THE INFLUENCE OF LIMESTONE PARTICLE SIZE IN LAYER DIETS ON BONE AND EGGSHELL CHARACTERISTICS

by

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DEDICATED TO MY FAMILY

- *To you Nicolene, for all your love, support and encouragement during my studies. Thank you for being my best friend, and always willing to listen when I needed it the most.*

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ACRONYMS AND ABBREVIATIONS

ADF	Acid detergent fibre
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
AvP	Available Phosphorus
BBS	Bone breaking strength
BE	Blunt end
BMC	Bone mineral content
BMD	Bone mineral density
BW	Body weight
Ca	Calcium
Ca^{2+}	Calcium ions
$CaCO_3$	Calcium carbonate
$Ca_{10}(PO_4)_6(OH)_2$	Calcium hydroxyapatite
Ca:P	Calcium to phosphorus ratio
Ca:tP	Calcium to total phosphorus ratio
Ca:AvP	Calcium to available phosphorus ratio
Cd	Cadmium
CLF	Cage layer fatigue
CLO	Cage layer osteoporosis
cm^2	centimetre squared
CO_3^{2-}	Carbonate ions
CT	Calcitonin
CV	Coefficient of variation
D_2	Ergocalciferol
D_3	Eholecalciferol
DM	Dry matter
EQ	Equator
EXC	Excreta limestone content
Fe	Iron
FFDM	Fat-free dry matter
FTV	Free thoracic vertebra
g	gram
g/h/d	gram per hen per day

GIT	Gastrointestinal tract
H^{1+}	Hydrogen ions
HCl	Hydrochloric acid
HSD	Honest significant difference
Hz	Hertz
IU	International unit
kg	kilogram
L	litre
LSD	Least significance difference
m	meter
m^2	meter squared
MB	Medullary bone
ME	Metabolisable energy
meq/l	milliequivalent per litre
mg	milligram
ml	millilitre
MJ	Megajoules
mm	millimetre
mm^2	millimetre squared
N	Newton
N/m^2	Newton per meter squared
NDF	Neutral detergent fibre
NPP	Non-phytate phosphorus
NRC	National Research Council
NSF	Non shell forming
Pers. Comm.	Personal communication
pH	Hydrogen ion concentration
PTH	Parathyroid hormone
PTM	Proximal tarsometarsus
RBV	Relative bioavailability
R.S.A.	Republic of South Africa
SAS	Statistical Analysis Systems
SE	Sharp end
s.e.	Standard error
SF	Shell forming
ST	Shell thickness

SWUSA	Shell weight per unit surface area
THK	Thick-shelled
THN	Thin-shelled
V	Vanadium
VLDL	very low density lipoprotein
vs.	versus
µg	microgram
µm	micrometer
^{o}C	degrees Celsius
25(OH)D_3	25-Hydroxycholecalciferol
1,25$(OH)_2D_3$	1,25-Dihydroxycholecalciferol

CHAPTER 1

GENERAL INTRODUCTION

Human population growth and rising income are two of the major factors that increased the consumer demand for dietary protein sources, especially in South Africa. An increase in income changes the food consumption patterns of mankind from carbohydrate to protein. Poultry meat and eggs are amongst the most affordable sources of animal protein in the world. However, there are quite a few factors affecting the sustainable profitability of poultry producers. Eggshell soundness is one of the external quality factors that influence the economic viability of egg producers worldwide.

The eggshell must satisfy several conflicting demands: on one hand, the shell must be strong enough to prevent it from being crushed during handling and transportation, yet it must not be too strong to prevent the hatchling from breaking out of the egg at the end of the incubation period (Hamilton, 1982). The modern egg producer has superimposed a further demand, in that the shell must resist numerous stresses or insults as it passes through egg handling equipment. Although the eggshell contributes only 11% to the total egg weight, it has a fundamental structural role to ensure that intact eggs reach the consumer and to prevent bacterial contamination of the inner egg contents (Parkhurst & Mountney, 1987). The genetic differences between strains of laying hens are probably the most important factor influencing shell thickness and the variation thereof. Dietary calcium is only one of a number of nutritional factors influencing shell quality. Other factors such as the age of hens, position of the egg in the clutch, the supply of vitamin D_3 and phosphorus as well as many management factors such as lighting programmes, high environmental temperatures, relative humidity and disease status of the birds also influence eggshell quality (Hamilton, 1982; Rose, 1997; Klasing, 1998; Hayes & Saunders, 2002; Butcher & Miles, 2005).

Hamilton (1982) reported that the estimated annual cost due to shell breakage is about US. $10 million in Canada and US. $100 million in the United States of America. The findings of Roland (1988) and Roberts & Leary (2000) indicated that shell breakage caused estimated financial losses amounting to approximately US. $477.9 million/year and Aus. $10 million/year for egg producers in the United States and Australia respectively. The reports of Hamilton *et al*. (1979) and Rose (1997) indicated that an average of 7 – 8% of the total eggs packed got broken during transportation from the egg producer to the consumer. The report of Crystal (2000) suggested that a staggering 14.3 – 21.3% of total eggs laid are cracked worldwide, which implies an enormous financial loss to the egg producer. The financial losses caused by egg breakages in South Africa can be moderately calculated to amount to approximately R58 437/day, considering that 17 million laying hens are on a production rate of 75% and 1% egg loss, at an egg price of R5.50/dozen (D.G. Borstlap, 2005, Pers. Comm., 211 Elston Ave., Benoni 1501, R.S.A.).

Bone quality is another indirect factor that could influence economic sustainability of egg production. Hayes & Saunders (2002) suggested that approximately 30% of layers suffer bone fractures during their lifetime, which is in accordance with the report of Gregory & Wilkins (1989) that 29% of battery caged hens had one or more bone fractures at end-of-lay. The same authors indicated that 98% of the carcasses from spent laying hens contain broken bones when they reach the end of the evisceration line, limiting the meat processor in the utilization thereof. Skeletal problems also compromise the welfare of birds and some of the consequences are: reduced growth, increased mortality and increased carcass downgrading due to lesions (Day, 1990). Animal activist groups exploit the welfare issues regarding poultry production to provoke a negative feeling among consumers and this aspect will become an increasingly important economic consideration for poultry producers in the future. Whitehead & Fleming (2000) defines osteoperosis in laying hens as a decrease in the amount of fully mineralized structural bone, leading to increased fragility and susceptibility to fracture. A severe consequence of osteoperosis is cage layer fatigue (CLF) which involves bone brittleness, paralysis and death, indicating the severe economic and welfare consequences for egg producers. Fleming *et al*. (1998b) and Whitehead & Fleming (2000) suggested that the provision of a particulated source of calcium to laying hens would help to prevent osteoporosis and/or decrease the severity thereof.

About 99% of the calcium in the skeleton occurs in the form of calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) together with non-crystalline phosphates and calcium carbonates ($CaCO_3$) (Larbier & Leclerq, 1994). Apart from the structural role of calcium, it serves as an essential constituent of living cells and tissue fluids and plays an important function in the activity of various enzyme systems, the transmission of nerve impulses, the contractile properties of muscle and hormonal activities. It also has a fundamental role in the coagulation of blood (Larbier & Leclercq, 1994; McDonald *et al.*, 2002). One of the most important functions of calcium in the laying hen is to provide structural integrity to the egg by depositing $CaCO_3$ crystals around the outer shell membranes, forming the eggshell.

