., 2006). Broken eggs cause economic damages since it cannot be sold as quality eggs, since the occurrence of hair cracks increase the risk for bacterial contamination of the broken egg (Charles & Strong, 1988). It was estimated that approximately 13-20% of the total eggs produced worldwide are cracked or lost before reaching the consumer (Roland, 1988). Worldwide, economic losses due to poor shell quality are estimated at approximately 500 million U.S. dollars per year (Etches, 1996). Hincke (2006) reported that up to 10 000 cases of *Salmonella* infected eggs are annually found in Canada due to cracked eggshells.

According to Sabri *et al*. (1999) eggshell quality embodies characteristics such as shell thickness, shell Ca content, shell weight, egg specific gravity and shell strength. Most good quality eggshells from commercial layers contain approximately 2.2 g Ca in the form of $CaCO_3$ (Butcher & Miles, 2003). Koelkebeck (1999) indicated that eggshell thickness is determined by the time that the developing egg spends in the shell gland and the rate of Ca deposition during shell formation. The deposition of $CaCO_3$ onto the shell requires a Ca concentration in the gland fluid of 4 to 12 times higher than that of the blood. The largest quantity of Ca used for shell formation is obtained from blood, bones and the gastrointestinal track (Etches, 1995; 1996). The thickness of an eggshell depends generally on shell weight in relation to egg surface area (Nordstrom & Ousterhout, 1982). The egg surface area is dependent on the physical size of the egg which could be calculated from egg weight. For shell thickness to increase, shell weight must increase, egg surface area must decrease, or a combination of these two changes must occur (Nordstrom & Ousterhout, 1982). Carter (1969) reported that an eggshell crack occurs if the strength of the shell is less than the strength of the environmental insults to which it is exposed. Shell strength is determined by shell thickness and the shell matrix organization (Butcher & Miles, 1995).

To maintain good eggshell quality during the later stages of production, it is necessary to include particulate Ca sources in the diets of older flocks to alleviate specific shell quality problems. The positive effects of large particles on eggshell quality could be due to the increased specific intake of Ca just prior to the onset of shell formation (Guinotte & Nys, 1991).

2.7.1.1 Factors affecting eggshell quality

Eggshell strength is influenced by various factors such as nutrition, strain of laying hen, age, environmental temperatures and diseases.

(a) Nutrition

The ability of hens to produce quality shells depends largely on the availability of Ca from ingested food (Farmer *et al*., 1983). However, determining the appropriate Ca level for optimum eggshell quality is a continuous challenge, particularly in older laying hens. The average Ca requirement for eggshell formation within a population of hens is greatest at approximately peak production. However, because the quantity of Ca deposited on the shell increase slightly with age, the Ca requirements for an individual hen for a particular egg on a particular day could increase with age (Roland, 1986a). The reason for this is that the ability of the hen to store Ca for future shell formation is limited (Lennards & Roland, 1981). Mineral requirements of egg laying hens are similar to mineral requirements of other poultry, with the exception of Ca (NRC, 1994). Roudybush & Grau (1987) suggested that the requirement for Ca during lay is at least 100 times the requirement of the same hen when she is not laying.

During the past two decades, a number of reports which appeared in the literature suggested that shell quality improvement may be obtained at higher dietary Ca levels than the NRC (1984) estimated requirements (Atteh & Leeson, 1985; Roland, 1986a; Keshavarz, 1987; Clunies *et al*., 1992a). NRC (1984) suggested a constant Ca intake of 3.75 g/hen/day throughout the production cycle. However, there is a wide difference of opinion regarding Ca requirements for optimum performance, varying from 3.25 to 5.17 g/hen/day (Roush *et al*., 1986; Frost & Roland, 1991; Keshavarz & Nakajima, 1993; Roland & Bryant 1994; Roland *et al*., 1996; McDonald *et al*., 2002; Ahmad *et al*., 2003). Hayes & Saunders (2002) stated that maximum shell thickness is attained only at high Ca concentration (>40 g Ca/kg Diet), which are above the normal inclusion levels of commercial layer diets. Realizing this variation, the NRC (1994) reduced the Ca requirement for commercial Leghorn from 3.75 g/hen/day to 3.35 g/hen/day. The laying hen is also not hundred percent efficient in extracting Ca from the available sources in the diet (Butcher & Miles, 2003). Mueller *et al*. (1964) reported that only 60-75% of eggshell Ca came directly from the feed, while the remainder is withdrawn from body stores. Therefore, the diet has to furnish in excess of 4 g Ca/hen/day (Butcher & Miles, 2003).

On the other hand Keshavarz (2000) studied whether Ca particle size and increased Ca levels higher than the NRC (1994) recommendation could have a beneficial effect on shell quality of

older hens. The results indicated that even when the Ca level is plentiful in the diet, the presences of Ca sources in particle form produce beneficial effects on shell quality. Scheideler (2004) indicated that large particle size $CaCO_3$ with corresponding lower *in vitro* solubility values increased Ca retention in the gizzard for layers, thereby providing a Ca source for ionization during peak shell production and/or non-feeding times. Zhang & Coon (1997) stated that the retention of Ca in the gizzard of laying hens for improved shell quality is dependent upon particle size, porosity of the Ca source, and the overall *in vivo* solubility of the source. Since the resident time of Ca sources in the digestive system is important and has a direct effect on eggshell quality, Keshavarz (2000) recommended that 50% of the supplemental Ca in the diet of laying hens should be provided in particle form with proper solubility (50% or higher).

Less than optimum Ca causes demineralization of bone, low serum Ca levels and subsequently low egg production with higher thin-shelled eggs and egg breakages (Washburn, 1982), while excess Ca cause a reduced feed intake, poorer egg production and shell quality (North, 1984). There are other nutrients, such as Vit. D_3, P, and Mn, which also affect shell quality negatively if they are not properly balanced in the diet (Canadian Poultry Consultants Ltd., 2006). Vit. D_3 is vital in the absorption and mobilization of Ca during shell synthesis (North, 1984). Chen & Chen (2004) reported that increasing Ca absorption is responsible for increased eggshell strength. High levels of P in the blood inhibit the mobilization of Ca from the bone (Canadian Poultry Consultants Ltd., 2006) and Mn interferes with the metabolism of Ca, causing poor shell quality if it is excessive in the diet.

(b) Strain of bird

There is no doubt the correct breed of bird is the first positive step towards minimizing shell breakages on the farm (Woolford, 1994). Anderson *et al.* (2004) stated that genetic differences in eggshell formation characteristics exist between species and between breeds, strains and families within the species. Desert inhabiting breeds (Sinai) were found to have shells that were less porous and thicker than that of commercial White Leghorns (Arad & Marder, 1982). Significant differences exist between breaking strength, shell thickness and percent cracks in eggs of eight commercial strains (Bowman & Challender, 1963). Potts & Washburn (1974) observed significant differences in shell strength among three White and three Brown egg strains. Potts & Washburn (1977) indicated that the addition of oyster shell (large particle Ca) on *ad libitum* basis to hens receiving a diet containing 2.9% Ca improved shell strength but did not alter differences between lines or strains.

(c) Age of hens

It is generally accepted that the decrease in egg production and the increase in egg size, without a concurrent and equal increase in shell weight is the reason for the decrease in shell quality of older hens (Ousterhout, 1980; Lee, 1982). Weatherup & Foster (1980) indicated that egg weight increase with hen age, reaching a plateau by the end of the laying cycle. Hen age also affects the proportion of yolk, albumen and eggshell (Fletcher *et al*., 1981; Akbar *et al*., 1983; Danilov, 2000). Egg specific gravity and eggshell thickness decrease with an increase in age (Luquetti *et al*., 2004), due to the fact that egg size increase more quickly than shell weight (Curtis *et al.,* 1985). Woolford (1994) reported that the ability of the hen to absorb and retain Ca, as well as the quantity of skeletal Ca available for shell formation decrease with age.

(d) Environmental temperatures

One of the primary effects of high environmental temperatures on poultry is a reduced feed intake (Grieve, 2007). A reduction in feed intake results in a decrease in daily intake of nutrients in order to reduce metabolic heat production and maintain homothermy. Laying flocks typically have a reduction in egg size, followed by a reduced egg production, and reduced eggshell quality (Grieve, 2007). At environmental temperature of 35^0C to 38^0C, the animal pants and develops alkalosis, which continues to worsen up to 41^0C. The blood pH increases from 7.5 to 7.6 (El Hadi & Sykes, 1982), due to the decrease in partial pressure of carbon dioxide (PCO_2) in the arterial blood and the increased concentration of bicarbonate (HCO_3) (Koelkebeck & Odom, 1985; Beckman, 1999). An increased respiration rate of birds during unusually hot conditions results in a depletion of CO_2 in the blood and as a result changes occur in the blood that decrease the rate at which Ca is delivered to the uterus for shell formation. These changes in PCO_2 as well as alkalosis have a negative effect on eggshell formation that is inevitably reflected in the quality of the shell. Voisey *et al*. (1979), Deaton *et al*. (1981) and Daghir (1995) showed that eggshell strength decrease when the laying hen is exposed to temperatures in excess of 32^0C. It is also found that shell strength of layers exposed to cyclic temperature is superior to those exposed to constant high temperatures (Wilson *et al.,* 1972; Miller & Sunde, 1975a).

(e) Disease

Disease in general has a negative impact on shell quality, among other production traits. However, not all the diseases which affect poultry cause a decline in eggshell quality. The stress caused by a disease challenge can reduce water and feed intake of the affected birds (Canadian Poultry Consultants Ltd., 2006), resulting in a Ca deficiency, and consequent shell problems

(Beckman, 1999). Other common viral diseases such as Egg Drop Syndrome (EDS), Avian Influenza (AI), Newcastle disease (ND) and Infectious Bronchitis (IB) caused severe negative effects on eggshell quality (Butcher & Miles, 2003). It has established that the EDS virus affects only the shell gland while ND and IB affect every portion of the reproductive tract (Butcher & Miles, 2003).

2.8 Calcium sources

Ca is an important nutrient for laying hens. It is necessary for proper eggshell formation and is also needed to maintain skeletal integrity (Saunders-Blades & Anderson, 2003b). The high rate of egg production of modern laying hens puts tremendous demands on the Ca metabolism process, and therefore requires a high quality Ca source (Saunders-Blades & Anderson, 2003b).

Several supplemental sources of Ca are used in layer diets, with the most common being oyster shell and limestone (Roland, 1986b). Other common sources include $CaSO_4$, aragonite, CaCl, $CaPO_4$ and bone meal (BOA, 1980), ranging in Ca content from about 16 to 38%. Both oyster shell and limestone sources contain relatively the same quantity of Ca. However oyster shell is about three times more expensive than limestone (Saunders-Blades & Anderson, 2003b), limiting the use thereof in commercial practice (Hunton, 2006).

Limestone is an ordinary kind of sedimentary rock, principally composed of $CaCO_3$ as combination of $CaCO_3$ or combination of $CaCO_3$ and $MgCO_3$. Limestone is a very broad term referring to the Ca mineral ingredient of sources that may vary from 20-40% Ca. Most often feed-grade limestone pertains to calcitic limestone. The two most fundamental types of limestone are high Ca (calcitic) and dolomitic limestone (lLC, 2006). The Association of American Feed Control Officials (AAFCO, 2005) classify calcitic Ca sources of limestone into two categories namely; $CaCO_3$ and ground limestone. $CaCO_3$ is a product which contains a minimum of 38% Ca, while ground limestone is an acceptable source of $CaCO_3$ and contains not less than 33% Ca. Dolomitic stone is a mixture of $MgCO_3$ and $CaCO_3$ and is categorized as either dolomitic limestone or Mg limestone (ILC, 2006). However, limestone deposits that contain significant portions of Mg should be avoided as it may cause diarrhoea and reduce production performance (Waldroup, 1995). Other factors of concern with respect to limestone usage as a Ca source for laying hens are heavy metal content, impurity, solubility and particle size.

2.8.1 Factors affecting the quality of limestone

2.8.1.1 Limestone purity

The real value of a feed-grade limestone is based on its purity and Ca content and contains on average about 97-99% Ca. It is been documented that the more pure the Ca-source is, the higher the bio-availability thereof will be (ILC, 2006). The biological availability of different limestone sources are as follows; $CaCO_3$ 100%, ground limestone 90-95%, magnesium limestone 80%, and dolomitic limestone 53% (ILC, 2006). Chrystal (2000) indicated that limestone impurities include clay minerals, heavy metals and other micro-elements such as silica, iron, aluminium, magnesium, manganese and sulphur. ILC (2003) added that cadmium and vanadium are heavy metals present in limestone as impurities.

Excess sulphur can lead to an increased Ca excretion and thus potential depletion of bone Ca resources (Summers, 2007). The quantity of silicon is inversely proportional to the quantity of Ca in the limestone (Chrystal, 2000). Excess of Mn, Mg and Al adversely affect Ca absorption (Georgievskii, 1982). Excess Fe, Cd and V leads to rickets in the immature chickens, as well as a reduced growth rate and egg production (ILC, 2003). Tisdale *et al.* (1985) stated that pure calcitic limestone has a rating of 100% while most agricultural limestone rate as 90-95% due to impurities.

2.8.1.2 Particle size

Identification of the ideal particle size of Ca supplements in commercial layer diets to promote eggshell and bone quality has been the subject of considerable research. Particle size encompasses both the size of the various feed ingredients used in poultry diets as well as the consistency of the particle size. Particle size impacts directly on the bird itself and the manner in which it utilizes the nutrients in its diet, both in the case of layers and broilers (Kleyn, 2003). In general, reducing the particle size of limestone increases the surface area that is available for digestion. However, it has been suggested that for some species of animals, the particle size must be sufficient enough to allow optimum retention time, thereby maximizing Ca availability (Cumming, 1994; Nys, 1999; Pavloski *et al.,* 2003; Hendrix Genetics, 2006; Hunton, 2006). This is evident for laying hens, where it is common practice to include large particle size limestone in the diet (ILC, 2000). Roland & Bryant (2000) indicated that there is no intrinsic benefit of Ca sources, other than the size of the particles which provide the animal with continuous supply of Ca when it is most needed especially during the night when the hen has no access to feed.

However, controversy still exists among different researchers regarding the beneficial effect of large particle Ca and optimum particle size to optimize bone and shell quality during different production stages. De Witt (2006) reported a non-significant effect of limestone particle size on bone and eggshell quality during the early laying period. Guinotte & Nys (1991) and Saunders-Blades & Anderson (2003b) reported that Ca particle size have no significant effect on laying hen performance and shell quality during the late laying period. Contrary to this, Maff (2000), Schreweis *et al*. (2004) and Karunajeewa (2006) reported a significant influence of Ca particle size on eggshell during the late laying period. Most studies indicated that large particle Ca supplements were superior in promoting bone and eggshell quality to fine granular Ca supplements.