The eggshell is composed of $CaCO_3$ crystals and contains small amounts of magnesium, phosphorus, sodium and potassium (Parkhurst & Mountney, 1987; Rose, 1997). The eggshell contains on average 2.0 - 2.5 g calcium (Larbier & Leclercq, 1994; Rose, 1997; Klasing, 1998) and a daily dietary provision of 4.0 - 5.0 g calcium is required for laying hens, if the digestive efficiency of calcium is considered to be 50%. However, such high inclusion levels of dietary calcium is not normal in practise and some of the calcium demands for eggshell formation must be met from endogenous sources, such as medullary bone. The proportion of shell in the egg slowly decreases over the laying period (Larbier & Leclercq, 1994; Rose, 1997) whilst the total egg weight increases. This results in thinner eggshells, contributing to the weakness of shells in older laying hens. The calcium requirements of laying hens are relatively low, except during the afternoon at the onset of shell formation, when the developing egg is in the uterus (Leeson & Summers, 1997; Marangos, 2004). Leeson & Summers (1997) and Hayes & Saunders (2002) stated that the hen has the capability to consume and select the correct amount of calcium according to her biological and production requirements. This specific appetite for calcium during the late afternoon may be met by adding a separate calcium source, such as large particles oyster shell or limestone to the diet. It has also been suggested that the feeding of an additional dietary calcium source during the afternoon may obviate the need to draw calcium reserves from medullary bone. This could be beneficial for eggshell thickness and by reducing the depletion of phosphorus from bones during calcium mobilization (Hayes & Saunders, 2002), the variation in shell thickness between eggs in a clutch could also be reduced.

Medullary bone is not thought to process much intrinsic strength (Knott *et al*., 1995), but the work of Fleming *et al*. (1996; 1998a,b) indicated that medullary bone do contribute to the overall bone strength. An increase in medullary bone results in an increased bone breaking strength, thus limiting some of the welfare issues regarding egg production by battery caged laying hens. Medullary bone represents 10 – 12% of total bone in the chicken skeleton and acts primarily as a calcium reservoir for eggshell formation (Fleming *et al*., 1998a; Marangos, 2004). During the dark hours, when shell formation occurs, calcium is supplied by medullary bone and Parkhurst & Mountney (1987) suggested that approximately 30 – 40% of the calcium deposited onto the eggshell is supplied by medullary bone when dietary calcium levels are less than 2.0%.

The findings of Rennie *et al.* (1997) and Fleming *et al*. (1998b; 2003) undoubtedly illustrated that the provision of particulate sources of calcium resulted in a decreased loss of cancellous bone and an increased accumulation of medullary bone in laying hens. Farmer *et al.* (1986)

suggested that the increased amount of available calcium in the digestive tract, originating from the large particles of limestone leads to a decreased mobilization of bone calcium from medullary bone. The increased size of particulate limestone extends the period of calcium absorption into the period of darkness when food consumption has ceased and leads to a greater availability of ionic calcium (Ca^{2+}) for shell and bone formation. This greater availability of Ca^{2+} may facilitate medullary bone formation, especially during the early part of lay (<25 weeks of age) and has a sparing effect on cancellous bone resorption (Fleming *et al.*, 1998b).

Leeson & Summers (1997) reported that optimum eggshell quality and bone development in young birds is dependant upon a consistent pattern of calcium solubility and that the rate of *in vivo* solubility is mostly affected by particle size and particle porosity. The work of Zhang & Coon (1997) suggested that the amount of either limestone or oyster shell retained in the gizzard are significantly affected by the particle size of the calcium source. The prolonged retention time of large particle calcium supplements resulted in an increased *in vivo* solubility and therefore an increased relative bioavailability (RBV) (Zhang & Coon, 1997). The beneficial effect of larger particle size calcium sources on eggshell quality had been attributed to the increased retention time of the larger particles in the gastrointestinal tract (GIT) (McDonald *et al.*, 2002; Marangos, 2004). This increased retention time promotes a more constant metering of calcium into the GIT and maintained higher blood level of Ca^{2+} during the night when shell formation occurs (Scott *et al.*, 1971; Evans, 1997; Leeson & Summers, 1997).

The use of different calcium sources in layer diets to improve eggshell quality has been studied for an extensive period of time (Roland, 1986). There has been considerable controversy in the past concerning the relative potency of limestone versus oyster shell as calcium source for laying hens (Evans, 1997; Leeson & Summers, 1997). Part of the inconsistency in dealing with various sources of calcium was the great variation in physical and chemical characteristics of these sources. After reviewing 44 papers comparing the effect of oyster shell and limestone particle size on eggshell quality, Roland (1986) concluded that larger particles of both sources are equally effective in improving eggshell quality. However, the report of Rabon & Roland (1985) suggested that the solubility of limestone particles of similar size from different sources could vary by 62%. Heavy metal impurities contribute to the differences observed between the limestone sources. Although the report of Bristol (2003) gave no clear indication of the heavy metal levels that influenced eggshell quality, he suggested that iron (Fe), cadmium (Cd) and vanadium (V) levels of 4500 mg

Fe/kg DM, 12 – 40 mg Cd/kg DM and 200 mg V/kg DM could be toxic to laying hens. Leeson & Summers (1997) also concluded that the variability of limestone solubility between sources provoked some concern in recent years suggesting that not only particle size, but also the calcium source had a mayor influence on the effectiveness of Ca^{2+} provision to the hen.

The inclusion of either limestone or oyster shell in poultry diets are mostly influenced by the availability and price of the specific source. However, chemical composition and quality (heavy metal impurity) of the calcium source will influence the final decision on inclusion. For instance, the high levels of magnesium in dolomitic limestone (100 g Mg/kg DM) precludes the use of it in poultry diets (Leeson & Summers, 1997; Klasing, 1998). Oyster shell is a much more expensive ingredient than limestone, but it offers the advantage of being clearly visible in the diet, thus enabling self-selection by birds (Leeson & Summers, 1997; Marangos, 2004). In South Africa, limestone is the most commonly used calcium source for supplementation of poultry diets (G. Maritz & F.P. Viljoen, 2006, Pers. Comm., Agri Lime, P.O. Box 20366, Protea Park 0305, R.S.A.). Crystal (2000) suggested that a price of R220/ton for limestone in South Africa is relatively cheap, depending on the calcium content of the specific source. The inclusion of limestone with 36% Ca in a layer diet, resulted in a price increase of R2.83/ton in layer feed, compared to limestone with a 38% Ca content, illustrating the effect of limestone calcium content on the profitability of the feed manufacturer (Crystal, 2000).

Many of the South African feed manufacturing companies are situated inland, relatively close to the grain producing regions of the country. Because of transportation costs, the use of oyster shell as a calcium source is not a viable option for these companies. According to the Animal Feed Manufacturers Association (AFMA, 2006), limestone usage in South Africa increased from 115 587 ton in the year 2001/2002 to 136 272 ton in the year 2004/2005. The percentage of limestone grit and powder used by the AFMA members, changed from 36.12% to 43.10% for limestone grit and 63.88% to 56.90% limestone powder in the years 2001/2002 and 2004/2005 respectively. Although these figures represents the usage of limestone in the ruminant as well as monogastric feed sectors, the increased usage of limestone grit is clearly noticeable. One of the largest limestone suppliers to the feed manufacturing sector in South Africa is situated between the towns of Rustenburg and Thabazimbi in the North West Province. This specific calcitic limestone deposit is characterized by a homogenous calcium content of 36% Ca. This company supplies limestone to approximately 80% of the AFMA members and 56% of non-registered members in the feed manufacturing industry (G. Maritz & F.P. Viljoen, 2006, Pers. Comm., Agri Lime, P.O. Box 20366, Protea Park 0305, R.S.A.).

Production is approximately 8 000 to 10 000 tons per month and limestone are sieved and classified according to the diameter of the particles. Limestone with a particle size of <1.0 mm (AL 1000), 1.0-2.0 mm (AL 2000) and 2.0-3.8 mm (Grit) are mostly used in South African poultry diets, according to the suppliers. The positive effect of large particle limestone on shell and bone quality, as illustrated in literature, could provide the egg producers experiencing poor eggshell quality, an inexpensive opportunity to improve the quality of their products. However, no data regarding the affects on either eggshell or bone qualities are available for this specific limestone source with its characteristic particle sizes.