The addition of large particle size Ca supplements does improve eggshell quality in high producing laying hens when substituted part of the pulverized Ca source (Brister *et al*., 1981; Roland & Bryant, 1999). The improvement in eggshell quality due to the substitution of large particle size was especially enhanced after 48 weeks of age (Brister *et al.,* 1981). Eggshell thickness, shell weight, specific gravity and shell weight per unit surface area (SWUSA) were significantly affected by particle size (Cheng & Coon, 1990b; Hendrix Genetics, 2006). Pavloski *et al.* (2003) found that birds fed large particle size Ca had a higher eggshell thickness than the group fed fine particles. The improvements in shell thickness were presumably caused by the increased amount of Ca absorbed, and its subsequent availability for deposition onto the shell. Guinotte *et al*. (1995), Zhang & Coon (1997), Pavloski *et al.* (2003) and Narvaez-Solarte *et al*. (2006) observed a significantly higher shell weight in hens fed large particle size limestone compared to hens fed pulverized limestone. Clunies *et al*. (1992a) stated that hens laying eggs with thick shells retained significantly more Ca than hens laying eggs with thinner shells on shell formation days.

Literature indicated that limestone products of different particle sizes (0.5 to 1.180 μ) are equally effective in supplying Ca and influencing performance and digestion in most animals including poultry (ILC, 2006). In the case of laying hens, large particle limestone (1.4 to 5.6 mm) appears to be more effective than smaller granulations in producing eggs with acceptable shell strength and quality (Kuhl *et al.,* 1977; Pavloski *et al*., 2003; ILC, 2006). Several researchers (Nys, 1999; Hunton, 2006; Lichovnikova, 2007) indicated that any Ca particle exceeding 1 mm in size will be retained in the gizzard,thus resulting in a slow release of Ca into the blood stream, while Hendrix-Genetics (2006) recommended a minimum of 2 mm and ILC (2006) a minimum particle size of 1.4 mm. Roland & Bryant (1999) suggested that replacing

50% of ground limestone by large particle size limestone is quite adequate to optimize eggshell quality. Pavloski *et al*. (2003) confirmed the above results by replacing 60% of pulverized limestone and found significantly higher eggshell breaking force, shell weight, shell thickness and low deformation than in the hens fed pulverized limestone.

2.8.1.3 Solubility

One of the biggest differences among Ca sources is solubility thereof (Saunders-Blades & Anderson, 2003a). Recent literature indicated that limestone solubility properties can be used to select the appropriate particle size for layer diets (ILC, 2006). Solubility is affected by composition and particle size of the Ca sources that have different effects on eggshell and bone quality of laying hens. Supplemental $CaCO_3$ must first be solubilized in the digestive tract before Ca^{2+} could be of any nutritional use to the bird (Bronner, 1993; Guinotte *et al.,* 1995).

Scott *et al*. (1971) reported that oyster shell particles were retained in the gizzard after 12 hours of fasting while pulverized limestone was not. These workers suggested that the gizzard may act as a "metering" system, holding back and gradually solubilizing large particles of oyster shell or limestone, allowing for a steady intestinal absorption of Ca during the period of shell formation. A ground or fine particle size Ca source, is solubilized at a more rapid rate, and therefore provides the laying hen with a dietary Ca source for a short period of time (Zhang & Coon, 1997). Therefore, information concerning the quantity of Ca supplement solubilized by the hen would be helpful in understanding why some types of Ca supplements improve eggshell quality more than others. Limestone solubility varies by as much as 60-100%, even when comparing similar particle sizes (Rabon & Roland, 1985; Cheng & Coon, 1990a). Terms such as amorphic, crystalline or marbelite are loosely applied to limestone to indicate softness of the product. It is often assumed that softer (or amorphic) limestone is more bio-available but this is not strictly true (Chrystal, 2000).

Zhang & Coon (1997) studied both *in vitro* and *in vivo* solubility of two limestone sources of varying particle size in 88 weeks old Leghorn hens. They reported that there was a negative correlation between *in vitro* and *in vivo* solubility and concluded that less soluble limestone *in vitro* is desirable in laying hens since it remains in the digestive tract for a longer period of time. Similar results were confirmed by De Witt (2006) studying both *in vitro* and *in vivo* solubilities of different limestone particle sizes at week 37 of age. Hendrix Genetics (2006) stated that the availability of Ca at the end of the dark period is improved by using a coarser Ca source with a low *in vitro* solubility. Cheng & Coon (1990a) found a high positive coefficient of regression between shell weight per unit of surface area (SWUSA) and limestone solubility. Koreleski &

Swiatkiewics (2004) reported that eggshell calcification of some laying hens continued through the night until lights-on in the morning. They suggested that at least 50% of the Ca source should to be in particulate form and another 50% in the highly soluble powder form, because those birds, which have not completed calcification, should have access to powdered Ca, which is very rapidly dissolved and absorbed.

The important role played by Ca in laying hens regarding egg production, as well as bone and eggshell quality was highlighted in the literature review as well as the important role of calcium particle size on the above mentioned traits. However, the literature revealed some inconsistencies regarding the influence of different Ca particle sizes on the above mentioned parameters, warranting some further investigation. Therefore, the following two chapters will be based on the influence of limestone particle size on bone and eggshell characteristics, during later stages of production in an attempt to address the critical need for information regarding a specific limestone source used in South Africa.

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CHAPTER 3

THE INFLUENCE OF LIMESTONE PARTICLE SIZE IN LAYER DIETS ON BONE AND EGGSHELL CHARACTERISTICS AT POST PEAK PRODUCTION

3.1 Introduction

Egg content is protected by a highly ordered calcareous eggshell that needs to be as strong as possible to maximize the number of intact eggs reaching the market. Egg breakage results in a large economic loss to the poultry industry. Roland (1988) estimated that 13-20% of the total eggs produced worldwide are cracked or lost before reaching the consumer. Roland (1977) indicated that shell-less, ultra thin and thin-shelled eggs account for another 8% of collected eggs. Egg breakage is a function of the quality (or strength) of the shell (Carter, 1970; Butcher & Miles, 2003). Calcium is the major macro mineral of the eggshell and consequently the main factor influencing its quality (Bolukbasi *et al.,* 2005). The ability of hens to produce quality shells depends largely on the availability of Ca from ingested feed (Farmer *et al*., 1983). The skeleton on the other hand functions as a Ca reserve into which dietary Ca is deposited for storage and from which Ca is withdraw for use in eggshell formation (Webster, 2002). The hen uses a specialized bone component called medullary bone for this purpose.

The hen's inability to maintain eggshell quality as she ages is a severe problem for commercial egg producers (Roland, 1977). As the hen ages, the number of eggs produced declines along with a reduction in eggshell quality (Franco-Jimenez & Beck, 2005). Shell quality is basically governed by the quantity of shell weight per unit surface area (SWUSA) of the egg (Ousterhout, 1980). The increase in egg size with age and the limited capacity of Ca deposition onto shell membranes, consequently results in a reduced thickness of the eggshell (Etches, 1996). Some of themost common explanations for the decline in shell quality (thickness of the eggshell) is that as the hen ages her ability to absorb Ca and to mobilize skeletal Ca decrease due to the fact that genetic potential for egg production through years of genetic selection increased at a faster rate than her ability to maintain adequate shell deposition (Petersen, 1965; Roland, 1979).

Webster (2002) reported that inadequate Ca supply to the bird creates a conflict between structural bone maintenance and eggshell formation because cortical and cancellous bone are resorbed for maintenance of medullary bone. Successive events of Ca deficiency will lead to decline in medullary bone and mineral density which will result in osteoporosis and a reduction of egg production and shell quality (Webster, 2002). Therefore, the role of

nutritional factors is probably the most relevant to poultry bone strength and eggshell quality. Nutritional management of birds is important in maximizing the mineralization of the skeleton, and ultimately minimizing the severity of osteoporosis, which occurs as the laying cycle progresses (Newman & Leeson, 1997). Dale (1999) suggested that the physical size of the supplemental Ca source, rather than the source itself was responsible for the improvement in bone and eggshell quality observed in laying hens.

Identification of the ideal particle size of Ca supplements for commercial laying hens to promote bone and shell quality towards the end-of-lay has been the subject of considerable research. However, much controversy still exists about this topic (Rao & Roland, 1989). Various studies investigating the effect of Ca particle size on laying hen's performance and shell quality have yielded conflicting results. De Witt (2006) found a non-significant effect of limestone particles on bone and eggshell quality during the early laying period. In other studies, Guinotte & Nys (1991) as well as Saunders-Blades & Anderson (2003) reported that Ca particle size had no significant effect on laying hen performance and shell quality during the late production period. Contrary to this, Maff (2000), Schreiweis *et al*. (2004), Karunajeewa (2006) and Lichovnikova (2007) reported a significant influence of Ca particle size on eggshell weight, thickness, and strength during the late laying period. Large particle Ca sources dissolve slowly in the digestive tract, and therefore Ca is made available to the bird during the evening and dark hours when shell is deposited (Scott *et al*., 1971). Grieve (2004) suggested that to maintain eggshell quality in older hens, an increase in dietary Ca is needed due to the lower efficiency of Ca absorption from the diet. In order to facilitate better Ca absorption, hens older than 40 weeks of age should be fed a Ca source with larger particle sizes (Nys, 1999; Pavloski *et al*., 2003; Grieve, 2004; Hendrix-Genetics, 2006). However, there is a wide variation in the literature regarding the minimum particle size required to optimize eggshell quality. Nys (1999) and Hunton (2006) suggested a minimum particle size of 1.0 mm whereas Hendrix-Genetics (2006) recommended a minimum of 2.0 mm and ILC (2006) a minimum particle size of 1.4 mm.

From the literature it seems that Ca requirements of layers become critical towards the end-of-lay due to the inability of older hens to maintain eggshell quality. Furthermore, conflicting and contradictory results are recognized in the available literature regarding the ideal particle size for optimum shell quality and bone stability during the late laying period. In South Africa a specific calcitic limestone is commonly used as a source of supplemental Ca in layer diets. Therefore the purpose of this study was to evaluate the effect of specific calcitic limestone of different particle sizes on egg production, bone strength and eggshell quality during the later stages of the laying period.

3.2 Materials and Methods

This study is the continuation of the work started by De Witt (2006), who studied the effect of limestone particle size during the period of 17-32 weeks of age. The current study continued immediately from week 33 up to week 70 of age. Therefore the materials and methods used during the current study have been adopted from the work of De Witt (2006). In order to investigate the effect of limestone particle size on bone and eggshell characteristics, a study was conducted at the Paradys Experimental Farm of the University of the Free State.

3.2.1 Experimental design

The experiment was a fully randomized design with three different limestone particle sizes as treatments. Limestone classified as small (<1.0 mm), medium (1.0-2.0 mm) and large (2.0-3.8 mm) represented the three treatment of this study, as illustrated in Figure 3.1.

Figure 3.1 Different limestone particle sizes; small (<1.0 mm), medium (1.0-2.0 mm) and large (2.0-3.8 mm)

3.2.2 Birds and husbandry

Sixty-nine Lohmann-Silver pullets were used in this experiment. Hens were kept in individual single-deck cages from 17 to 70 weeks of age even though the present study was conducted from 33 to 70 weeks of age. They were kept in a naturally ventilated laying house with no climate control systems and provided with 15.5 hours light/day during the experimental period. Twenty-three birds were randomly assigned to each treatment. The battery cages (Figure 3.2) were equipped with individual feed troughs, water nipples, perches and galvanized trays for excreta collection. On reception, all birds were already debeaked and vaccinated according to the prescription of the supplier. Feed and water were provided for *ad libitum* consumption.

Figure 3.2 Individual battery cage system

3.2.3 Calcium supplement

The calcitic limestone of different particle sizes obtained from Agri Lime (Pty.) Ltd. in the North West province South Africa was used as Ca supplement in this study. According to the supplier this specific Ca source contains 36% Ca on dry matter (DM) basis. The micro-element composition according to the supplier of the calcitic limestone is shown in Table 3.1.

Table 3.1 The chemical analysis of the micro-element concentration of the limestone source on a dry matter basis

Micro element	**Concentration** (mg/kg DM)
Silica (Si)	15000
Magnesium (Mg)	4900
Iron (Fe)	4000
Aluminum (Al)	900
Manganese (Mn)	710
Fluorine (F)	86
Copper (Cu)	20
Zinc (Zn)	10
Vanadium (V)	<13
Cadmium (Cd)	<10
Lead (Pb)	<5
Arsenic (As)	<7

According to Chrystal (2000) commercial limestone is available at registered Ca levels of 38%, 36%, 34%, and 32% and it is available in different particle sizes. Agri Lime (Pty.) Ltd. use the following particle size classification; Al 1000 (0-1.0 mm), Al 2000 (1.0-2.0 mm) and Grit (2.0-3.8 mm).

3.2.4 Diet composition

The complete layer diet was formulated according to commercial layer diet standards to contain 3.6% Ca DM. The physical and calculated chemical composition of the diet is set out in Tables 3.2 and 3.3 respectively. Limestone (9.44%) was included in the basal diet in order to attain 3.6% dietary Ca across all treatments. A paddle type feed mixer (Figure 3.3) was used for mixing the basal diet with limestone.

Table 3.2 Physical composition of the complete layer diet on an air-dry basis

Physical composition	**%**
Yellow maize	64.23
Soya oil cake (47%)	16.84
Sunflower oil cake (37%)	3.99
Limestone	9.44
Full fat soya (36%)	3.19
Mono calcium phosphate	1.23
Fine salt	0.43
Premix E[1]	0.40
Natuphos 500 (Phytase 500 High Inclusion)[2]	0.06
Bicarbonate of soda	0.06
Choline powder[3]	0.02
Methionine	0.10

[1] Commercial mineral/vitamin premix (Inclusion levels of nutrients are confidential)
[2] Commercial phytase enzyme was included in the diet to give a minimum of 300 FTU (Phytase units) using Natuphos 500 High Inclusion with an enzyme concentration of 500 FTU
[3] Choline powder was added to provide 0.03% Choline

The complete layer diet was prepared by mixing 14.16 kg limestone/treatment with 150 kg of the basal diet. In order to ensure proper mixing of the limestone particles and basal diet, 50 kg of the basal diet was mixed with 5 kg of the limestone for approximately five minutes. Another 50 kg of basal diet and 5 kg of the limestone were added and mixed for the same duration of time. After adding the final basal diet and limestone into the feed mixer, the

complete diet (164.16 kg) was mixed for another 10 minutes. The same mixing procedure was carried out across all treatments. Mixing of diets was done on monthly basis to prevent peroxidation of the nutrients.

Table 3.3 Calculated chemical composition of the complete layer diet on an air-dry basis

Calculated chemical composition	**%**
Moisture	9.89
Protein	15.50
Fat	3.17
Ash	13.20
NDF[1]	8.18
ADF[2]	4.09
Crude fibre	2.76
Calcium	3.60
Phosphorus	0.58
Available phosphorus	0.29
Calcium to available phosphorus ratio	12.41
Calcium to phosphorus ratio	6.17
Chlorine	0.30
Sodium	0.18
Potassium	0.60
Magnesium	0.26
AME[3] (MJ/kg)	12.85
Arginine	1.01
Isoleucine	0.64
Lysine	0.78
Methionine and cystine	0.67
Methionine	0.38
Threonine	0.57
Tryptophane	0.18

[1] Neutral detergent fibre
[2] Acid detergent fibre
[3] Apparent metabolizable energy (MJ/kg)

Figure 3.3 Paddle type feed mixer

3.2.5 Experimental measurements

3.2.5.1 Performance of laying hens

Daily egg production records were summarized on weekly basis and expressed as percentage production. The cracked and shell-less eggs were also numerated on daily basis for production purposes, but not included for eggshell quality determination. The procedure of Ahmad & Balander (2003) was used to determine hen-day egg production where the number of eggs produced was divided by the number of live birds in each treatment.

All individual eggs produced weekly at the age of 54, 58, 62 and 70 weeks were marked, weighed and summarized at the end of a specific week. The mean egg weight for a specific collection period was calculated from total weekly egg weight. The mean egg content was calculated according to the procedure of Narushin (1977) by deducting the mean shell weight from the mean egg weight for each week expressed in gram. Egg output was determined in the former weeks according to Rose (1997) where the number of eggs produced by an individual hen (weekly) is multiplied by her weekly mean egg weight.

Individual feed intake was determined every week for the entire experimental period. Weekly feed intake records were used to calculate the average daily feed intake for individual hens. Feed conversion ratio (FCR) expressed in gram feed per gram egg (g/g) was determined according to the procedure of Abdallah *et al*. (1993) with some alterations. It was determined by dividing the mean weekly feed intake by the mean weekly egg weight during week 54, 58, 62 and 70 of age. Hens were weighed individually as illustrated in Figure 3.4 at the age of 34, 38 ,42 ,46, 50, 54, 58, 62, 66 and 70 weeks.

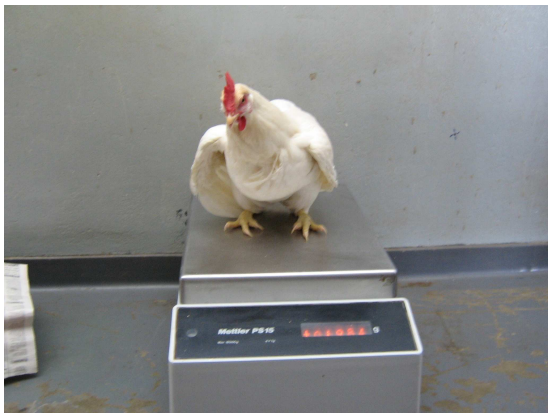

Figure 3.4 Weighing of hens

3.2.5.2 Eggshell quality

Eggshell quality characteristics were determined on all the eggs collected at week 54, 58, 62 and 70 of age. Eggs were broken out and the shells washed according to the procedure of Strong (1989) and Kul & Seker (2004) under slightly flowing water to clean any adhering materials. The shells were then air-dried at room temperature for one hour before shell thickness was measured. The eggshell thickness measurements were done according to the procedure described by De Ketelaere *et al*. (2002), Ehtesham & Chowdhury (2002), Ahmad & Balander (2003) and Kul & Seker (2004) using a shell thickness meter (micro-meter) (Figure 3.5) with the accuracy of 0.01 mm, whereby three replicates of each measurement were done on the blunt end, equator and sharp end regions of each egg, resulting in a total of nine measurements on an individual egg.

Figure 3.5 Measuring shell thickness

The eggshell dry weight and ashing were determined according to the procedure described by Clunies *et al*. (1992). Eggshells were carefully crushed into dry pre-weighed crucibles and

oven-dried at 105 ^{0}C for 12 hours. The eggshell and crucibles were weighed after oven-drying to determine the dry weight of the shells. The eggshells were then demineralized in a muffle furnace at 550^{0}C for 16 hours and ash weight was determined after cooling in a desiccators. Both egg weight and shell weight were used in the subsequent determination of other eggshell quality variables by using the following formulae:

Eggshell Ca content (g) = SW (g) x 0.373 (Simons, 1986)
Percentage eggshell (%) = (SW/EW) x 100 (Orban & Roland, 1990)
Egg surface area (ESA) = 3.9782 W $^{0.7056}$ where W = egg weight (mg) (Carter, 1975)
Shell weight per unit surface area (SWUSA) (mg/cm^2) = SW/ESA (Wells, 1967)
SW = shell weight
EW = egg weight

3.2.5.3 Bone characteristics

Bone characteristics measurements were evaluated in 10 hens per treatment at week 70 of age. All hens were weighed and sacrificed by cervical dislocation and the carcass subjected to overnight refrigeration at -20^{0}C before the removal of both left and right tibia bones and right humerus from each hen. Scalpel and scissors were used to remove meat from the bones (Figure 3.6) and to remove fibula bone from the tibia as shown in Figure 3.7. After cleaning the bones from all adhering materials, all bones were weighed full fat.

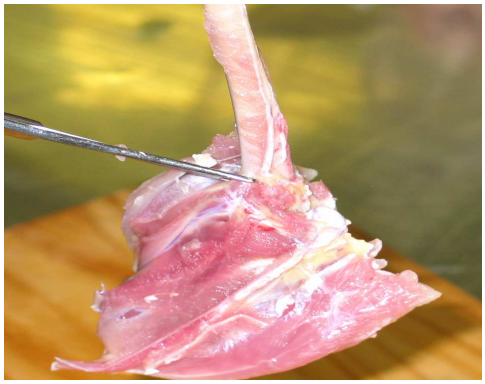

Figure 3.6 Removal of meat from tibia bone

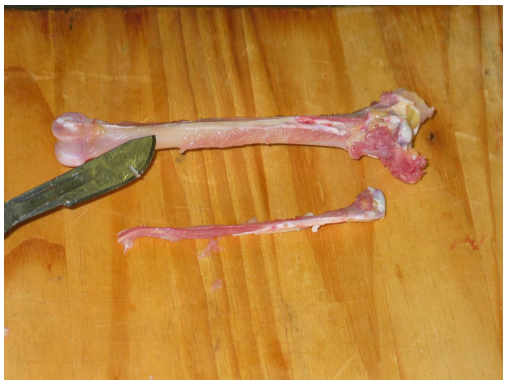

Figure 3.7 Removal of fibula bone

Bone dimensional characteristics (width and length) of both tibia and humeri bones were determined according to procedure described by Zhang & Coon (1997) using a Vernier Caliper to the nearest 0.01 mm as shown in Figures 3.8 and 3.9. After the completion of bone dimension measurements, all bones were stored individually in plastic bags in a freezer at -18^{0}C for later measurements of bone breaking strength, stress and bone ash percentage.

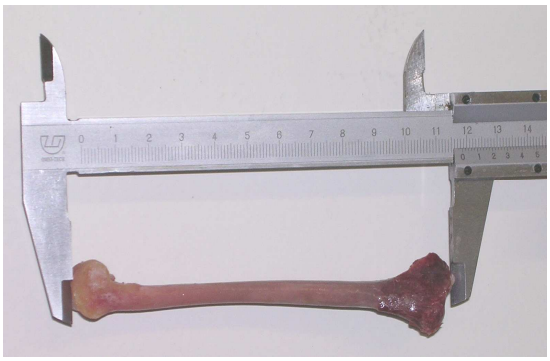

Figure 3.8 Tibia length measurement

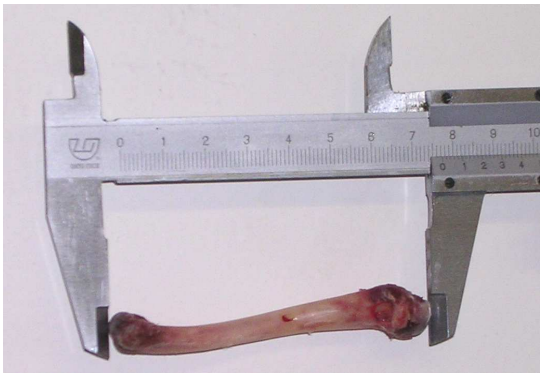

Figure 3.9 Humerus length measurement

(a) Bone ash

Bone ash determination was limited to the left tibia bone. The procedure of Al-Batshan *et al.* (1994) was used to determine fat free bone ash. Frozen left tibia bones were thawed and cut into three pieces to allow fitting into the thimble of the Soxhlet extractor. Bones were fat extracted in 98% hexane for four hours. The fat-free tibias were weighed after drying at 105^0C for 24 hours in an oven. The ash content of the fat-free, moisture-free bones was determined according to the procedure described by McCoy *et al*. (1996) at 550^0C in a muffle furnace for 24 hours. The percentage ash was determined as the ratio of ash to dry, fat-free tibia weights.

(b) Bone breaking strength

Bone breaking strength was determined on the right tibia and humerus bone using procedure outlined by Fleming *et al*. (1998 a, b). Bones were thawed and prior to the breaking test, each bone was marked at the midpoint. The outside diameter was measured both perpendicular to and parallel to the direction of the applied force, using dial calipers. The breaking strength of the bone was measured using an Instron tensile/compression machine. The centre point of

each bone was placed between two 10 mm diameter restraining bars which were set 30 mm apart. Then the bone was gradually subjected to an increasing force at 30 mm/minute on a three-point stress test until it broke. A computer controlled Instron machine recorded force readings every 0.02 seconds with the help of an HBM MVD 25010 signal conditioning. After breaking, diameter measurements were made inside and outside the midshaft of the bone, both perpendicular to and parallel to the direction of the applied force to calculate the area moment of inertia. Bone breaking strength in Newton (N/m^2) was also used in the estimation of bone stress according to Crenshaw *et al.* (1981),by applying the following formula.

Stress (kg/cm^2) = [Force (kg) x length (cm) x C (cm)] / [4 x moment of inertia (cm)]

Where: Force = Breaking strength (kg)

Length = 0.03 cm

C = Radius of bones (cm)

Moment of inertia = (π x $Radius^4$)/ 4

3.2.5.4 Statistical analysis

Bone and eggshell quality data were subjected to one-way analysis of variance using the General Linear Model (GLM) procedures of SAS (SAS, 1999), with limestone particle size as fixed treatment and hens as the experimental units. Statistical significance was based on 5% probability level. Tukey's studentized range (HSD) test outlined under the GLM procedure of SAS (1999) was used to separate the means. Pearson's correlation method was used to determine the relationship between individual bone and eggshell traits.

3.3 Results and Discussion

3.3.1 Performance parameters

The performance parameters data, which include feed intake, body weight, egg production, feed conversion ratio and egg output are shown in Tables 3.4 and 3.5 and in Figures 3.10, 3.11 and 3.12. There is also supplementary information in Table 3.6 on particle size, genotype, age of hens and Ca content of diets used by different researchers. It is clear from Table 3.6 that these factors differ among researchers. Therefore the comparison of results of various studies with each other should be done with caution.

According to Table 3.4 limestone particle size generally had no significant ($P>0.05$) effect on performance parameters during the entire experimental period (late laying period, beyond 33 weeks of age). Although feed intake of hens (Figure 3.10) during certain weeks had shown some significant differences, it was not constant and supported by the mean intake values for the entire experimental period (Table 3.4). These non-significant results for the late laying

period are in agreement with that of Rabon *et al*. (1991) and Scheideler (1998) for feed intake, Guinotte & Nys (1991) for egg production and feed conversion ratio and Maff (2000) for egg output. These researchers large particle sizes (Table 3.6) ranged between 0.07 and 4.75 mm in total which compare more or less with that of the current study (<1.0-3.8 mm).

Table 3.4 The effect of limestone particle size on the mean performance of hens during the experimental period (33-70 weeks) (Mean±s.e.)

			Significance	
Parameter	**Particle size (mm)**	**Mean±s.e**	**P[1]**	**CV[2](%)**
Feed intake	<1.0	101.98±1.20		
(g/hen/day)	1.0-2.0	100.07±1.79	0.6229	6.5
	2.0-3.8	101.43±1.22		
Body weight	<1.0	1961.67±31.76		
(g/hen)	1.0-2.0	1947.25±36.82	0.4189	7.2
	2.0-3.8	2003.08±22.13		
Egg production (%)	<1.0	82.65±0.02		
	1.0-2.0	82.31±0.02	0.3991	10.9
	2.0-3.8	79.25±0.02		
Egg output	<1.0	352.53±8.79		
(g/week)	1.0-2.0	342.39±0.02	0.0599	11.6
	2.0-3.8	323.40±0.02		
FCR[3]	<1.0	1.67±0.03		
(g feed/g egg)	1.0-2.0	1.65±0.03	0.5025	8.7
	2.0-3.8	1.70±0.03		

[1] (P>0.05) = non-significant
[2] Coefficient of variation
[3] Feed conversion ratio (g feed/g egg)

In contrast with the results of the present study Guinotte & Nys (1991) observed a significantly higher feed intake and body weight with the use of large particle limestone. Karunajeewa (2006) reported significantly higher egg production of White Leghorn and Australorp hens receiving large particle limestone between 49 and 72 weeks of age. These contradictory results could probably be attributed to the different particle sizes used in the various studies or the genetic differences between strains of laying hens. Karunajeewa (2006) used Ca particle sizes which ranged between 3.0-6.0 mm (Table 3.6) while the range of Guinotte & Nys (1991) was also wider (1.18-4.75 mm) than that used by other researchers mentioned above.

Table 3.5 The effect of limestone particle size on egg output and feed conversion ratio during the experimental period (54, 58, 62 and 70 weeks) (Mean±s.e.)

		Particle size (mm)			Significance	
Parameter	**Week**	**<1.0**	**1.0-2.0**	**2.0-3.8**	**P[1]**	**CV[2](%)**
Egg output	54	350.40±9.79	338.36±10.10	332.32±11.21	0.4596	13.6
(g/week)	58	353.32±16.72	339.22±11.31	333.94±10.65	0.5643	17.6
	62	367.80±11.81	353.13±11.06	341.16±12.52	0.2879	15.1
	70	347.41±9.55	344.54±7.69	308.61±16.99	0.1481	16.6
FCR[3]	54	1.64±0.04	1.62±0.04	1.70±0.04	0.2543	10.3
(g feed/g egg)	58	1.70±0.03	1.66±0.04	1.72±0.03	0.5885	9.7
	62	1.70±0.07	1.63±0.05	1.76±0.04	0.3015	15.7
	70	1.65±0.04	1.70±0.04	1.70±0.05	0.7229	10.9

[1] (P>0.05) = non-significant
[2] Coefficient of variation
[3] Feed conversion ratio (g feed/g egg)

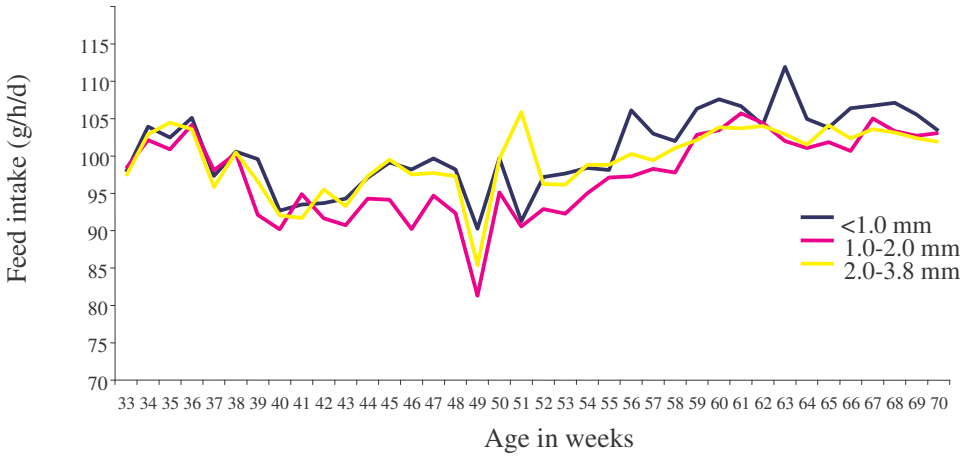

Figure 3.10 The effect of limestone particle size on the weekly feed intake of hens during the later stages of lay