During telephonic interviews, most of the feed manufacturing companies in South Africa, supplied the author with inconsistent information regarding the different particle size limestone used and/or the ideal ratio of small and large particles preferred by their clients. In most of the responses regarding the ratio distribution of limestone particles, the ideal mixtures used by the different feed suppliers were in the range of 40 - 60% of either small or large particles limestone. These uncertainties regarding the ideal limestone particle size and ratios of small and large particle mixtures for optimum eggshell and bone quality characteristics, as well as the fact that none of these data were available for this specific limestone source, necessitates this study.

This dissertation consists of a general introduction (Chapter 1), a literature review (Chapter 2), three separate articles on the experiments conducted (Chapters 3 – 5) and finishes with the general conclusions of the comprehensive unit (Chapter 6). Although great care has been taken to avoid unnecessary repetition, some repetition has been inevitable.

In Chapter 3, the *in vivo* and *in vitro* solubility of the specific limestone source, differing in particle size and size distribution ratios of particles, was determined. The influence of limestone particle size and size distribution ratios of limestone particles on bone quality characteristics were investigated in Chapter 4. In Chapter 5, the influence of limestone particle size and size distribution ratios of limestone particles on egg production and eggshell quality characteristics at peak production and end of lay were determined. The general conclusions of all three experiments (Chapters 3 – 5) are summarised in Chapter 6.

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CHAPTER 2

LITERATURE REVIEW

During the last fifty years, there was remarkable genetic progress the domestic chicken (*Gallus domestics*), in both the meat and egg laying type of chicken. At the same time, poultry nutrition, house designs and management practices had to be changed and adapted to exploit the genetic potential. Unfortunately, some management practices could not keep up with the genetic progress and caused biological limitations to certain aspects of poultry production. In the case of the laying hens, the increment in egg production and decreased body weight resulted in cage layer fatigue and poor eggshell quality in older hens. In spite of countless attempts to improve shell quality, the general agreement from all sectors of the research and production communities are that shell quality is still a major economic problem. Problems with eggshell quality must be studied in conjunction with the change in bone qualities as well as the molecular biology of calcium during calcification.

2.1 Functions of calcium

Calcium is the most prevalent mineral in the body and is required in a greater amount than any other mineral. Calcium alone constitutes more than a third of the total mineral content of an adult bird (Klasing, 1998). The skeleton contains about 98% of the calcium in the fowl, mostly in the form of calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) with small amounts of noncrystalline calcium phosphate and calcium carbonate ($CaCO_3$). The calcium requirements of growing chicks are the consequence of the rapid skeletal mineralization, while in the laying hen, most of the dietary calcium is used for shell formation. The mineral fraction of the egg comprises only 1% of the egg weight but plays an important role in the formation of strong eggshells. The shell forms the major portion of the mineral content of the egg and could constitute of 2.0 – 2.5g of Ca.

Apart from its structural functions, calcium is essential for the activities of numerous enzyme systems, as well as the transmission of nervous impulses and muscle contractions. Calcium is also important for the coagulation of blood and the regulation of the heartbeat. According to McDonald *et al.* (2002), blood plasma of mammals usually contains 80 – 120 mg Ca/litre, while the blood plasma of chickens contains 300 – 400 mg Ca/litre, illustrating the importance and comprehensive calcium metabolism process in poultry.

2.2 Calcium metabolism

About 25% of the calcium in the blood of laying hens circulates as free ionic calcium (Ca^{2+}), while the remainder is bound to proteins such as albumin, or complexes with citrate, phosphate or sulfate (Klasing 1998). The low level of calcium in the plasma (≈ 5 meq/l in non-laying birds) and cell cytosol (≈ 1 meq/l) is precisely regulated, because of their important role in intracellular communication, macromolecule interactions and blood clotting. This regulation is accomplished by the vitamin D endocrine system, consisting of the parathyroid hormone (PTH), calcitonin (CT) and vitamin D that control the rate at which calcium is absorbed from the intestines, deposited or mobilized from bones and excreted by the kidneys (Wideman, 1987; Norman & Hurwitz, 1993).

The metabolic activity of birds in an intensive poultry production system is very high. During one year of egg production, good laying hens will produce more than 10 times their body weight in the form of eggs. In six weeks of life, broilers increase their body weight more than 40 times. Mineral metabolism, particularly calcium metabolism, can be even more intensive as illustrated by the fact that during one year of production the laying hen will deposit 30 to 40 times the calcium present in her skeleton, in the form of eggshells. In broilers, the body calcium and phosphorus increase more than 60 times during six weeks of life (Simons, 1986). During the 20 hours that are required to form an eggshell, 25 mg of calcium must be deposited on the egg every 15 minutes (Butcher & Miles, 2004). This amount of calcium is the total amount of calcium available in the circulatory system of a normal laying hen at any given time. The importance of adequate dietary calcium for optimum metabolism and eggshell quality is thus obvious.

2.3 Digestion and absorption

The concentration of minerals in different feedstuffs is extremely variable. The solubility of a mineral, the utilization for specific metabolic processes and the rate of endogenous excretion following absorption are dependent upon the chemical and physical form in which minerals are found in the diet. Other inherent factors such as level of fibre, chelators, other minerals and the pH of the gastrointestinal tract (GIT), markedly affect the digestion and absorption of minerals. Therefore, the term mineral relative bioavailability (RBV) is used to express the nutritional value of dietary minerals in a manner that considers both solubility and the metabolic fate of the mineral. The RBV of calcium supplements is often compared with that of $CaCO_3$ and the response criterion is usually bone ash content. The increase in bone ash that results from incremental increases in calcium is used to calculate the RBV. However, it should be realized that the term 'bioavailability' is a relative and not an absolute term. A mineral source such as $CaCO_3$ may by definition have a RBV of 100% when only 50% is actually absorbed from the digestive tract. For minerals the solubility, or true availability, is always less than the RBV, but the dietary requirements are set on RBV and not on digestibility or metabolic basis (Klasing, 1998). Augspurger & Baker (2004) found that the RBV among $CaCO_3$, oyster shell, citrate and citrate-malate as calcium sources were similar, while Soares (1995) suggested that the RBV of common calcium supplements is as follows: $CaCO_3$ 100%; eggshell 100%; oyster shell 100%; bone meal 100%; dolomitic limestone 66%; limestone 89%; calcium sulfate 90%; defluorinated phosphate 94%; and lucerne 88%.

2.3.1 Factors affecting calcium absorption

Several factors have an influence on the rate as well as the amount of Ca^{2+} absorbed. Van der Klis (1993) suggested that some of the factors which may affect gastrointestinal absorption of calcium are; dietary levels of the mineral, physical and chemical form, rate of passage, viscosity of digesta, chelating agents and mineral interactions, pH of the GIT as well as protein, fat and carbohydrate interactions.

2.3.1.1 Dietary calcium levels

The most common and relative cheapest sources of calcium supplementation are limestone and oyster shell. The dietary level of calcium is certainly one of the major factors that influence calcium absorption. Larbier & Leclercq (1994) suggested that a high dietary level of calcium lowers the absorption of the mineral and *vice versa*. Dietary calcium levels greater than 40 g/kg can reduce the palatability of the diet and resulted in lower feed consumption as well as decreased absorption of other minerals. It was found that the level of available calcium in the diet is inversely proportional to the absorbability of calcium across the wall of the small intestine (Rao & Roland, 1990).