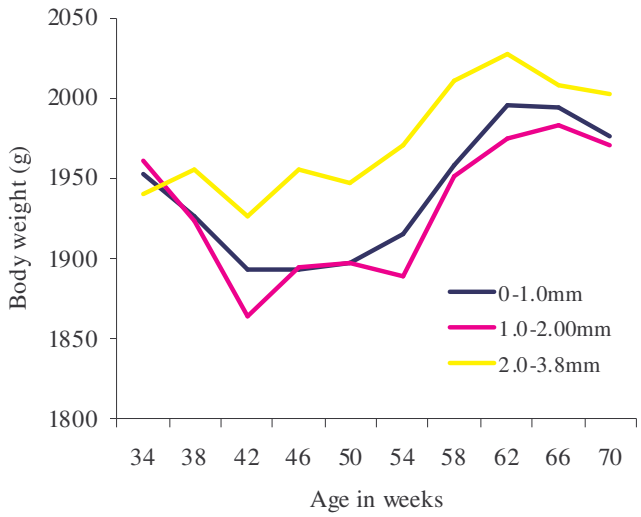

Figure 3.11 The effect of limestone particle size on the monthly body weight of hens during the later stages of lay

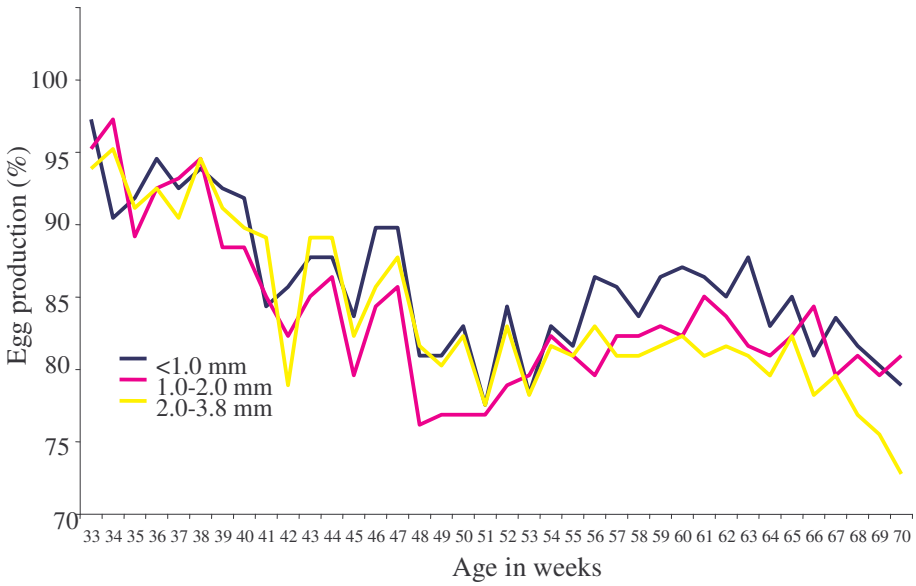

Figure 3.12 The effect of limestone particle size on the weekly egg production of hens during the later stages of lay

Table 3.6 Variables used in different studies, regarding limestone particle size

Authors	Particle size (mm)	Genotypes of hens	Age (weeks)	Dietary Ca content (%)
Cheng & Coon (1990)	Small 0.15-0.50			
	Medium 1.02-1.68	DeKalb-DX	36	2.00-4.50
	Large 2.38-3.36			
Guinotte & Nys (1991)	Small 0.075-0.15	ISA-Brown	64-77	3.60
	Medium 0.3-1.17			
	Large 1.18-4.75			
Rabon *et al*. (1991)	Small 74µ-1.41	DeKalb	79	2.75
	Large 2.00-4.70			
Fleming *et al.* (1998b)	Small 2.5	ISA-Brown	15-70	3.53
	Large 4.0			
Scheideler (1998)	Small 0.07-0.25	Babcock B300	108	3.80
	Medium 0.42-0.84			
	Large 2.00-4.00			
Maff (2000)	Small 0.50-2.75	Lohmann-Brown	20-70	4.19
	Large 2.00-4.00			
Rao & Raju (2004)	Small 2.00-4.00	White Leghorns	24-37	2.75
	Medium 4.00-6.00			3.25
	Large 6.00-8.00			3.75
Karunajeewa (2006)	Small <1.00	White Leghorn & Australorp	24-72	3.00
	Large 3.00-6.00			3.50
De Witt (2006)	Small <1.00	Lohmann-Silver	17-32	3.60
	Medium 1.00-2.0			
	Large 2.00-3.80			
Currrent study	Small <1.0	Lohmann-Silver	33-70	3.60
	Medium 1.00-2.00			
	Large 2.00-3.80			

3.3.2 Egg characteristics

3.3.2.1 Egg weight and contents

The influence of limestone particle size on egg weight and-content results is set out in Table 3.7 and 3.10. According to these results, it is clear that the dietary treatment did not have any significant (P<0.05) effect on egg weight and content during the experimental period. These results are in accordance with the findings of Cheng & Coon (1990), Scheideler (1998) and Rao & Raju (2004). Contrary to these results Guinotte & Nys (1991) reported that large particle limestone resulted in a heavier egg weight than small particle limestone. From Table 3.6 it seems that factors like the range between small and medium particles, as well as genotype and age of hens could influence the results of various studies. Differences in particle sizes within a specific particle size (small, medium or large) could also play a role. It is clear that variables used in different studies hamper a meaningful comparison between studies.

Table 3.7 The effect of limestone particle size on egg weight and content (Mean±s.e.)

		Particle size (mm)			Significance	
Parameter	**Weeks**	**<1.0**	**1.0-2.0**	**2.0-3.8**	**P[1]**	**CV[2](%)**
Egg weight (g)	54	60.38±0.91	59.07±1.01	58.28±0.65	0.2323	6.7
	58	60.17±0.86	59.06±0.96	59.07±0.75	0.5826	6.6
	62	61.84±0.88	60.57±1.05	59.07±0.75	0.1810	6.8
	70	62.89±0.88	61.04±0.99	61.42±1.19	0.4139	7.6
Egg content (g)	54	54.61±0.85	53.35±0.93	52.62±0.61	0.2210	6.9
	58	54.33±0.81	53.34±0.90	53.25±0.69	0.5738	6.9
	62	55.99±0.81	54.70±0.95	53.57±0.72	0.1312	6.9
	70	57.03±0.81	55.23±0.90	55.52±1.11	0.3603	7.8

[1] (P>0.05) = non-significant
[2] Coefficient of variation

3.3.2.2 Eggshell quality

Eggshell quality embodies characteristics such as shell weight, shell percentage, shell Ca, SWUSA, shell thickness and shell breaking strength. The effects of limestone particle size on eggshell characteristics results are displayed in Tables 3.8, 3.9, 3.10 and 3.11. According to these results limestone particle size had no significant (P>0.05) effect on eggshell characteristics during the entire experimental period except on eggshell thickness. From Table 3.8 it is evident that eggshell thickness results have shown some response to dietary treatment during certain weeks. These results were however not constant and supported by the mean shell thickness values for the entire experimental period (Table 3.11). These non-significant (P>0.05) results are in agreement for the late laying period with that of Scheideler (1998) and Maff (2000). These researchers large particle size (Table 3.6) was similar to the one used in

the current study. Contrary to these results Guinotte & Nys (1991), Rabon *et al*. (1991) and Karunajeewa (2006) found a significant (P<0.05) positive influence of large Ca particle size on eggshell quality during the later stages of lay. Cheng & Coon (1990) observed significantly (P<0.05) thicker eggshells during the later stages of lay with the use of large Ca particle size. Hens fed large particles had an average shell thickness of 0.302 mm in comparison to 0.280 mm for hens fed small limestone particle size. The results of the present study are unexpected since dietary treatment is likely to have a more pronounced effect on eggshell quality during the later production stages when Ca absorption is less efficient. These seemingly contrary results could probably be explained in some instances by different Ca particle sizes and Ca contents used in the diets as shown in Table 3.6. An interaction of large particles with suboptimal Ca level in the diets used by Cheng & Coon (1990), Rabon *et al*. (1991) and Karunajeewa (2006) could be responsible for the different results. The results of the present study where an optimum Ca level of 3.6% was used, supports the statement of Rao *et al*. (1992) that partial substitution of fine limestone by larger particles in layers may not promote better eggshell quality under optimal dietary Ca conditions. Differences in particle size, variation within a specific particle size, range between the lowest and highest particle size, Ca-content of the diet, genotype and age of hens could also be responsible for contrary results among studies. As already stated these confounded effects among various studies hampers meaningful comparisons, discussions and conclusions.

Table 3.8 The effect of limestone particle size on eggshell thickness during the experimental period (Mean±s.e.)

		Particle size (mm)			Significance	
Parameter	**Week**	**<1.0**	**1.0-2.0**	**2.0-3.8**	**P[1]**	**CV[2](%)**
Blunt end (mm)	54	0.37±0.00[a]	0.36±0.00[b]	0.36±0.00[b]	0.0351	6.0
	58	0.36±0.00	0.36±0.00	0.36±0.00	0.7879	6.6
	62	0.35±0.00	0.36±0.00	0.36±0.00	0.4604	7.5
	70	0.36±0.00	0.37±0.00	0.36±0.00	0.8790	10.3
Equator (mm)	54	0.40±0.00[a]	0.39±0.00[b]	0.39±0.00[b]	0.0231	4.9
	58	0.37±0.00	0.38±0.00	0.39±0.00	0.1381	6.9
	62	0.37±0.00[a]	0.39±0.00[b]	0.40±0.00[b]	0.0011	7.7
	70	0.38±0.00	0.39±0.00	0.39±0.00	0.8027	9.2
Sharp end (mm)	54	0.39±0.00[a]	0.37±0.00[b]	0.37±0.00[b]	0.0348	6.5
	58	0.36±0.00	0.38±0.00	0.37±0.00	0.3401	8.4
	62	0.36±0.00	0.38±0.00	0.38±0.00	0.0811	9.3
	70	0.36±0.00	0.37±0.00	0.38±0.00	0.6026	10.6

[a,b] Mean in rows with different superscripts differ significantly (P<0.05)
[1] (P>0.05) = non-significant
[2] Coefficient of variation

Table 3.9 The effect of limestone particle size on eggshell quality during the experimental period (Mean±s.e.)

		Particle size (mm)			Significance	
Parameter	**Weeks**	**<1.0**	**1.0-2.0**	**2.0-3.8**	**P[1]**	**CV[2](%)**
Shell weight	54	5.77±0.08	5.71±0.11	5.67±0.07	0.6545	6.9
(g)	58	5.83±0.11	5.72±0.09	5.82±0.08	0.6314	7.5
	62	5.84±0.11	5.88±0.13	5.84±0.09	0.9706	8.6
	70	5.86.011	5.81±0.16	5.91±0.12	0.8762	10.3
Eggshell	54	9.58±0.09	9.68±0.13	9.73±0.10	0.6224	5.1
(%)	58	9.71±0.15	9.70±0.13	9.87±0.10	0.5534	5.9
	62	9.45±0.13	9.70±0.13	9.84±0.11	0.0925	5.9
	70	9.31±0.14	9.51±0.14	9.63±0.16	0.4046	8.1
Eggshell Ca	54	2.15±0.03	2.13±0.04	2.11±0.03	0.6546	6.9
(g)	58	2.18±0.04	2.13±0.04	2.17±0.03	0.6316	7.5
	62	2.18±0.04	2.19±0.01	2.17±0.03	0.9706	8.6
	70	2.18±0.04	2.16±0.01	2.20±0.05	0.8762	10.3
Shell ash	54	0.26±0.00	0.27±0.00	0.26±0.00	0.0796	7.8
(g)	58	0.25±0.00	0.24±0.00	0.25±0.00	0.8182	9.7
	62	0.25±0.00	0.25±0.00`	0.24±0.00	0.4149	9.2
	70	0.25±0.00	0.25.0.00	0.25±0.00	0.4777	9.8
ESA[3]	54	71.80±0.76	70.68±0.85	70.04±0.55	0.2377	4.7
(cm^2)	58	71.62±0.72	70.68±0.82	70.70±0.63	0.5832	4.7
	62	73.02±0.73	71.94±0.83	70.98±0.65	0.1816	4.8
	70	73.89±0.74	72.34±0.83	72.65.0.98	0.4049	5.4
SWUSA[4]	54	80.47±0.69	80.80±1.03	80.89±0.80	0.9363	4.8
(mg/cm^2)	58	81.47±1.20	80.93±1.00	82.42±0.81	0.5796	5.7
	62	79.99±1.10	81.60±1.18	82.30±0.92	0.3089	6.0
	70	79.20±1.19	80.19±1.76	81.28±1.26	0.5876	8.1

[1] (P>0.05) = non-significant
[2] Coefficient of variation
[3] ESA = Egg surface area
[4] SWUSA = shell weight per unit surface area

Table 3.10 The effect of limestone particle size on mean eggshell quality during the experimental period (Mean±s.e.)

Parameter	Particle size (mm)	Mean± s.e	Significance	
			P[1]	CV[2](%)
Egg weight	<1.0	61.32±0.82		
(g)	1.0-2.0	59.93±0.95	0.3219	6.5
	2.0-3.8	59.59±078		
Egg content	<1.0	55.52±0.76		
(g)	1.0-2.0	54.12±0.87	0.2263	6.6
	2.0-3.8	53.64±0.72		
Shell weight	<1.0	5.83±0.09		
(g)	1.0-2.0	5.78±0.12	0.9396	7.7
	2.0-3.8	5.81±0.08		
Eggshell	<1.0	9.51±0.11		
(%)	1.0-2.0	9.65±0.14	0.3468	5.7
	2.0-3.8	9.76±0.11		
Eggshell Ca	<1.0	2.17±0.04		
(g)	1.0-2.0	2.16±0.04	0.9367	7.7
	2.0-3.8	2.17±0.04		
Eggshell ash	<1.0	0.25±0.01		
(g)	1.0-2.0	0.26±0.01	0.8331	8.3
	2.0-3.8	0.25±0.01		
ESA[3]	<1.0	72.58±0.69		
($cm^{2)}$	1.0-2.0	71.41±0.80	0.3223	3.3
	2.0-3.8	71.12±0.65		
SWUSA[4]	<1.0	80.28±0.93		
(mg/cm^2)	1.0-2.0	80.88±1.17	0.6111	5.6
	2.0-3.8	81.67±0.84		

[1] (P> 0.05) = non-significant
[2] Coefficient of variation
[3] Egg surface area
[4] SWUSA = shell weight per unit surface area (mg/cm^2)

Table 3.11 The influence of limestone particle size on mean eggshell thickness during the experimental period (Mean±s.e.)

Parameter	Particle size (mm)	Mean± s.e	Significance	
			P[1]	CV[2](%)
Blunt end	<1.0	0.36±0.00		
(mm)	1.0-2.0	0.36±0.00	0.9465	6.8
	2.0-3.8	0.36±0.00		
Equator	<1.0	0.38±0.00		
(mm)	1.0-2.0	0.39±0.00	0.3526	6.5
	2.0-3.0	0.39±0.00		
Sharp end	<1.0	0.37±0.00		
(mm)	1.0-2.0	0.36±0.00	0.6999	8.0
	2.0-3.8	0.37±0.00		

[1] (P> 0.05) = non-significant
[2] Coefficient of variation

3.3.3 Bone parameters

3.3.3.1 Bone dimensional properties

Bone dimensional properties are used as the measure of skeletal development in laying hens. The effect of limestone particle size on bone dimensions are illustrated in Table 3.12. Limestone particle size had no significant (P>0.05) effect on length, width and weight of the tibia and humerus respectively, laying hens at the end-of-lay. These results are in agreement with the results of Maff (2000) who reported that limestone particle size had no significant influence on tibia length of Lohmann-Brown hens at 72 weeks of age. This researchers large particle size of 2.00-4.00 mm (Table 3.6) was very close to the one used in the current study but the dietary Ca level (4.19%) was higher. Tibia length in the present study did not respond to dietary treatment and this may be due to the fact that structural bone formation largely ceased at sexual maturity (period when ovulation begins, <25 weeks of age) and that new bone formation seemed to be confined to the medullary bone (Almeida Paz *et al*., 2006). On the other hand De Witt (2006) who studied the effect of limestone particle on bone dimensions at 37 weeks of age on the same hens used in the current study reported that an increase in limestone particle size resulted in a significant (P<0.05) decrease in tibia length and weight as well as humerus length. The different results could have been due to age differences. Guinotte *et al.* (1995) indicated that in young birds (3 weeks of age) coarse limestone particles decreased intestinal Ca retention and bone mineralization. This suggests insufficient solubilization process of the large particles in young birds, possibly because of reduced digestive transit time.

Table 3.12 The effect of limestone particle size on bone dimensions (Mean±s.e.) of layer hens at 70 weeks of age

Parameters	Particle size (mm)			Significance	
	<1.0	1.0-2.0	2.0-3.8	P[1]	CV[2](%)
Right tibia					
Weight (g)	10.14±0.35	10.36±0.35	10.06±0.72	0.9106	15.7
Length (mm)	116.49±1.68	118.86±1.12	114.30±4.09	0.4900	7.2
Width (mm)	6.42±0.07	6.45±0.08	6.48±0.08	0.8527	3.6
Right humerus					
Weight (g)	3.93±0.20	3.73±0.21	3.82±0.17	0.7842	15.5
Length (mm)	78.10±0.73	78.31±0.72	78.44±0.73	0.9473	2.8
Width (mm)	6.15±0.10	6.09±0.12	6.18±0.09	0.8025	5.1

[1] (P>0.05) = non-significant
[2] Coefficient of variation

The detrimental effect of large particle size on tibia and humerus length and/or weight observed by De Witt (2006) at 37 weeks of age was, however, not reflected at 70 weeks of age. Therefore the results of the present study did not confirm the statement of Almeida Paz *et al*. (2006) that structural bone formation largely ceased at sexual maturity (< 25 weeks of age).

3.3.3.2 Bone mechanical properties

Bone strength is the toughness or ability to endure stress; therefore it is related to ultimate load or stress at which the bone will break. The influence of limestone particle size on bone breaking strength properties results are illustrated in Table 3.13. According to these results, tibia bone breaking strength (P=0.0107) and stress (P=0.0391) were significantly affected by limestone particle size at end-of-lay. An increase in limestone particle size resulted in an increase in both tibia breaking strength and stress. These results were also confirmed by Moran *et al.* (1970), Miller & Sunde (1975) and Cheng & Coon (1990) that an increase in Ca particle size resulted in an increase in tibia bone breaking strength. The practical benefit of large particle size Ca on bone breaking stress was also confirmed by Guinotte & Nys (1991) who found a significant effect of large particle size limestone on bone ultimate stress (P<0.05), yield stress (P<0.05) and stiffness (P<0.001) of ISA-Brown laying hens between 64 and 77 weeks of age. Fleming *et al.* (1998b) working with ISA-Brown laying hens between 15 and 70 weeks of age reported that the effect of limestone in particulated form on bone breaking strength was more pronounced later during the laying period. Accordingly De Witt (2006) found that particle size had no significant (P<0.05) influence on bone breaking strength at week 37 of age. Fleming *et al.* (1998b) confirmed that adding limestone to diets in the form of particles rather than as a powder has a beneficial effect in decreasing the severity of some of the characteristics of osteoporosis. This may be associated with greater availability of Ca in enhancing medullary bone formation, thereby inhibiting structural bone resorption as well as contributing directly to bone strength. Rennie *et al.* (1997) stated that particulate Ca sources extend the period of Ca absorption into darkness when food consumption has ceased leading to a greater availability of Ca for shell and bone formation.

The humerus bone breaking strength and stress were not statistically (P>0.05) affected by limestone particle size. However, humerus bone breaking strength (Table 3.13) increased non-significantly (P>0.05) with an increase in limestone particle size between 50 and 70 weeks of age.

Table 3.13 The effect of limestone particle size on bone mechanical properties (Mean±s.e.) of layer hens at 70 weeks of age

	Particle size (mm)			Significance	
Parameters	**0-1.0**	**1.0-2.0**	**2.0-3.8**	**P**	**CV[1](%)**
Right tibia					
Breaking strength (N/m^2)	266.29±21.1[a]	331.96±26.8[ab]	395.01±29.7[b]	0.0107	24.5
Stress (kg/cm^2)	98.64±8.6[a]	121.21±10.5[ab]	143.57±13.5[b]	0.0391	28.4
Right humerus					
Breaking strength (N/m^2)	234.11±24.2	252.24±18.5	256.54±20.88	0.7457	25.8
Stress (kg/cm^2)	97.22±8.5	110.70±10.0	108.94±12.8	0.6646	31.4

[a,b] Mean in rows with different superscripts differ significantly (P<0.05)
[1] Coefficient of variation

These results are consistent with the findings of Fleming *et al.* (1998b) who reported that limestone particle size did not affect humerus bone breaking strength of ISA-Brown hens at 15, 25, 50 and 70 weeks of age. In accordance with the present study particulate limestone resulted in a non-significant higher humerus breaking strength than powdered limes

3.3.3.3 Bone ash percentage, bone index and percentage bone

Bone ash concentration is used as indicator of bone status in mineral nutrition of poultry while bone weight/length index is used in the determination of morphometric properties of the bones. The effect of limestone particle size on percentage bone, bone ash and bone index of tibia and humerus bones of laying hens in late is illustrated in Table 3.14. These results demonstrated that limestone particle size did not have a significant ($P>0.05$) effect on any of these parameters at the end of the laying period. These results are in disagreement with the results of Guinotte & Nys (1991) who reported that particulated Ca sources yielded significant higher bone ash (54.1%) than ground sources (50.7%) in ISA-Brown laying hens during the late laying period. De Witt (2006) also reported during the early laying period (37 weeks of age) that large limestone particle size induced a significant ($P<0.05$) increase in bone ash of Lohmann-Silver laying hens. The different results between the early and late laying period should be interpreted with caution because earlier when discussing bone dimension results it was concluded that large limestone particle size had a negative impact on Ca utilization in young birds as reflected by tibia and humerus dimensions. However, the increased bone ash percentage during the early laying period probably suggests that hens on the large particle size treatment had more bone ash because they were producing less shell and keeping more structural bone rather than mobilizing it. The study of De Witt (2006) on different particle sizes revealed, however, no differences in shell weight, eggshell percentage and shell thickness during the early stages of lay. Therefore the higher bone ash content during early lay did not support the opinion that large limestone particle size has a negative effect on Ca utilization in young birds. De Witt (2006) also came to the conclusion that the use of bone dimensions to predict responses to the effect of limestone particle size yielded inexplicable results.

The fact that humerus bone percentage and bone weight/length index were not responsive to the dietary treatments is to be expected because bone dimensional traits (Table 3.12) used as their determinants were also not statistically ($P>0.05$) significant. A significant ($P<0.01$) correlation between different bone indices and their determinants were also observed; namely humerus weight and percent humerus ($r^2=0.80$), humerus weight and humerus index ($r^2=0.98$) and percent humerus and humerus index ($r^2=0.84$).

Table 3.14 The effect of limestone particle size on bone ash percentage, bone index and percentage bone (Mean±s.e.) of layer hens at 70 weeks of age

	Particle size (mm)			Significance	
Parameters	**0-1.0**	**1.0-2.0**	**2.0-3.8**	**P[1]**	**CV[2](%)**
Right tibia					
Percentage bone[3]	0.50±0.01	0.50±0.01	0.51±0.04	0.9893	14.6
Ash[4] (%)	59.46±1.29	61.40±0.77	61.27±0.73	0.2878	4.6
Bone index[5]	86.89±2.11	87.01±2.33	86.72±4.46	0.9980	11.6
Humerus					
Percentage bone[6]	0.20±0.01	0.18±0.01	0.19±0.01	0.3663	12.1
Bone index[7]	50.25±2.24	47.57±2.34	48.63±2.19	0.7207	14.3

[1] (P>0.05) = non-significant
[2] Coefficient of variation
[3] Percentage tibia bone = (Tibia weight/Body weight) x 100
[4] % Tibia ash = (Tibia ash weight / Fat free tibia weight) x 100
[5] Tibia bone index = (Tibia weight /tibia length) x 100
[6] Percentage humerus bone = (Humerus weight/Body weight) x 100
[7] Humerus bone index = (Humerus weight / Humerus length) x 100

3.4 Conclusions

According to the production and shell quality results of the present study it seems that limestone particle size had no influence during the late stages of lay. Bone dimensional properties, bone indexes and ash percentage were also not influenced during the late laying period. However, dietary treatment resulted in a beneficial influence on bone mechanical properties, where large particle limestone resulted in a higher bone breaking strength and stress. The inability of dietary Ca particle size to influence feed intake and egg production may be due to the specific particle size used in the current study. The large particle size used in the current study appeared to be relatively smaller in comparison to large particles used in other similar studies where positive results on feed intake and egg production were obtained.

Another factor to be considered is that the different particle sizes used in the present study were not available in a precise particle size. Specific particle sizes were in the form of distribution ratios that is liable to variation within a specific particle size and this could probably influence the results. This also applies for other similar studies. This aspect needs further investigation. It seems, however, that there is no intrinsic benefit of limestone particle size when the diet and the limestone source provided sufficient available Ca to the layer hens with the exception of bone mechanical properties at the end of lay.

De Witt (2006) also evaluated this specific Ca source during early stages of lay and concluded that particle size had no significant effect on above mentioned parameters. Therefore limestone particle size overall seems to have a non-significant influence on production, bone and shell quality with the exception of bone mechanical properties at the end of the laying period.

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CHAPTER 4

THE INFLUENCE OF LIMESTONE PARTICLE SIZE DISTRIBUTION RATIOS IN LAYER DIETS ON BONE AND EGGSHELL CHARACTERISTICS AT POST PEAK PRODUCTION

4.1 Introduction

Calcium (Ca) is an important nutrient for laying hens. It is necessary for proper eggshell formation and is also needed to maintain skeletal integrity (Saunders-Blades & Anderson, 2003). Korver (2004) indicated that the large Ca demand for eggshell formation compounds the inability of the actively laying hen to deposit new structural bone. Kuhl (2000) concluded that eggshell quality is greatly dependent on the skeletal condition of the laying hen. For these reasons Ca has been added to laying hen diets for a number of years. Despite this, egg producers still suffers financial losses each year from poor shell quality and from the loss of hens due to poor bone quality related syndromes such as osteoporosis. Sustained high rates of egg production create a tremendous metabolic demand for Ca especially during post peak egg production (Webster, 2003). Bain (1997) indicated that the highest incidence of cracked eggs occurs mainly in the last third quarter of the laying period (after 53 weeks of age). The laying hen's ability to mobilize skeletal Ca decreases with age while the number of eggs produced declines along with a reduction in eggshell quality (Franco-Jimenez & Beck, 2005) and therefore the laying hens require a high quality Ca source in order to meet these requirements.

A number of Ca sources are being used to meet the requirement of dietary Ca for birds. However, Roland & Bryant (2000) conformed that there is no intrinsic benefit of Ca sources, other than the size of the particles that provide Ca when it is most needed especially during the night when the hen has no access to feed. Whitehead (2004) stated that provision of dietary Ca source in particulate form rather than powdered form improves both shell and bone quality in older laying hens thus making the birds less dependent upon bone mobilization to provide Ca for eggshells. Historically, the positive benefits affecting eggshell quality and various bone parameters have been shown with feeding larger particle sized limestone (Cheng & Coon, 1990). These researchers showed that *in vitro* solubility measurements on large particulate Ca provided a more accurate basis for feeding recommendations than just particle size alone. Further, large particle size $CaCO_3$ with corresponding lower *in vitro* solubility values increased Ca retention in the gizzard for layers (Zhang & Coon, 1997), thus providing a source for Ca ionization and availability for eggshell formation during peak shell production non-feeding times.

However, confusion still exist among poultry producers about the optimum blend of fine to large particle $CaCO_3$ to use during different stages of egg production. Guinotte *et al*. (1995) indicated that laying hens diets usually include coarse particles of Ca in substitution of 50-66% of fine particles to improve eggshell quality. Hunton (2006) recommended that the distribution ratio containing 25% large particles is the minimum for eggshell quality. For the early laying stages, Webster (2003) and Saunders-Blades & Anderson (2003) recommended that Ca particle size distribution ratio of 33% coarse and 67% fine are quite adequate to optimize bone and eggshell quality. For the peak production period, Scheideler (2004) stated that a blend of 65% fine and 35% large is optimum while the blend of 50% fine to coarse particles supported good eggshell quality after peak egg production. For the later stages of lay, Roland & Bryant (1999) indicated that there is no need to completely eliminate fine Ca source from the laying hens diet but rather 50% inclusion rate of both fine to large particle size can alleviate shell quality problems in aging hens. Pavloski *et al*. (2003) also during the later stages of lay found a significant higher shell mass, shell thickness and eggshell breaking force and lower eggshell deformation with 80% large particle distribution ratio. On the other hand, dietary calcium in particulate form promotes better sustained mineralization of medullary bone so that less resorption of structural bone occurs (Webster, 2003). This effect can benefit bone quality to the end of the laying cycle. Fleming *et al.* (1998b) reported that particulate limestone resulted in the improvement of tibial radiographic densities and bone breaking strength and keel radiographic densities later during the laying period. Whitehead & Fleming (2000) indicated that feeding large limestone particles resulted in improved bone strength in older hens.

Given the different recommendations on limestone particle size distribution in layer diets, plus high metabolic demand for Ca during the post peak egg production, further research is of utmost importance. Therefore the influence of particle size distribution ratios of a specific calcitic limestone commonly used in South Africa as a supplemental Ca source in layer diets on bone and eggshell quality during the later stages of the laying period was investigated.

4.2 Materials and Methods

To investigate the effect of limestone particle size distribution ratios in layer diet on bone and eggshell quality characteristics, a study was conducted using the calcium source described in Chapter 3, paragraph 3.2.3

4.2.1 Experimental design

The experiment was a fully randomized design with five limestone particle sizes distribution ratios as treatments. The different distribution ratios were made up of a mixture of both small particle size (<1.0 mm) and large particle size (2.0-3.8 mm) to give the following ratios of fine to large particles namely; 0, 25, 50, 75 and 100%. The limestone samples of five different limestone ratios used during the present study are illustrated in Figure 4.1.