2.3.1.2 Site of absorption

Calcium is absorbed in the ionic form (Ca^{2+}) and therefore factors that influence the solubility and ionization of calcium consequently had an effect on the absorption thereof (Guinotte *et al*., 1995; Soares, 1995). Inorganic forms of calcium, such as $CaCO_3$, limestone, oyster shell and calcium phosphates, are readily solubilized by the acidic environment of the proventriculus and gizzard and hydrated calcium salts are often more soluble than the anhydrous forms. Most calcium absorption occurs in the upper small intestine (duodenum and jejunum) (Hurwitz & Bar, 1970; Van der Klis *et al*. 1990), where the digesta is still acidic following digestion in the ventriculus (gizzard). Underwood & Suttle (1999) reported that limited absorption of calcium occurs in the lower GIT of laying hens. The secretion and absorption of calcium by the different GIT segments in laying hens is dependant on the stage of eggshell formation (Hurwitz & Bar, 1965; Waddington *et al*., 1989).

Calcium is transported across the intestinal membranes by a saturable, active (transcellular) process and a non-saturable (paracellular) process. The active process could be affected by the nutritional and physiological status of the bird and could therefore increase significantly if a hen is exposed to calcium restrictions. Klasing (1998) describes active calcium transport in the following four steps, namely: (i) Energy-dependent uptake of Ca^{2+} across the enterocyte membrane, (ii) binding of Ca^{2+} to calbindin within endocytic vesicles, (iii) fusion of vesicles with lysosomes and (iv) the movement of lysosomes along microtubules and exocytosis of the contents at the basal lateral membrane. When calcium intake is adequate or higher than the requirements, the majority of calcium absorption occurs by passive absorption in the jejunum and ileum.

2.3.1.3 Phosphorus interaction

The absorption of phosphorus in the GIT seems to be similar, but not dependent on the absorption of calcium. Hurwitz & Bar (1970) determined that phosphorus absorption in broilers is the most efficient in the duodenum and upper jejunum, with no absorption occurring in the lower GIT. In laying hens, phosphorus absorption occurs throughout the entire GIT, but the rate of absorption declines in the lower GIT. In addition to its function in bone formation, phosphorus is required during carbohydrate and fat metabolism. Phosphorus also plays an important role in follicular development and low levels of phosphorus could cause a cessation in lay and/or defective laying cycles (Marangos, 2004). Phosphorus is also a key component of many important compounds in the body such as phospholipids, phosphoproteins and the high energy phosphate bonds in adenosine triphosphate (ATP) and adenosine diphosphate (ADP).

Calcium and phosphorus are required in sufficient quantities, as well as the correct ratio, to ensure optimal skeletal development and absorption of both minerals. The calcium to phosphorus (Ca:P) ratio of bone is slightly greater than 2:1 and changes little over time (Klasing, 1998). Much controversy still exists about the Ca:P ratio in diets of poultry. According to Sainsbury (2000) the Ca:P ratio should be between 1:1 and 2:1, but not outside this range, while Shafey (1993) found that a Ca:P ratio of diets between 1.4:1 and 4:1 are well tolerated if vitamin D_3 is adequate. However, Hayes & Saunders (2002) regard the principle of a 2:1 Ca:P ratio of not much validity in growing birds. They suggest that if both minerals are provided adequately, the Ca:P ratio will have less impact than when deficiencies in any of the two occurs. Qian *et al.* (1997) found that in the practical utilization of microbial phytase and vitamin D_3, the dietary calcium to total phosphorus ratio (Ca:tP) is more critical than the dietary concentrations of Ca or P. Qian *et al.* (1997) suggested that dietary Ca:tP ratios formulated in the range of 1.1:1 to 1.4:1 appear to provide the best efficacy of supplemental phytase and vitamin D_3 in broilers.

The phosphorus requirements of birds at any age are increased by high dietary calcium levels or a vitamin D_3 deficiency. Excess phosphorus tends to reduce eggshell strength as well as the absorption of calcium and therefore it is important to regulate dietary phosphorus provision to laying hens (Larbier & Leclercq, 1994; Simons, 1986). However, high levels of dietary calcium decrease the absorption of phosphorus by forming precipitates in the intestines. Larbier & Leclercq (1994) suggested that a dietary phosphorus deficiency reduced egg output, but have little effect on egg weight and that the P-requirements of laying hens are considerably lower than the Ca-requirements. The solubility as well as the absorption of both Ca and P is depressed by an excess of either one of the minerals.

Most common forms of inorganic phosphorus found in foods are readily absorbed from the diet. More than half of the organic phosphorus in the seeds of plants is poorly utilized by birds, because it is a component of phytic acid (Klasing, 1998). Before phytic acid can nutritionally be utilized, it must be hydrolyzed enzymatically by phytases to produce phosphoric acid and orthophosphate salts. The phytase activity present in the small intestine of poultry is insufficient to permit complete utilization of the phosphorus in phytic acid form, where it complexes with minerals such as calcium. Phytic acid also forms complexes with other minerals such as magnesium, potassium, manganese, iron and zinc to reduce their availability.

Edwards (1993) reported that the utilization of phytate phosphorus was greatly enhanced by the addition of 5-10 μg of $1,25(OH)_2D_3$/kg DM in the presence or absence of supplemental phytase. Augspurger & Baker (2004) also found that supplemental phytase enhanced calcium utilization from a basal diet, but it did not improve utilization of calcium that was provided as $CaCO_3$. The report of Mohammed *et al*. (1991) and Qian *et al*. (1997) suggested that supplemental vitamin D_3 resulted in a significant increase in phytate phosphorus solubility as well as Ca and P retention in the body. The research of Kemme *et al.* (1997) illustrated that Ca absorption and retention increased in pigs when diets is supplemented with phytase and is in accordance with the work done by Qian *et al*. (1997) and Augspurger & Baker (2004) on poultry. These improvements were negatively influenced by a wide Ca:tP ratio and positively influenced by higher levels of vitamin D_3 as illustrated by Kemme *et al.* (1997) and Augspurger & Baker (2004).

2.3.1.4 Vitamin D

Vitamin D is a crucial nutrient companion for calcium and phosphorus and plays an essential role in the utilization of both minerals. Chickens can synthesize this vitamin from sunlight, but even if they are kept under natural extensive or semi-intensive systems, the amount synthesized is totally unreliable and insufficient. Vitamin D is a required component of the endocrine system of birds and regulates calcium and phosphorus homeostasis, bone mineralization and eggshell formation (Klasing, 1998). Cholecalciferol (vitamin D_3) is only found in animal material, while ergocalciferol (vitamin D_2) is found in plant material. Sainsbury (2000) and Hayes & Saunders (2002) stated that ergocalciferol has no vitamin D activity for birds and must not be fed to poultry because of its poor utilization characteristics. Vitamin D_2 does not bind to plasma vitamin D-binding protein with sufficient affinity to prevent its rapid conjugation and excretion in the bile (Klasing, 1998). This metabolic loss results in a bioavailability of only 7-10% for poultry. Because vitamin D is unstable and decomposes during the manufacture and storage of feeds, it is usually supplemented at levels of three to ten times the nutritional requirements.

The requirements for vitamin D increases if; (i) dietary calcium levels are low, (ii) the Ca:P ratio is impaired and (iii) when dietary phosphorus are present in the form of phytic acid. Edwards *et al.* (1990) and Xu *et al.* (1997) have found that levels of vitamin D_3 well above the requirements of poultry and the use of more active metabolites such as 25-hydroxycholecalciferol ($25(OH)D_3$), or 1.25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), to be effective for treating or preventing bone disturbances such as tibial dyschondroplasia and osteomalacia. The study of Qian *et al*. (1997) also demonstrated that the addition of vitamin D_3 to corn-soybean diets for broilers increased calcium retention by 5-12%. A unique vitamin D-binding protein is synthesized in laying hens. This binding protein had a higher affinity for D_3 than for $25(OH)D_3$ and forms a complex with phosvitin to deliver D_3 to the follicle for deposition into egg yolk (Klasing, 1998). When dietary calcium is deficient, high levels of PTH activate the renal 1-hydroxylase enzyme and increase the circulating levels of $1,25(OH)_2D_3$. These two hormones act in concert to mediate a variety of actions that increase plasma calcium concentration, including increased calcium absorption from the small intestine as well as calcium mobilization from bone and increased re-absorption of calcium from the kidney tubules.

During a phosphorus deficiency, growth hormones mediates an increase in 1α-hydroxylase activity, causing high 1,25$(OH)_2D_3$ in the absence of high levels of PTH. This hormone combination causes mobilization of calcium and phosphorus from bones, while calcium is excreted to maintained normal plasma calcium and phosphorus. The tight regulation of plasma calcium and phosphorus levels requires the stringent regulation of 1,25$(OH)_2D_3$ synthesis and the regulation of 1,25$(OH)_2D_3$ catabolism via 24-hydroxylation. Cholecalciferol is present in the yolk of the egg and utilized by the embryo throughout development. The vitamin D endocrine system becomes competent in the chicken embryo at 6-8 days of incubation with the activation of the mesonephric kidney. Both genomic and non-genomic mechanisms are involved in the endocrine actions of 1,25$(OH)_2D_3$.

Symptoms of vitamin D deficiency are usually similar to those of a calcium deficiency. In young growing chickens, early deficiency symptoms are mainly a slow growth rate and an awkward gait. There is also a tendency for growing birds to rest frequently in a squatting position and to display apparent pain when walking. As the deficiency advances, rickets become evident, ribs develop beading at their junctures with the spinal column and long bones are easily bent due to insufficient calcification (Klasing, 1998). The epiphyseal plate of long bones becomes wide and degenerative due to the failure of cartilage producing cells to mature and this leads to their accumulation rather than replacement by osteoblasts. Hens with a vitamin D deficiency lay eggs with poor shell quality, exhibits hypertrophy of the parathyroid gland and develop osteomalacia. The high levels of parathyroid hormone that accompany vitamin D deficiency cause osteodystropia fibrosa, characterized by demineralization of the medullary bone and infiltration of fibrous connective tissue. Both the rate of lay, as well as the hatchability of eggs diminishes (Larbier & Leclercq, 1994). Embryos from eggs with inadequate vitamin D_3 have impaired calcium transport from the eggshell via the chorioallantoic membrane while bone calcification is also impaired. In severe incidences of deficiencies, chicks may die at the end of incubation or they may be unable to pip the shell. Deficient embryos often have a malformed upper mandible or incomplete formation of the beak (Shen *et al.*, 1981).

Excessive intake of vitamin D_3 or its metabolites causes disruptions in calcium and phosphorus metabolism (Soares, 1995). The relative toxicity of the vitamins follows the same pattern as their bioactivity namely: $D_2 < D_3 < 25(OH)D_3 < 1,25(OH)_2D_3$ (Klasing, 1998). Although vitamin D_3 and 25$(OH)D_3$ have little metabolic activity themselves, their affinity for the vitamin D-transport protein causes the displacement of 1,25$(OH)_2D_{3,}$ which are used to activate calcium mobilization. Elevated rates of calcium absorption and mobilization from bone cause abnormally high blood calcium levels (hypercalcemia), resulting in soft tissue calcification, cellular degeneration and inflammation (Klasing, 1998). Kidney tubule calcification often results in a fatal buildup of excretory products. Vitamin D toxicity is exacerbated by high dietary levels of either calcium or phosphorus, especially in the growing chick. Although hens are more resistant to vitamin D toxicity than growing chicks, toxic levels of vitamin D may be transferred into the egg. Vitamin D toxicosis in the embryo occurs normally in the late stages of embryonic development, due to excessive mobilization of shell calcium.

2.3.1.5 Fats

Dietary fats serve as carriers of fat-soluble vitamins and some fat is necessary for normal absorption of these vitamins. Jacob *et al*. (2003) claimed that a deficiency of linoleic acid will adversely affect egg production, because absorption of fat-soluble vitamins A, D, E and K are restricted. Diets that are high in free fatty acids may reduce the availability of calcium by forming insoluble calcium soaps that are assimilated with difficulty. The fat soluble vitamin D could be adversely affected by an impaired fat metabolism, which will lead to reduced calcium absorption and utilization.

2.3.1.6 Gastrointestinal pH

The digestive system of a growing chicken is extremely sensitive for dietary calcium levels during the first 2 - 3 weeks of age. Manganese and zinc forms an insoluble complex which renders it unavailable to the young chick when an intestinal pH of 6.5 or higher is reached (Moreki, 2005). The optimal pH for phosphorus absorption is 6 and a pH higher than 6.5 markedly decreased the absorption of phosphorus. Hydrochloric acid lowers the pH of the digestive tract, especially in the proventriculus and ventriculus (gizzard) and favours the dissociation and absorption of calcium. Excess free fatty acids in the diet cause the pH to decrease in the GIT, which results in an alteration of calcium and phosphorus absorption.

2.3.1.7 Genotype and age of hen

Calcium demands differ between breeds, strains within the same breed, age of birds, as well as the physiological status of birds. Calcium absorption is much greater in the laying hen during eggshell formation (dark hours) than during ovulation at daytime. A major problem in laying hens is the severe decrease in eggshell quality associated with age because of the decrease in 1α-hydroxylase activity with age (Abe *et al*., 1982; Elaroussi, 1994). These decreases in eggshell quality of older hens lead to higher occurrences of egg breakage during production and processing and decreased profitability for the egg producer. Elaroussi *et al*. (1994) found that one of the possible causes of the increased rate of cracked or soft-shelled eggs associated with older laying hens is related to the decrease in the renal $25(OH)_3$-1α-hydroxylase activity which resulted in an impairment of the biosynthesis of $1,25(OH)_2D_3$, confirming the results of Petersen (1965) who suggested that the ability of the hen to absorb calcium from the digestive system and to mobilize calcium from the medullary bone are reduced with age. The study of Elaroussi *et al*. (1994) demonstrated that both young and old laying hens eventually adapt to dietary Ca restrictions in terms of increased $1,25(OH)_2D_3$ production. However, both the rapidity and magnitude of the response is decreased in older hens compared to younger hens and Elaroussi *et al*. (1994) concluded that younger laying hens have a greater adaptive response to calcium restriction than older hens.

An increased calcium demand during the laying cycle is accommodated by an appropriate increase in intestinal Ca-absorption and a decrease in renal Ca-excretion (Elaroussi *et al*., 1994). Both renal 1α-hydroxylase activity and plasma $1,25(OH)_2D_3$ concentrations are significantly higher during the active stage of eggshell calcification than in other stages. During reproductive activity in the female chicken, endogenous estrogen mediates changes in the function of the kidney that involve the two major Ca-regulating hormones, namely PTH and $1,25(OH)_2D_3$. The number of PTH receptor sites and the activity of PTH-dependent adenylate cyclase are elevated in the kidney of a mature reproductive hen, relative to either the mature male or immature chicken of either sex.

2.4 Calcium requirements for maintenance

Calcium requirements may be divided into two components, namely maintenance and production requirements. Klasing (1998) reported that the amount of dietary calcium needed to maximize bone or eggshell mineralization and strength (production) are greater than that needed for other functions (maintenance). Therefore the amount of dietary calcium needed for production purposes are typically used as the response criterion for setting the requirements.

Maintenance calcium requirements are those Ca^{2+} needed to replace the small amounts of calcium, lost from endogenous sources each day (Klasing, 1998). Most of the endogenous Ca losses occur in the excreta. Loss through this route is dependant upon other dietary factors, especially phosphorus level and diet acidity. Maintenance calcium requirements for birds are not generally known but are less than 0.2% of the dietary levels in adult chickens and may be less than 0.02% if dietary phosphorus levels are low (Klasing, 1998). In another opinion, Larbier & Leclerq (1994) suggested that the calcium requirement for maintenance of a mature bird could be as low as 50 mg/day/kg body weight. Dietary calcium provision could be calculated by dividing total requirements by the coefficient of Ca utilization, which range between 50% and 60%.