Figure 4.1 Inclusion levels (%) of large particles limestone (2.0-3.8 mm) into the five distribution ratios

4.2.2 Birds and husbandry

One hundred and fifteen, 17 weeks Lohmann-Silver pullets were randomly allocated to five treatments which resulted in twenty-three hens per treatment. All the hens were subjected to similar conditions as those outlined in Chapter 3 in terms of housing, diets and general husbandry practices.

4.2.3 Experimental measurements

The same experimental measurements used in the particle size study in Chapter 3 paragraph 3.2.5.1 for performance parameters, paragraph 3.2.5.2 for eggshell quality and paragraph 3.2.5.3 for bone characteristics were used in this study without any modification.

4.2.4 Statistical analysis

Bone, production and shell quality data were subjected to one-way analysis of variance using the General Linear Model (GLM) procedures of SAS (SAS, 1999), with limestone distribution ratios as fixed treatments and hens as the experimental units. Statistical significance was based on 5% probability level. Tukey's studentized range (HSD) test outlined under the GLM procedure of SAS (1999) was used to separate the means. Pearson correlation method was used to determine the relationship between different bone parameters and eggshell thicknesses of the different regions of the egg.

4.3 Results and Discussion

4.3.1 Performance parameters

The effect of limestone particle size distribution ratios on performance parameters results are shown in Table 4.1 and 4.2 and in Figure 4.2 to 4.4. According to these results it seems that the dietary treatment in general had no significant (P>0.05) influence on performance traits including body weight, egg production, feed conversion ratio and egg output during the entire experimental period. Feed intake results (Figure 4.1) on the other hand have shown some inconsistent, significant (P<0.05) response to the dietary treatment during some weeks. This was however not evident in the mean performance parameters data in Table 4.2. These non-significant results are in accordance with the findings of Brister *et al*. (1981), Keshavarz *et al.* (1993), Saunders-Blades & Anderson (2003), Pizzolante *et al*. (2006) and Lichovnikova (2007) during the later stages of lay. From Table 4.3 it is evident that these researchers used different ranges of distribution ratios, limestone particle sizes, genotype of hens, ages and Ca-content in the diets.

However, the present study results are in disagreement with the results of Saunders-Blades & Anderson (2003) on feed intake. These researchers compared fine particles (0.50-1.00 mm) with a distribution ratio of 67:33 fine to large (>0.40 mm) ratios and found a significant higher feed intake compared to birds receiving 100:0 fine to large ratios. From Table 4.3 it seems that factors like a larger particle size (>4.0), genotype and age of hens could be responsible for the contrary results of the two studies. In Chapter 3 it was speculated from results in the literature that larger particles in the diet compared to the current study could increase feed intake.

Table 4.1 The effect of different limestone particle size distribution ratios on egg output and feed conversion ratio during the experimental period (Mean±s.e.)

		Large particles (%)					Significance	
Parameters	**Weeks**	**0**	**25**	**50**	**75**	**100**	**P[1]**	**CV[2](%)**
Egg output	54	350.40±9.79	340.35±11.5	338.53±10.8	352.02±9.83	332.32±11.21	0.6476	14.1
(g/week)	58	353.32±16.72	329.71±12.7	352.54±12.3	354.24±10.64	333.94±10.65	0.4831	17.1
	62	367.80±11.81	346.46±9.56	335.09±18.32	349.77±9.80	341.16±12.52	0.4492	16.8
	70	347.41±9.55	345.79±10.09	314.68±14.4	341.87±10.9	308.61±16.99	0.1271	18.5
FCR[3]	54	1.64±0.04[a]	1.74±0.04[ab]	1.69±0.03[ab]	1.80±0.04[b]	1.70±0.04[ab]	0.0312	9.8
(g feed/g egg)	58	1.70±0.03	1.74±0.04	1.73±0.04	1.76±0.04	1.72±0.03	0.7848	10.1
	62	1.70±0.04	1.73±0.09	1.68±0.05	1.75±0.04	1.76±0.04	0.8456	16.6
	70	1.65±0.04	1.67±0.04	1.67±0.03	1.74±0.03	1.70±0.05	0.5334	10.5

[a,b] Means in rows with different superscripts differ significantly (P<0.05)
[1] (P>0.05) = non-significant
[2] Coefficient of variation
[3] Feed conversion ratio (g feed/g egg)

Table 4.2 The influence of different limestone particle size distribution ratios on the mean performance of hens during the experimental period (Mean ± s.e.)

Parameter	Large particles (%)	Means ± s.e.	Significance	
			P[1]	CV[2](%)
Feed intake	0	101.98±1.20		
(g)	25	100.87±1.35		
	50	101.42±1.17	0.4066	6.0
	75	104.16±1.54		
	100	101.44±1.22		
Body weight	0	1961.67±31.76		
(g)	25	1998.13±41.38		
	50	1965.20±35.03	0.8908	8.3
	75	1990.08±41.71		
	100	2003.08±22.13		
Egg production	0	82.65±0.02		
(%)	25	81.80±0.02		
	50	80.27±0.02	0.2713	10.4
	75	84.52±0.01		
	100	79.24±0.02		
Egg output	0	356.15±8.65		
(g/week)	25	340.58±6.89		
	50	335.21±9.44	0.1457	10.5
	75	349.47±6.00		
	100	330.88±7.83		
FCR[3]	0	1.67±0.03		
(g feed/g egg)	25	1.72±0.04		
	50	1.69±0.02	0.2635	8.2
	75	1.76±0.03		
	100	1.70±0.03		

[1] (P>0.05) = non-significant
[2] Coefficient of variation
[3] Feed conversion ratio (g feed/g egg)

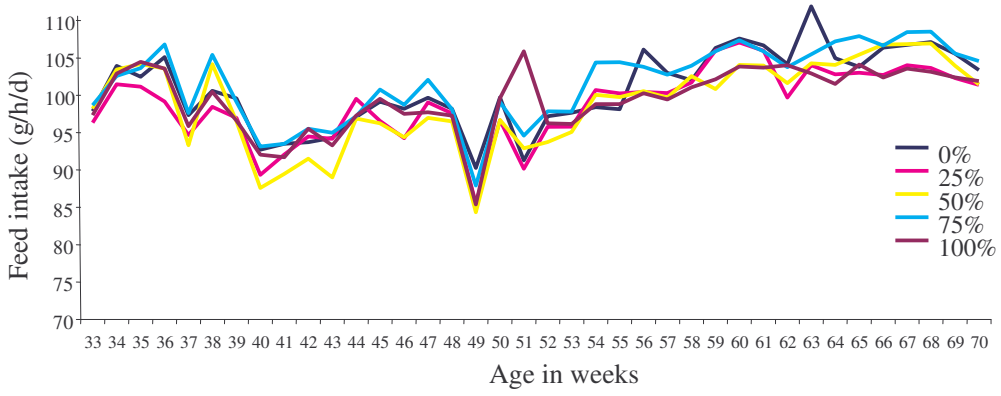

Figure 4.2 The effect of limestone distribution ratios (% large particles inclusion) on the weekly feed intake of layers during the later stages of lay

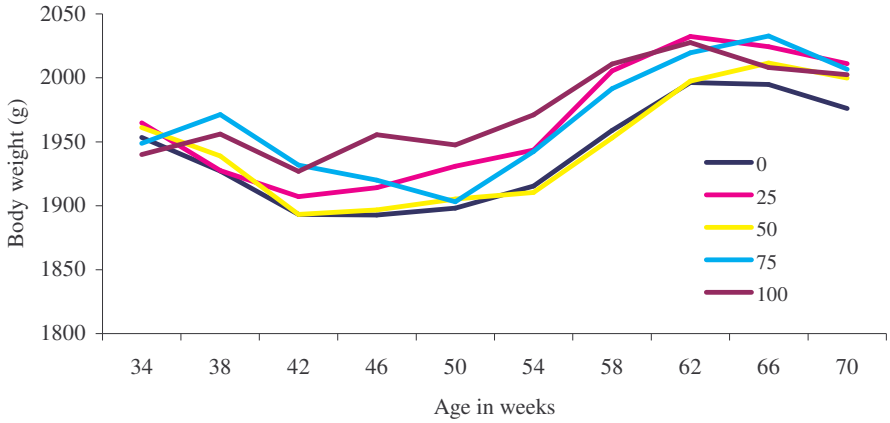

Figure 4.3 The effect of limestone distribution ratios on the monthly body weight of hens during the later stages of lay

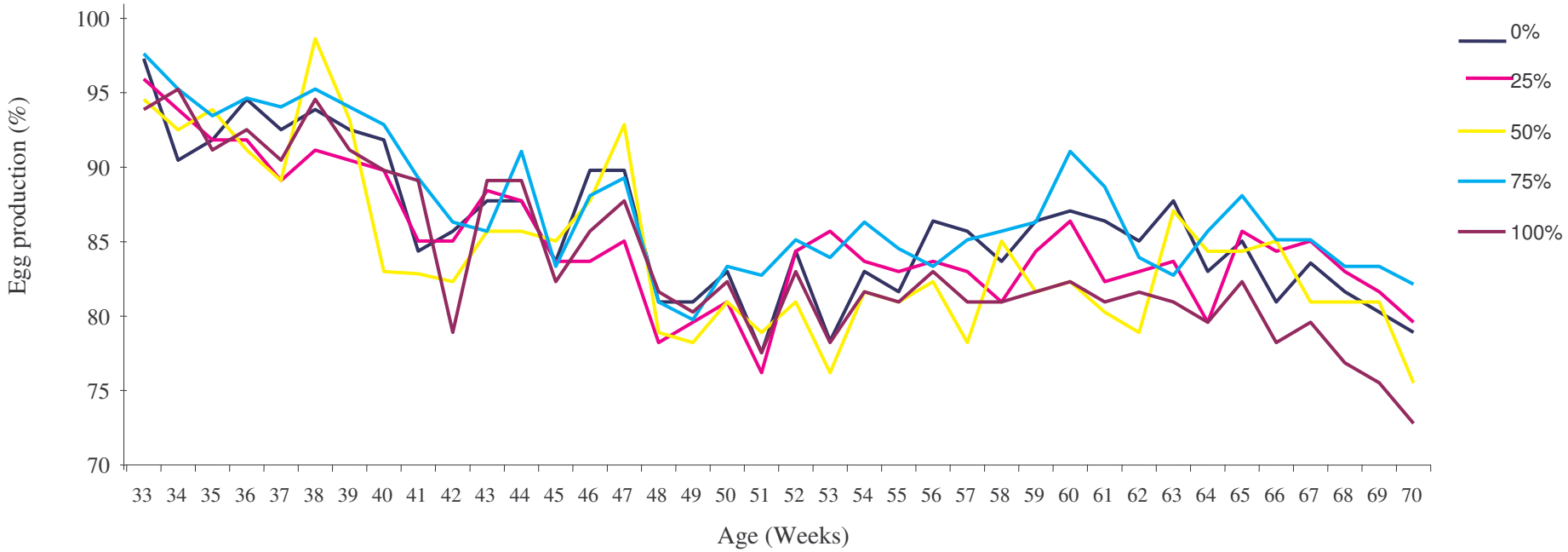

Figure 4.4 The effect of limestone distribution ratios (% large particles inclusion) on the weekly egg production of hens during the later stages of lay

Table 4.3 Variables used in different studies, regarding limestone particle size distribution ratios

Authors	Distribution ratios	Particle size (mm)	Genotypes of hens	Age (weeks)	Dietary Ca content (%)
Brister *et al*. (1981)	100:0 fine:large	Small 0.08-0.85	Babcock B 3000V	24-72	3.50
	67:33 fine:large	Large 2.00-2.38			4.00
Rabon *et al*. (1991)	50:50 fine:large	Small 74μ-1.41	DeKalb	79	2.75
		Large 2.00-4.70			
Keshavarz (1991)	100:0 fine:large	Small 0.15-0.84	Babcock B300	30-46	3.80
	67:33 fine:large	Large 2.36-4.75			
	0:100 fine:large				
Keshavarz *et al*. (1993)	100:0 fine:large	Small 0.15-0.84	Babcock B300	22-62	3.00-4.00
	67:33 fine:large	Large 2.36-4.75			
Saunders-Blades & Anderson (2003)	100:0 fine:large	Small 0.50-1.00	DeKalb	19-74	
	67:33 fine:large	Large >4.00			
Pavloski *et al*. (2003)	100:0 fine:large		Hissex	58-62	3.61
	40:60 fine:large				
	20:80 fine:large				
Scheideler (2004)	100:0 fine:large	Small 0.28 mm	Hy-line Brown	18-70	3.25-3.95
	75:25 fine:large	Large 1.40-5.60			3.60-4.55
	65:35 fine:large				
	50:50 fine:large				
	40:60 fine:large				
	30:70 fine:large				
De Witt (2006)	100:0 fine:large	Small 0-1.00	Lohmann-Silver	17-32	3.60
	75:25 fine:large	Medium 1.00-2.00			
	50:50 fine:large	Large 2.00-3.80			
	25:75 fine:large				
	0:100 fine:large				
Pizzolante *et al*. (2006)	100:0 fine:large	Small 0.185	Hy-line Brown	83	3.50
	70:30 fine:large	Large 2.83			4.00
	50:50 fine:large				
Lichovnikova (2007)	29:71 fine:large	Small 1.00-2.00		56-57	3.97
	32:68 fine:large	Large 2.00-5.00			4.46
	50:50 fine:large				
Currrent study	100:0 fine:large	Small 0-1.00	Lohmann-Silver	33-70	3.60
	75:25 fine:large	Large 2.00-3.80			
	50:50 fine:large				
	25:75 fine:large				
	0:100 fine:large				

4.3.2 Egg characteristics

4.3.2.1 Egg weight and contents

The effects of limestone particle size distribution ratios on egg weight and content results are illustrated in Table 4.4 and Table 4.7. It is evident from these results that limestone particle size distribution ratios in general had no significant (P>0.05) effect on egg weight and content during the late laying period. Several researchers (Rabon *et al.,* 1991; Keshavarz, 1991; Pavloski *et al*., 2003; Saunders-Blades & Anderson, 2003; Scheideler, 2004) confirmed that different Ca distribution ratios have no significant effect on egg weight and content during the later stages of lay. De Witt (2006) also reported that limestone particle size distribution ratios had no significant influence on egg weight and contents of Lohmann-Silver hens during the early laying stages.

In contrast with these results, Lichovnikova (2007) found a significantly (P<0.05) lower egg weight (61.7 g) with a 71% compared to a 50% large particle ratio (63.9 g). There is no obvious explanation for these different results from Table 4.3. The dietary Ca level (4.46%) of Lichovnikova (2007) was higher than that used in the current study. It is, however, doubtful whether this factor could influence the results as the Ca-needs of the hens was satisfactory met in the present study. This researcher large particle size also had a higher variation (2.00-5.00 mm) than that of the current study but lower than other researchers (1.40-5.60 mm) in Table 4.3. Factors like breed (production) and limestone source could probably also have played roles.

Table 4.4 The effect of different limestone distribution ratios on egg weight and egg contents during the experimental period (Mean±s.e.)