Elaroussi *et al*. (1994) reported that calcium homeostasis is achieved by balancing the efficiency of intestinal Ca-absorption, renal Ca-excretion and bone mineral metabolism to the calcium requirement of the bird. The main hormones controlling these balances are PTH, CT and 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) produced by the renal conversion of 25-hydroxycholecalciferol ($25(OH)D_3$) through the activity of the enzyme 25-hydroxycholecalciferol-1-hydroxylase (1α-hydroxylase).

2.5 Calcium requirements for bone formation

Bone is made up of calcium hydroxyapatite phosphate crystals, deposited onto an organic collagen matrix. There are several different types of bone in the laying hen. The main types that provides structural integrity is cortical- and cancellous- (or trabecular) bone (Whitehead & Fleming, 2000). These bones are formed during growth, but with sexual maturity a third type of nonstructural bone, called medullary bone is formed. Bone formation consists of a constant remodeling process in which osteoclast cells resorb areas of bone and replace them by osteoblasts that deposit new bone.

Longitudinal bone growth is initiated by the accretion of cartilage in the epihyseal growth plates on each end of the long bones (Leach & Gay, 1987). These cartilages are degraded by infiltrating osteoblasts, which in turn deposit collagen and hydroxyapatite within the template previously created by cartilage. Bone width is increased by the deposition of calcium phosphate onto a collagen matrix located on the bone periosteal surface by osteoblasts (Klasing, 1998). The bone remodeling process, permits simultaneous increases in diameter and length of bones. The closely coupled processes of bone re-absorption and accretion result in a continual turnover of calcium, phosphorus and other minerals, making them available to buffer dietary shortages.

The amount of calcium needed for growth had been determined by empirical methods, which establish the minimal dietary level that maximizes bone ash and bone breaking strength. The calcium requirements for pullets are relatively low during the growing period, but with onset of egg production, the need is increased at least four times and this is mainly due to the needs for shell formation (Jacob *et al*., 2003).

2.6 Calcium requirements for eggshell formation

Ahmad *et al*. (2003) recorded a wide difference of opinion in literature, regarding calcium requirements for optimum performance, varying between 3.25 and 5.17g/h/d. They found that egg production differs significantly ($P<0.05$) between the lowest (2.5% Ca) and the highest (5.0% Ca) levels of calcium intake, while an increased dietary calcium level resulted in a significant linear improvement of specific gravity. Ahmad *et al*. (2003) also found that dietary calcium had no significant effect on egg weight and feed consumption and concluded that strain, age, individual feed consumption, absorption capacity and egg size are some of the factors which might influence the variation in calcium requirements of hens. According to the NRC (1994), the Ca-requirements of white-egg breeders and layers at a feed intake of 100 g/h/d are 3.25% and of brown-egg layers at a feed intake of 110 g/h/d are 3.60%.

The calcium requirements for egg production could be estimated from the number of eggs laid, the amount of calcium in those eggs and the pattern of egg laying (e.g. daily, or alternate days) and its been suggested that the amount of calcium deposited could vary between 1.7 and 2.2 g Ca/egg (Marangos, 2004). The average eggshell contains about 0.3% phosphorus, 0.32% magnesium and traces of sodium, potassium, zinc, manganese, iron and copper. Klasing (1998) suggested the possibility to calculate daily calcium requirements for various fowl species at a known production rate if the following assumptions are made namely; (i) 99% of the Ca in an egg is found in the shell and the shell constitutes of 95% ash, (ii) dietary Ca is 70% digestible and (iii) the metabolic fecal and urinary losses of Ca are trivial.

The assumption that faecal, urinary and metabolic losses are trivial is known to be valid for poultry and Japanese quail, where endogenous calcium losses account for less than 0.2% of the requirements (Klasing, 1998). Wideman (1987) suggested that calcium availability for eggshell formation is the controlling parameter for urinary calcium and phosphorus excretion. Urinary phosphorus excretion increased, while urinary calcium excretion decreased, during eggshell formation when bone minerals are released. It was also suggested by Wideman (1989) that diets high in calcium and low in phosphorus resulted in a very high urinary calcium excretion.

2.7 Dietary calcium supplementation

Grains and their by-products are generally low in calcium. Grains seldom contain more than 1 g Ca/kg DM and grain by-products seldom more than 1.5 g Ca/kg DM. Underwood & Suttle (1999) suggested that the supplementation of vegetable protein sources, to increase the calcium content of the diet would be ineffective, because of the low Ca concentrations in these feeds (2.0 - 4.0 g Ca/kg DM). Animal protein sources like fish-, meat-, blood-, feather- and bone meal are good sources of calcium (50 - 100.0 g Ca/kg DM). These sources can however, be scarce and expensive. However, grain and vegetable protein sources are important determinants of the absorption and availability of calcium in non-ruminant diets. The reason for this is that the phytate contained by these sources forms insoluble complexes with inorganic as well as with organic calcium, which hinders the absorption of Ca^{2+}.

Between 1921 and 1963 at least 13 articles were published comparing oyster shell and limestone as calcium sources in layer diets and their effect on eggshell quality. Of these articles, 10 reported that fine granular limestone was equal or better than large particles of oyster shell for eggshell quality, while three reported that oyster shell resulted in better eggshells than limestone (Roland, 1986). From 1970 to 1985 at least 21 articles were published comparing the response of laying hens fed oyster shell and fine granular limestone on egg quality. Fourteen of these articles reported that oyster shell was better than fine particles of limestone, while seven reported that fine particles of limestone and oyster shell are of equal value for eggshell quality. The studies mentioned above were not true comparisons between oyster shell and limestone, because large particles of oyster shell were compared to fine particles of limestone. From 1972 to 1982, at least 12 articles were published in which large particles of oyster shell were compared to similar sized particles of limestone. Of these studies, 10 reported an equal response when the same size particles were compared, while two reported that oyster shell gave better results than limestone (Roland, 1986). It is clearly noticeable that the controversy regarding the ideal calcium source and specific particle size remain factors for consideration in formulating poultry diets.

2.7.1 Particle size

Zhang & Coon (1997) suggested that the retention time of calcium supplements influences the availability of Ca^{2+} and that Ca retention in the GIT is dependent upon particle size, porosity and the overall *in vivo* solubility of the source. The availability of Ca^{2+} and performance differences due to the particle size of the calcium sources is most evident at marginal dietary calcium levels.

In a literature review, Roland (1986) suggested that most research workers used approximately 66% of the $CaCO_3$ source as large particles and 33% as finely ground. Roland (1986) suggested that commercial egg producers should obtain the best results using 33% to 66% larger particles of $CaCO_3$. Marangos (2004) suggested that the particle size needs to be a minimum of 0.9 to 1.0 mm in diameter to be retained in the gizzard for more efficient utilization during the time of eggshell formation. Bristol (2006) illustrated that large limestone particles (1.4-5.6 mm) appear to be more effective than smaller particles (0.005-1.18 mm) in producing eggs with acceptable shell qualities and strength. In another opinion, Marangos (2004) suggested that the inclusion of limestone sources with larger and less soluble particle sizes (2.0-4.0 mm) as a major (60-70%) portion of the total source, increases the retention time of the limestone in the gizzard, which resulted in an improved shell strength. Scheideler *et al*. (2005) illustrated that a mixture of 50% fine and 50% large particles of limestone resulted in optimum eggshell quality. However, the ideal limestone particle size and digestive utilization thereof differ between broiler chickens and laying hens, according to Guinotte *et al*. (1991).