		Large particles (%)					Significance	
Parameters	**Week**	**0**	**25**	**50**	**75**	**100**	**P[1]**	**CV[2](%)**
Egg weight	54	60.38±0.91	58.20±0.93	59.24±0.78	58.36±0.72	58.28±0.65	0.2605	6.3
(g)	58	60.17±0.86	58.77±0.98	59.20±0.81	59.06±085	59.07±0.75	0.8185	6.7
	62	61.84±0.88	59.76±0.98	60.82±086	59.63±0.77	59.41±0.78	0.2368	6.6
	70	62.89±0.89	61.05±1.21	61.01±1.07	60.48±0.84	61.42.1.19	0.5443	7.9
Egg content	54	54.61±0.85	52.65±0.87	53.36±0.72	52.87±0.68	52.62±0.61	0.2893	6.5
(g)	58	54.33±0.81	53.04±0.90	53.40±0.74	53.45±0.79	53.25±0.69	0.8212	6.9
	62	55.99±0.81	53.95±0.92	54.99±0.79	53.93±0.73	53.57±0.72	0.1942	6.8
	70	57.03±0.81	55.32±1.15	55.31±0.98	54.70±0.79	55.52±1.11	0.5222	8.1

[1] (P>0.05) = non-significant
[2] Coefficient of variation

4.3.2.2 Eggshell quality

The avian eggshell is an excellent subject for the study of Ca bio-mineralization in laying hens. The effects of limestone distribution ratios on eggshell quality parameters (shell weight, shell thickness, shell percentage, shell Ca, shell ash, SWUSA) results are presented in Table 4.5, 4.6, 4.7 and 4.8. According to the weekly results in Tables 4.5 and 4.6, eggshell thickness was the only eggshell parameter that responded to the dietary treatment, but this significance was not reflected on the mean eggshell thickness results in Table 4.8. Therefore, it is evident from these results that limestone particle size distribution ratios in general have no significant ($P>0.05$) influence on eggshell quality parameters during the later stages of lay. De Witt (2006) during early laying period found that different particle size distribution ratios of large and small limestone particles had no significant effect on eggshell parameters of Lohmann-Silver hens. The non-significant results were not expected due to the fact that the late stage of lay is the most sensitive period when the dietary treatment usually is expected to give the most prominent response. This is because the eggs are larger and Ca homeostasis may be less efficient.

Contrary, to these results several researchers (Brister *et al*., 1981, Pavloski *et al*., 2003, Scheideler, 2004 and Lichovnikova, 2007) reported that Ca particle size distribution had a significant ($P<0.05$) effect on eggshell quality at the later stages of lay. These researchers used different particle sizes of small and large particles to combine different ratios as shown in Table 4.3. Lichovnikova (2007) using a 29:71 distribution ratio of fine to large particles found a significant ($P<0.05$) higher egg shell weight, eggshell weight ratio and eggshell strength than 50% large particle ratio between 56 and 57 weeks of age. Pavloski *et al*. (2003) also confirmed that the 60 to 80% replacement of small particle with large particle limestone in Hissex laying hens diet resulted in the highest eggshell weight, shell breaking strength and lowest shell deformation at week 62 of age. Differences in particle size within a specific particle size (small or large), breed and age could probably contribute to the different results of various studies. From the information in Table 4.3 it is difficult to identify one single factor to explain the contrary results between various studies.

Table 4.5 The effect of different limestone distribution ratios on eggshell quality characteristics during the experimental period (Mean±s.e.)

Parameters	Week	Large particles (%)					Significance	
		0	25	50	75	100	P[1]	CV[2](%)
Eggshell weight (g)	54	5.77±0.08	5.55±0.10	5.61±0.10	5.49±0.15	5.67±0.07	0.3594	8.8
	58	5.83±0.11	5.73±0.11	5.81±0.10	5.61±0.12	5.82±0.08	0.4905	8.7
	62	5.84±0.11	5.81±0.15	5.82±0.10	5.70±0.11	5.84±0.09	0.8828	9.1
	70	5.86±0.11	5.73±0.13	5.70±0.10	5.78±0.13	5.91±0.12	0.7849	10.3
Eggshell (%)	54	9.58±0.09	9.55±0.13	9.47±0.12	9.42±0.25	9.73±0.10	0.6877	7.7
	58	9.71±0.15	9.75±0.14	9.82±0.13	9.50±0.19	9.87±0.10	0.4244	7.1
	62	9.45±0.13	9.74±0.22	9.57±0.12	9.57±0.17	9.84±0.11	0.4520	7.6
	70	9.31±0.14	9.42±0.19	9.35±0.19	9.57±0.19	9.63±0.16	0.6559	8.7
Eggshell Ca (g)	54	2.15±0.03	2.07±0.04	2.09±0.04	2.05±0.06	2.11±0.03	0.3595	8.8
	58	2.18±0.04	2.14±0.04	2.17±0.02	2.05±0.06	2.17±0.03	0.4906	8.7
	62	2.18±0.04	2.17±0.05	2.17±0.04	2.13±0.04	2.17±0.03	0.8829	9.1
	70	2.18±0.04	2.14±0.05	2.13±0.05	2.16±0.05	2.20±0.05	0.7849	10.3
Eggshell ash (g)	54	0.26±0.00	0.25±0.00	0.26±0.00	0.27±0.00	0.26±0.00	0.2517	9.0
	58	0.25±0.00	0.25±0.00	0.24±0.00	0.25±0.00	0.25±0.00	0.5240	9.7
	62	0.25±0.00	0.24±0.00	0.24±0.00	0.26±0.00	0.24±0.00	0.2768	10.0
	70	0.25±0.00	0.24±0.00	0.24±0.00	0.25±0.00	0.25±0.00	0.4926	10.8
Egg surface area (cm^2)	54	71.80±0.76	69.95±0.80	70.84±0.66	70.09±0.06	70.04±0.55	0.2682	4.5
	58	71.62±0.72	70.43±0.83	70.81±0.68	70.68±0.72	70.70±0.63	0.8156	4.7
	62	73.02±0.73	71.27±0.83	72.16±0.72	71.16±0.65	70.98±0.65	0.2400	4.6
	70	73.89±0.74	72.33±1.02	72.31±0.90	71.88±0.70	72.65±0.98	0.5418	5.6
SWUSA[3] (mg/cm^2)	54	80.47±0.69	79.36±1.00	79.13±1.08	78.33±2.03	80.89±0.80	0.6113	7.5
	58	81.47±1.20	81.28±1.11	81.99±1.08	79.30±1.55	82.42±0.81	0.3826	6.9
	62	79.99±1.10	81.55±1.79	80.61±1.04	80.11±1.39	82.30±0.92	0.6884	7.5
	70	79.20±1.19	79.28±1.49	78.75±1.64	80.40±1.57	81.28±1.26	0.7354	8.5

[1] (P> 0.05) = non-significant
[2] Coefficient of variation
[3] SWUSA = shell weight per unit surface area

Table 4.6 The effect of different limestone particle size distribution ratios on eggshell thickness during the experimental period (Mean±s.e.)

		Large particles (%)					Significance	
Parameters	**Weeks**	**0**	**25**	**50**	**75**	**100**	**P[1]**	**CV[2](%)**
Broad End	54	0.37±0.00	0.36±0.01	0.36±0.01	0.35±0.00	0.36±0.00	0.1685	8.8
(mm)	58	0.36±0.00	0.36±0.00	0.36±0.00	0.35±0.00	0.36±0.00	0.8402	8.8
	62	0.35±0.00	0.36±0.00	0.37±0.00	0.36±0.00	0.36±0.00	0.7864	8.2
	70	0.36±0.00	0.36±0.00	0.36±0.00	0.37±0.00	0.36±0.00	0.6885	10.8
Equator	54	0.40±0.00a	0.37±0.00b	0.37±0.00b	0.37±0.00b	0.39±0.00	0.0012	7.9
(mm)	58	0.37±0.00	0.38±0.00	0.38±0.00	0.37±0.00	0.39±0.00	0.3365	7.5
	62	0.37±0.00a	0.39±0.00	0.38±0.00a	0.38±0.00a	0.40±0.00b	0.0024	7.3
	70	0.38±0.00	0.37±0.00	0.37±0.00	0.38±0.00	0.39±0.00	0.2908	9.0
Sharp End	54	0.39±0.00	0.37±0.00	0.37±0.00	0.36±0.00	0.37±0.00	0.2439	9.2
(mm)	58	0.36±0.00	0.36±0.00	0.38±0.00	0.36±0.00	0.37±0.00	0.2439	9.2
	62	0.36±0.00	0.36±0.00	0.38±0.00	0.36±0.00	0.38±0.00	0.2087	9.6
	70	0.36±0.00	0.35±0.00	0.37±0.00	0.37±0.00	0.38±0.00	0.5105	10.9

[a,b] Means in rows with different superscripts differ significantly (P<0.05)
[1] (P>0.05) = non-significant
[2] Coefficient of variation

Table 4.7 The effect of different limestone particle size distribution ratios on mean eggshell quality characteristics during the experimental period (Mean±s.e.)

	Large particles (%)					Significance	
Parameters	**0**	**25**	**50**	**75**	**100**	**P[1]**	**CV[2](%)**
Egg weight (g)	61.32±0.82	59.44±0.99	60.06±0.83	59.38±0.74	59.60±0.78	0.4474	6.4
Egg content (g)	55.52±0.76	53.76±0.92	54.33±0.75	53.72±0.60	53.64±0.72	0.3683	6.5
Shell weight (g)	5.83±0.09	5.71±0.11	5.73±0.11	5.64±0.12	5.82±0.82	0.6902	8.5
Eggshell (%)	9.51±0.11	9.62±0.14	9.55±0.13	9.52±0.20	9.76±0.11	0.7297	6.9
Eggshell calcium content (g)	2.17±0.04	2.12±0.04	2.13±0.04	2.11±0.05	2.17±0.03	0.6903	8.5
Eggshell ash (g)	0.25±0.01	0.25±0.01	0.24±0.01	0.26±0.01	0.25±0.01	0.5021	9.1
Egg surface area (cm^2)	72.58±0.69	70.99±0.84	71.53±0.69	70.95±0.62	71.13±0.65	0.4474	4.5
SWUSA[3]	80.28±0.93	80.37±1.13	80.11±1.10	79.53±1.59	81.67±0.84	0.7738	6.8

[1] (P> 0.05) = non-significant
[2] Coefficient of variation
[3] SWUSA = shell weight per unit surface area

Table 4.8 The influence of different limestone particle size distribution ratios on mean eggshell thickness during the experimental period (Mean ± s.e.)

Parameter	Large particles (%)	Mean±s.e.	Significance	
			P[1]	CV[2](%)
Broad end (mm)	0	0.36±0.00		
	25	0.36±0.00		
	50	0.36±0.00	0.9993	8.3
	75	0.36±0.00		
	100	0.36±0.00		
Equator (mm)	0	0.38±0.00		
	25	0.38±0.00		
	50	0.38±0.00	0.3636	7.0
	75	0.38±0.00		
	100	0.39±0.00		
Sharp end (mm)	0	0.37±0.00		
	25	0.36±0.00		
	50	0.37±0.00	0.7871	9.2
	75	0.36±0.00		
	100	0.37±0.00		

[1] (P> 0.05) = non-significant
[2] Coefficient of variation

4.3.3 Bone parameters

4.3.3.1 Bone dimensional properties

Bone dimensional properties are used as measure of growth and development in laying hens and it is critical to develop nutritional and management strategies that can reduce structural bone loss in laying hens. The effect of limestone particle size distribution ratios on bone dimensions data is illustrated in Table 4.9. According to these data the right tibia and humerus weight, length and width of hens at 70 weeks of age were not significantly (P>0.05) modified by different limestone distribution ratios. Bone dimensions characteristics results were quite variable among different distribution ratios treatments. This may be due to the fact that variation in body weight account for >98% of the variability in tibia length and weight (Applegate & Lilburn, 2002). These results are consistent for the late laying period with the results of Keshavarz *et al*. (1993) who observed that Ca particle size distribution ratio had no significant (P>0.05) effect on bone dimensional properties. De Witt (2006) also reported non-significant results of limestone particle size distribution ratios on Lohmann-Silver bone dimensional characteristics at 37 weeks of age.

Table 4.9 The effect of different limestone distribution ratios on bone dimensions (Mean±s.e.) of layer hens at 70 weeks of age

Parameters	Large particles (%)					Significance	
	0	25	50	75	100	P[1]	CV[2](%)
Right tibia							
Weight (g)	10.14±0.35	10.61±0.32	10.81±0.16	10.96±0.24	10.06±0.72	0.4446	12.1
Length (mm)	116.49±1.68	118.83±1.16	120.27±1.03	117.89±1.30	114.30±4.09	0.3727	5.8
Width (mm)	6.42±0.07	6.47±0.02	6.51±0.08	6.59±0.09	6.48±0.08	0.8579	5.1
Right Humerus							
Weight (g)	3.93±0.20	3.76±0.18	3.80±0.13	3.98±0.18	3.82±0.17	0.8716	13.9
Length (mm)	78.10±0.73	78.14±0.67	78.44±0.67	78.11±0.69	78.44±0.73	0.9915	2.8
Width (mm)	6.15±0.10	6.07±0.10	6.19±0.10	6.21±0.07	6.18±0.09	0.7986	4.4

[1] (P>0.05) = non-significant
[2] Coefficient of variation

4.3.3.2 Bone mechanical properties

Bone mechanical properties such as breaking strength and stress are important to determine the bioavailability of minerals and establishing their requirements. The influence of limestone particle size distribution ratios on bone mechanical properties is shown in Table 4.10. There was a significant effect of limestone particle size distribution ratios on both bone breaking strength (P=0.0101) and stress (P=0.0293) of the right tibia bone at week 70 of age. Limestone particle size distribution ratio of 100% large particles yielded a higher bone breaking strength and stress compared to the treatment without any large particles. The diets with 25, 50 and 75% large particles showed however no clear trend. Therefore no particular limestone particle distribution ratio within the range of 25 to 75% large particles seems to influence bone breaking strength and stress. The results of present study are in contrast with the results of De Witt (2006) who found a non-significant (P>0.05) effect of limestone particle size distribution ratios on bone breaking strength and stress at 37 weeks old for Lohmann-Silver hens. It seems that large particle limestone during the laying period had a beneficial effect on bone breaking strength and stress at the end of the laying period (70 weeks). No leg breaks or abnormalities were however encountered in any of the experimental treatments. The difference between bone mechanical properties in De Witt (2006) study and the current study are consistent with Fleming *et al.* (1998b) who hypothesised that limestone particle size affect bone mechanical properties significantly later in the laying cycle.

Humerus bone breaking strength and stress results (Table 4.10) were not statistically (P>0.05) different among different distribution ratios. These results are consistent with the findings of De Witt (2006) who indicated that humerus bone breaking strength and stress were not significantly (P>0.05) affected by limestone distribution ratios at week 37 of age. Schreiweis *et al.* (2004) indicated that bone breaking strength and the amount of medullary bone found in the humerus were correlated (r^2=0.71, P<0.002). Although considered as a pneumatic bone, the humeri of some birds have varying concentration of marrow and medullary component (Fleming *et al*., 1996; Whitehead & Fleming, 2000). Fleming *et al.* (1998a) concluded that the medullary component of the humerus bone may contribute to the overall fracture resistance of bone, although not to the same degree as structural bone.

Table 4.10 The effect of different limestone distribution ratios on bone mechanical properties (Mean±s.e.) of laying hens at 70 weeks of age

Parameters	Large particles (%)					Significance	
	0	25	50	75	100	P	CV[1](%)
Right tibia							
Breaking strength (N/m^2)	266.29±21.1[a]	290.44±21.2[ab]	265.90±31.2[a]	308.60±30.1[ab]	395.01±29.7[b]	0.0101	27.8
Stress (kg/cm^2)	98.64±8.61[a]	107.30±9.6[ab]	96.37±12.2[a]	106.05±10.3[ab]	143.57±13.5[b]	0.0293	31.3
Right Humerus							
Breaking strength (N/m^2)	234.11±24.2	218.25±25.2	226.40±23.9	271.79±25.8	256.54±20.9	0.4882	30.9
Stress (kg/cm^2)	97.22±8.5	97.56±12.2	94.54±11.8	113.28±13.4	108.94±12.8	0.7623	36.6

[a,b] Means in rows with different superscripts differ significantly (P<0.05)
[1] Coefficient of variation

4.3.3.3 Bone ash percentage, bone index and percentage bone

Bone traits such as bone ash percentage are commonly used as measures of assessing Ca and P requirements of poultry. The influence of limestone particle size distribution ratios on bone percentage, ash and index is shown in Table 4.11. According to these results no significant (P>0.05) differences occurred due to limestone particle size distribution ratios on any of these parameters. These results are in agreement with that of Keshavarz *et al*. (1993) that limestone particle size distribution ratios had no significant (P>0.05) influence on tibia ash percentage during the later laying stages. In contrast to the current study Scheideler (2004) observed that limestone particle size distribution had a significant effect on bone ash percentage during the late laying period of Hy-Line W-36 and W-98 hens. This researcher noted a significant higher bone ash percentage with a 100:0 (fine:large) distribution ratio. However, Bristol (2003) indicated that small limestone particles are rapidly dissolved and utilized within a few hours after ingestion. This then results in a reduced Ca availability during shell calcification, which makes birds more dependent upon bone mobilization to provide Ca for eggshells. Therefore the results of Scheideler (2004) were contrary to what is generally believed and are difficult to explain. Waldroup (1995) stated that the response to Ca source or particle size is sensitive to dietary Ca levels, being of greater concern when dietary Ca levels are minimal. Therefore, as stated before, both tibia and humerus characteristics (Table 4.11) were not significantly affected by limestone particle size may be because this particular calcitic limestone provided sufficient available Ca^{+2} to offset the effect of particle size.

Table 4.11 The effect of different limestone distribution ratios on percentage bone, bone index and bone ash percentage (Mean±s.e.) of laying hens at 70 weeks of age

Parameters	Particle size (mm)					Significance	
	0	25	50	75	100	P[1]	CV[2](%)
Right tibia							
Tibia bone[3] (%)	0.50±0.01	0.53±0.01	0.55±0.01	0.54±0.01	0.51±0.04	0.4674	11.7
Ash (%)[4]	59.46±1.29	62.83±0.48	61.59±0.63	61.47±0.60	61.27±0.73	0.0643	3.8
Bone index[5]	86.89±2.11	89.18±1.97	89.85±1.15	92.92±1.25	86.72±4.46	0.4185	8.8
Right humerus							
Humeri bone[6] (%)	0.20±0.01	0.19±0.01	0.19±0.01	0.20±0.01	0.19±0.01	0.9269	13.8
Bone index[7]	50.25±2.24	48.01±2.09	48.45±1.70	50.96±2.12	48.63±2.19	0.8217	13.0

[1] (P>0.05) = non-significant
[2] Coefficient of variation
[3] Percentage tibia bone = (Tibia weight/Body weight) x 100
[4] % Tibia ash = (Tibia ash weight / Fat free tibia weight) x 100
[5] Tibia bone index = (Tibia weight /Tibia length) x 100
[6] Percentage humeri bone= (Humeri weight/Body weight) x 100
[7] Humerus bone index = (Humerus weight / Humerus length) x 100

4.4 Conclusions

From the results of the present study, it seems that the different limestone particle size distribution ratios had no influence on egg production, eggshell quality and some bone characteristics. However, the distribution ratio of 100% large compared to 100% fine resulted in a significant ($P<0.05$) higher bone breaking strength and stress at week 70 of age. Contrary to these two extremes large limestone particle size distribution ratios ranging from 25 to 75% seems to have no effect on bone breaking strength and stress. De Witt (2006), working with the same animals during the early stages of lay, found no effect of different limestone particle size distribution ratios on bone strength and stress at week 37. This indicates that bone mobilization occurs during the later stages of lay to provide Ca for eggshells. Furthermore it is evident from the literature that limestone particle size distribution ratio would give the most prominent results when dietary Ca levels are low or marginal. Therefore, it is clear that the specific limestone particle size distribution ratios of 25-75% large particles did satisfy Ca needs of layer hens during the later stages of lay except for bone breaking strength and stress.

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CHAPTER 5

GENERAL CONCLUSIONS

Calcium plays a vital role in bone and eggshell formation in laying hens. However, the physical form of Ca supplemental sources could be of utmost importance because it determines the availability of Ca^{2+} for utilization by laying hens. Small particulate limestone has a greater surface area exposure per given weight, and hence, a higher solubility in comparison to large particle size $CaCO_3$ with reduced surface area exposure. Highly soluble Ca sources will most likely pass through the gastrointestinal tract too quickly, and hence, may not be available for absorption at the time when peak eggshell formation is taking place. Therefore it could be important to use large particle size Ca sources in layer diets that will be available on a slower, metered basis to ensure availability of Ca^{2+} during times of peak shell formation.

The present study investigated the effect of limestone particle size and distribution ratios of a specific calcitic limestone source on bone and eggshell quality. It seems from the available literature that studies regarding limestone particles included variables like different particle sizes, variation within a specific particle size, range between the lowest and highest particle size, particle size distribution ratios, Ca-content of the diet, genotype and age of hens. These variables used in different studies do not allow meaningful comparison among studies, discussions and final conclusions regarding limestone particle size and/or distribution ratios in layer diets. Therefore the results of the present study apply only for the specific variables used.

From the results of the present study it seems that limestone particle size and/or distribution ratios did not influence feed intake, body weight, and egg production of layer hens. According to some literature, larger particle sizes than those used in the present study (2.0-3.8 mm), could have a beneficial influence on the feed intake of layer hens. However, in agreement with the current study, most studies in the available literature revealed no influence of particle size on egg weight and content.

Dietary treatment also did not have a significant effect on bone parameters at the end-of-lay period, except on bone mechanical properties where an increase in limestone particle size resulted in an increase in tibia bone breaking strength and stress. In contrast with large limestone particle sizes, distribution ratios of fine and large particle sizes ranging from 25 to 75, did not displayed any influence on tibia breaking strength and stress at 70 weeks of age. This

increase in bone strength and stress in response to large limestone particles was more pronounced towards the end-of-lay period. On the other hand tibia and humerus weight and/or length could be negatively influenced by an increase in limestone particle size during the early stages of lay (e.g. 37 weeks of age), without any effect at 70 weeks of age. It seems that continuous bone formation throughout the production cycle cancelled these detrimental effects that arise during the early laying period.

According to the performance and eggshell quality results, it seems that the limestone source used in this study provided sufficient available Ca^{2+} to the laying hens, with the exception of bone mechanical properties at the end-of-lay. These performance and eggshell quality results were obtained irrespective of limestone particle sizes and/or distribution ratios and it seem that adequate dietary Ca is the most important determining factor of these specific traits. However, large limestone particles may be beneficial under commercial circumstances when dietary Ca levels are lower than anticipated due to poor mixing of raw materials and/or nutrient segregation. Large particles limestone is also important in ensuring an increased tibia bone breaking strength and stress at the end-of-lay, especially for high producing hens.

A further factor to be considered in future research is the variation in the physical particle size (e.g. 2.0-3.8 mm) within a specific particle size category. This factor could influence the results and are supported to some extent by the fact that different distribution ratios of fine and large limestone particle size had no influence on tibia breaking strength and -stress at 70 weeks of age. However, the effect of more defined limestone particle sizes on egg production and eggshell quality characteristics warrants further research.

ABSTRACT

A specific calcitic limestone source that is widely used in South African poultry diets was evaluated during two concurrent studies. During the first study, the effect of limestone particle size on bone quality, egg production and eggshell quality was determined. Limestone was classified according to particle sizes as small (0-1.0 mm), medium (1.0-2.0 mm) and large (2.0-3.8 mm).

During the second study, the effect of different distribution ratios of small and large particle sizes of limestone on bone quality, egg production and eggshell quality characteristics was determined. Small (0-1.0 mm) and large (2.0-3.8 mm) particles limestone from the first study were mixed to obtain the following five distribution ratios used in the second study namely; 0, 25, 50, 75, 100% small or large particles.

The experimental protocol for both studies was the same. Initially one hundred and thirty eight, 17 weeks old Lohmann-Silver pullets, were obtained from a commercial egg producer and randomly allocated to six treatments (n=23/treatment). All birds were kept in individual metabolic cages for the duration of the study. The influence of limestone particle size and distribution ratios of particles on feed intake, body weight and egg production was determined for weeks 33 up to week 70 of age. During weeks 54, 58, 62 and 70 of age, the effect of limestone particle size and distribution ratios of particles on eggshell quality characteristics such as shell weight, percentage eggshell, eggshell calcium egg surface area, shell weight per unit surface area (SWUSA) and shell thickness was determined. At 70 weeks of age, the effect of limestone particle size and distribution ratios of limestone particles on bone dimensions (length, width and weight), bone mechanical properties (breaking strength and stress) and percentage bone ash was determined.

The results of the limestone particle size study indicated that different limestone particle sizes did not have a significant influence on feed intake (P=0.6229), body weight (P=0.4189), egg production (P=0.3991), egg output (P=0.0599) and feed conversion ratio (P=0.5025). Accordingly different limestone particle sizes did not show any significant effect on mean eggshell characteristics such as shell weight (P=0.9396), percentage eggshell (P=0.3468), eggshell calcium (P=0.9367), egg surface area (P=0.3223), SWUSA (P=0.6111) and shell thickness (P=0.6663) during the entire experimental period. At 70 weeks of age large particles limestone resulted in a significant higher tibia bone breaking strength (P=0.0107) and stress

(P=0.0391). No significant (P>0.05) influence of limestone particle size was found on bone length, width, weight, percentage ash and index at week 70 of age.

The results of the second study illustrated that different ratios of small and large limestone particles had no significant effect on feed intake (P=0.4066), body weight (P=0.8908), egg production (P=0.2713), egg output (P=0.1457) and feed conversion ratio (P=0.2635) during the entire experimental period. No statistical differences were detected due to a mixture of different ratios of coarse and fine limestone particles on mean eggshell characteristics such as shell weight (P=0.6902), percentage egg shell (P=0.7297), eggshell calcium (P=0.6903), egg surface area (P=0.4474), SWUSA (P=0.7738) and eggshell thickness (P=0.7167) during the entire experimental period.

It was concluded that the different limestone particle sizes and ratios of small and large particles limestone in the diets used during the present study, generally had no significant influence on bone and eggshell quality characteristics of Lohmann-Silver laying hens during the later stages of lay, except on bone mechanical properties.

OPSOMMING

'n Spesifieke kalsitiese kalksteenbron wat algemeen in Suid-Afrika in pluimveerantsoene gebruik word, is tydens twee gelyklopende studies geëvalueer. Tydens die eerste studie is die invloed van partikelgrootte op beenkwaliteit, eierproduksie en eierdopkwaliteit bepaal. Die kalksteen is volgens partikelgrootte as fyn (0-1.0 mm), medium (1.0-2.0 mm) en grof (2.0-3.8 mm) geklassifiseer.

Tydens die tweede studie is die invloed van partikelgrootte-verspreiding van fyn en growwe kalksteenpartikels op beenkwaliteit, eierproduksie en eierdopkwaliteit bepaal. Fyn (0-1.0 mm) en growwe (2.0-3.8 mm) kalksteenpartikels van die eerste studie is met mekaar gemeng om die volgende vyf partikelgrootte-verspreidings van 0, 25, 50, 75 en 100% fyn of growwe partikels te verkry.

Die eksperimentele prosedure vir beide studies was dieselfde. Een honderd agt en dertig, Lohmann-Silver lêhenne (17 weke oud), is vanaf 'n kommersiële eierprodusent verkry en ewekansig in ses behandelings ingedeel (n=23/behandeling). Die invloed van partikelgrootte en partikelgrootte-verspreiding op voerinname, liggaamsgewig en eierproduksie is vanaf week 33 tot 70 ouderdom bepaal. Gedurende die ouderdom van week 54, 58, 62 en 70 is die invloed van partikelgrootte en partikelgrootte-verspreiding op eierdopkwaliteit-eienskappe soos dopgewig, persentasie dop, kalsiuminhoud, eieroppervlakte, eierdopgewig per eenheid oppervlakte en eierdopdikte bepaal. Gedurende week 70 is die invloed van partikelgrootte en partikelgrootte-verspreiding op been-metings (lengte, dikte en gewig), meganiese-eienskappe (sterkte en spanning) en persentasie been-as bepaal.

Die resultate van die kalksteen partikelgrootte studie het geen betekenisvolle invloed op voerinname (P=0.6229), liggaamsgewig (P=0.4189), eierproduksie (P=0.3991), eieruitset (P=0.0599) en voeromset-verhouding (P=0.5025) getoon nie. Ooreenkomstig het verkillende kalksteen partikelgroottes geen betekenisvolle effek op gemiddelde eierdopeienskappe soos eierdopgewig (P=0.9396), persentasie eierdop (P=0.3468), eierdopkalsium (P=0.9367), eieroppervlakte (P=0.3223), dopgewig per eenheid oppervlakte (P=0.6111) en dopdikte (P=0.6663) gedurende die eksperimentele periode uitgeoefen nie. Op 70 weke ouderdom het growwe partikelgrootte kalksteen 'n betekenisvolle hoër tibia breeksterkte (P=0.0107) en -spanning (P=0.0391) tot gevolg gehad. Geen betekenisvolle (P>0.05) invloed van partikelgrootte op beenlengte, -breedte, -gewig, -persentasie as en -indeks is op week 70 waargeneem nie.

Die resultate van die partikelgrootte-verspreidingstudie het geïllustreer dat verskillende verhoudings van fyn en growwe kalksteen partikels geen betekenisvolle effek op voerinname (P=0.4066), liggaamsgewig (P=0.8908), eierproduksie (P=0.2713), eieruitset (P=0.1457), en voeromset-verhouding (P=0.2635), gedurende die eksperimentele periode uitgeoefen het nie. Geen betekenisvolle verskille as gevolg van ‘n mengsel van fyn en growwe kalksteen is op die gemiddelde eierdopeienskappe soos dopgewig (P=0.6902), persentasie eierdop (P=0.7297), eierdopkalsium (P=0.6903), eieroppervlakte (P=0.4474), dopgewig per eenheid oppervlakte (P=0.6111) en dopdikte (P=0.7167) gedurende die eksperimentele periode waargeneem nie.

Daar is tot die slotsom gekom dat verskillende kalksteen partikelgrootte en partikelgrootte-verspreiding van fyn en growwe kalksteen in die dieet wat tydens die huidige studie gebruik is, oor die algemeen been- en eierdopkwaliteit van Lohmann-Silver lêhenne gedurende die laat lêperiode, met die uitsondering van meganiese-beeneienskappe, nie betekenisvol beïnvloed het nie.