2.7.1.1 Laying hens

Roland *et al*. (1974), Muir *et al*. (1975) and Kuhl *et al*. (1977) reported that limestone particle size had no effect on shell thickness, egg-breaking strength and specific gravity respectively, whereas other authors found that shell quality improved significantly with particulated limestone (Meyer *et al*., 1973; Watkins *et al*., 1977, Cheng & Coon, 1990b). The solubility of limestone could be responsible for these contradictory reports. The work of Anderson *et al*. (1984) on broilers is in contrast with that of Guinotte & Nys (1991), Rao & Roland (1990) and Cheng & Coon (1990b) on laying hens. These authors found that large particle size calcium supplements resulted in an improved bone ossification, eggshell quality and performance, whereas Anderson *et al.* (1984) reported no significant improvements of bone quality with the use of large limestone particles. Zhang & Coon (1997) suggested that the increased Ca availability from larger limestone particles could be the result of a slower rate of passage through the GIT of mature hens, allowing absorption of Ca^{2+} when needed for shell formation. This suggested that larger particles of calcium sources are released more slowly which is important for the continuity of shell formation, especially in the dark period when no feed consumption occurs (Jacob *et al*., 2003). The greater retention time of larger limestone particles allows the limestone to stay in an acidic environment for a longer period of time where the acidity would increase the opportunity to dissociate $CaCO_3$ into Ca^{2+}, producing more available calcium for absorption. An increased amount of available calcium in the digestive tract at night would lead to a decreased mobilization of bone calcium reserves and Farmer *et al*. (1986) indicated that maintaining adequate calcium reserves in the GIT would reduce the skeletal dependence thereof. The beneficial effect on shell quality by replacing a partly pulverized limestone with particulated limestone is illustrated by Scott *et al*. (1971) and Brister *et al*. (1981). In another opinion, Zhang & Coon (1997) suggested that the gizzard have an upper limit for the amount of ingested limestone particles that could be retained. Layers fed diets with large limestone particles, or less soluble particles, may reach a saturation state in the gizzard and the continuous intake of the same diet may cause an increased output of undissolved limestone.

Guinotte & Nys (1991) reported that particulated limestone resulted in higher shell weights compared to ground limestone or particulated oyster shell and that egg production was not affected either by origin of calcium source or particle size. In contrast with the results of Guinotte & Nys (1991), Roberts & Leary (2000) reported that although particulated calcium resulted in a slight improvement of eggshell quality, a decrease in egg production was recorded. The positive effect of coarse limestone on increased egg weight observed by Guinotte & Nys (1991) are in contrast with the observations of Muir *et al*. (1976) and Watkins *et al*. (1977) who reported that large particles limestone resulted in no significant improvement of egg weight. The NRC (1994) suggested that the conditions under which large particle size calcium consistently improve eggshell strength should be identified, because of all the inconsistencies regarding the ideal particles size and particle distribution in literature.

2.7.1.2 Broilers

Hillman *et al*. (1976) reported that small particle size limestone resulted in an increased weight gain, feed efficiency and calcium availability to broiler chickens at low dietary calcium levels. The report of Guinotte *et al*. (1991) also suggested that small particles of different calcium sources resulted in an increased feed consumption and better feed conversion in broilers, whereas the inverse is true in laying hens (Guinotte & Nys 1991). In contrast Anderson *et al*. (1984) found that it is not advantageous to use fine particle size calcium in broiler diets, because it produced lower tibia ash values when fed at high levels. They also found that the calcium and phosphorus imbalance was more serious when small particles of calcium sources were used in broiler diets. In contrast with the work of Anderson *et al*. (1984), Guinotte *et al*. (1991) suggested that coarse particle size was associated with a decrease in broiler performance and that the poor availability of coarse particles of calcium cause a decrease in bone ossification. The higher transit time of coarse particles compared with ground particles, due to gizzard retention and their lower solubility in combination with the physiological shorter transit time in growing chicks may explain the negative effect of coarse particles on calcium retention (Guinotte *et al*., 1991). Anderson *et al*. (1984) suggested that chicks could pass excess calcium of medium particle size through the digestive tract more rapidly than small particle size calcium, therefore limiting the overall utilization thereof. Guinotte *et al*. (1991) concluded that the depressive effects of coarse particle size on calcium retention, weight gain, feed efficiency, tibial morphometry and mineralization suggested that it is better to use ground calcium supplements in broiler diets. The conflicting results of limestone particle size on the growth and production performances of broiler and laying hens therefore seems to remain a much debatable subject.

2.7.2 Calcium source

Various studies had shown that factors such as calcium source, pH of the GIT, retention time in the GIT, particle size and the influence of other nutrients contribute to Ca-solubility (Ajakaiye *et al*., 1997). The chemical structure of calcium sources such as limestone and oyster shell have a great influence on the solubility and absorption of Ca^{2+}. The use of *in vivo* and *in vitro* solubility to predict the RBV of calcium sources had been used with different degrees of success.

Calcium carbonate is obtained from mineral (limestone) or animal deposits (oyster shells) and differs in physical and chemical composition (Reid & Weber, 1976). Limestone had been used for many years to increase soil pH. Effectiveness of limestone for this purpose is related to how fast it neutralizes diluted acid. Fine particles of limestone neutralize acid faster and are more effective in increasing soil pH and are wrongly termed as “more reactive” (Anderson *et al*., 1984). Periodically dolomitic limestone, which is primarily used in the steel industry, is offered to the feed industry (Leeson & Summers 1997; Jacob *et al*., 2003). Dolomitic limestone contains at least 10% magnesium (Jacob *et al*., 2003) which complexes and competes with calcium for absorption sites. The consequence of feeding dolomitic limestone is an induced calcium deficiency and Roland (1986) reported that dolomitic limestone reduced eggshell strength due to the high magnesium content.

Rabon & Roland (1985) reported that the solubility of limestone particles of similar size from different sources varied by 62%. Ajakaiye *et al.* (1997) using seven different calcium sources and reported that particle size as well as source had a significant effect on solubility. The results of Zhang & Coon (1997) indicate that the amount of limestone retained in the gizzard is significantly affected by the limestone source, particle size and dietary calcium level. In contrast, Augspurger & Baker (2004) found that the RBV of calcium was not affected by the source.

The results of Ahmad & Balander (2003) which suggested that egg production was improved with the use of oyster shell are consistent with the results of Hamilton *et al*. (1985). However Cheng & Coon (1990a) did not find any difference in egg production with different calcium sources. The influence of calcium source on egg production is thus subjected to contradictions. Although the egg production results of Cheng & Coon (1990a), differs from that of Ahmad & Balander (2003) their results with egg weights are in agreement with each other and both studies reported that neither the calcium source, nor a reduced phosphorus level affected egg weights. Keshavarz & Nakajima (1993) reported that supplementation of a diet with oyster shell resulted in a greater specific gravity and improved eggshell quality and their results are consistent with the reports of Scott *et al*. (1971), Keshavarz & McCormick (1991) and Ahmad & Balander (2003). Guinotte & Nys (1991) reported that ground or particulated seashells and ground oyster shells are equal to particulated limestone as calcium supplements for eggshell quality and that the calcium retention of the different sources varied parallel with the surface area of the specific source. It therefore seem that the inconsistencies regarding the effect of calcium source on egg production and eggshell quality still remain a problem that needs further investigation.

2.8 Bone characteristics

2.8.1 Functions of bone

Bones have an important structural as well as metabolic role in poultry. Structurally, bone supports the musculature of the bird and therefor growth and development are intimately associated with overall body (skeletal) growth. Metabolically, bone provides a labile pool of calcium and phosphorus that could be utilized during nutritional shortages. Bone also provides protection for the soft tissue and vital organs in the body cavity and permits locomotion of the bird (Bouxsein & Augat, 1999).

2.8.2 Types of bone

The two forms of bone present in domestic fowl are structural and medullary bone. Structural bone can be subdivided into compact (cortical) and cancellous (trabecular) bone, both of which are lamellar. Bone growth in the immature chicken occurs through the formation of structural bone (Fleming *et al*., 1998b). The peak mass of structural bone is reached at the onset of follicular activity and then declines slowly throughout the laying period (McCoy *et al*., 1996).

2.8.2.1 Cortical bone

Cortical bone is characterized by the haphazard organization of collagen fibres and is thought to have little instrinsic strength (Knott *et al*., 1995). Roach (2000) indicated that cortical bone is a temporary bone that will either be resorbed or replaced with lamellar bone via the remodeling process. Cortical bone is produced when osteoblasts produce osteoid rapidly in processes such as new bone formation during fracture healing or during fetal bone development. The collagen fibre is deposited in an irregular, loosely intertwined pattern in the osteoid, where it is eventually remodeled to form lamellar bone. Longitudinal bone growth proceeds faster at the proximal plate than the distal plate and bone close to the proximal end of the shaft is therefore likely to be newer and less mineralized than at the distal end (Williams *et al*., 2000).

2.8.2.2 Cancellous bone

Cancellous bone consists of a delicate scaffolding of interconnecting fine bone plates and trabeculae which are surrounded by spaces containing marrow. Cancellous bone could be found in many parts of the skeleton, especially in the centre of cranium bones, and it forms the bulk of the bone substance at the ends of long bones. Kenney (2000) illustrated that cancellous bone had a large surface area and a greater blood supply than cortical bone, making it more responsive to changes in circulating hormones such as oestrogen, PTH, CT and testosterone, which can affect bone metabolism. Cancellous bone is characterized by a regular parallel alignment of collagen into lamellae. Whitehead *et al*. (1998) describe cancellous bone as a type of structural bone that provides internal skeleton support.

2.8.2.3 Medullary bone

At the onset of sexual maturity, females of many avian species deposit medullary bone (MB) in the marrow cavity of long bones. MB is non-structural and has a spongy appearance, which is characterized by the haphazard organization of collagen fibres in its matrix. The femur and tibiotarsus are rich in MB, while the humerus seldom contains any MB. MB represents 10 - 12% of total bone and acts as a reservoir of available calcium for use during eggshell formation (Marangos, 2004). Dietary calcium supplies about 60 - 75% of the calcium requirements for eggshell formation, whereas the other 25 - 40% comes from MB (Schreiweis *et al*., 2003). Therefore, the skeleton may become severely depleted as it serves as an alternative source of calcium provision during dietary deficiencies.

A daily cycle had been described in chickens, where MB calcium is accreted in the morning and used at nighttime. The mobilization at night occurs due to the lack of dietary inputs and the high Ca demands of the shell glands during eggshell deposition. The concentration of MB varies from partial filling around the periphery of the cortical cavity to the complete filling thereof. Calcium is also drawn from the cortical bone to maintain MB weight. Over a period of time a daily negative balance of MB reserves occurs and this had been related to a decline in shell quality with age (Marangos, 2004). If dietary calcium is insufficient, the mass and density of MB declines and it becomes brittle. Fleming *et al*. (1998a) stated that the measurements of humeral bone breaking strength are highly correlated with the amount of humeral MB. The results of Knott *et al*. (1995) suggested that MB has little intrinsic strength compared to cortical- and cancellous bone, although the mineral composition of MB is similar to that of cortical- or cancellous bone. It was previously thought that large quantities of MB could be detrimental for bone strength. It had been suggested that the partitioning of available calcium during the laying period may favour formation of highly labile MB at the expense of the more structural cancellous and cortical bone types. Fleming *et al*. (1996) suggested that humeral MB bone have a considerable heritable component. The results of Fleming *et al*. (1998a) show unequivocally that the amount of MB had a direct relationship with humeral breaking strength in laying hens and suggested that although MB may not have any intrinsic strength, it could contribute to the fracture resistance of the surrounding cortical bone. MB itself is isotropic in structure, being turned over so fast that there is no time for the orientation of collagen in response to functional loading, as in the case of the more anisotropic cancellous bone (Fleming *et al*., 1998b).

2.8.3 Effect of calcium deficiency on bones

Gregory & Wilkins (1989) reported that 29% of end-of-lay battery caged hens in the United Kingdom had one or more broken bones during their lifetime. These fractures occurred either during their time in cages or during depopulation and transport to processing factories. They found that by the time that the carcasses reached the end of the evisceration line, 98% of the carcasses contained broken bones. Whitehead & Wilson (1992) reported that since bone fragility in commercial laying hens had first been recognized, the intensity of the problem had increased and suggested that possible changes in genetic factors and husbandry systems could be responsible for these increases. They attributed most of the osteopenia in commercial hens to osteoporosis and suggested that cage layer fatigue (CLF) should more properly be referred to as "cage layer osteoporosis" (CLO).

2.8.4 Osteoporosis

Osteoporosis in laying hens is defined as a decrease in the amount of fully mineralized structural bone, leading to an increased fragility and susceptibility to fracture (Whitehead & Fleming, 2000). The origin of osteoporosis is not well defined, but it is suggested that the problem is partly genetic, resulting from the breeding of light-weight, energy efficient birds, that maintain a high rate of lay over a prolonged period of time (Whitehead & Fleming, 2000).

2.8.4.1 Development of osteoporosis

Whitehead & Fleming (2000) illustrated that osteoporotic hens show evidence of widespread loss of structural bone throughout the skeleton. This loss of structural bone starts when the hens reach sexual maturity and continues throughout the laying period. At onset of sexual maturity the rise in circulating oestrogen resulted in a switch of bone formation from structural to medullary bone and the continued re-absorption of structural bone leads to osteoporosis (Fleming *et al.*, 1998b; Whitehead & Fleming, 2000). As osteoporosis progresses, cortical bone thickness decreases and the cohesive system becomes thinner.

Patterns of bone loss with age vary among different bones and a striking change occurrs during the first ten weeks of sexual maturity. A marked loss of cancellous bone occurrs in both the proximal tarsometatarsus (PTM) and the free thoracic vertebra (FTV), suggesting that the major development of osteoperosis occurs in these bones a few weeks after the onset of egg production (Whitehead & Fleming, 2000). After 25 weeks of age, further loss of cancellous bone from the FTV is smaller, but losses of cancellous bone and accumulation of medullary bone continue in the PTM, although at reduced rates.

The increase in humeri radiographic density and breaking strength observed between 15 and 25 weeks of age could be ascribed to the development of MB in bones that are normally pneumatized together with the absence of any appreciable loss of structural bone over this time period (Fleming *et al.*, 1996). The results of McCoy *et al.* (1996) indicated that bone strength increased with age. However, in contrast, Whitehead & Wilson (1992) suggested that the loss of bone throughout the normal laying period of hens is a natural phenomenon. Fleming *et al.* (1998a) suggested that the overriding loss of strength in osteoporotic avian bone is due to the loss of bone mass. Strategies aiming at minimizing fracture incidence should therefore concentrate on preventing structural bone loss over the lifetime of the hen.

2.8.4.2 Consequences of osteoporosis

Because cortical bone re-absorption is confined to endoseal surfaces (Whitehead & Fleming, 2000), the decline in structural cortical and trabecular bone components have no influence on the external dimensions of long bones. The loss of trabecular and cortical bone could lead to the exposure of the spinal column and it is presumed that pressure on exposed nerves accounts for the paralysis observed during CLF (Whitehead & Fleming, 2000). However, the thinning of cortical bone and loss of trabecular integrity resulted in bones becoming weaker and more susceptible to fractures if subjected to trauma. The ischium, humerus, keel and furculum showed the highest fracture incidences, with frequently observed breaks in the pubis, ulna and femur (Gregory & Wilkins, 1989). Spinal bones could be severely affected by osteoporosis, but fractures are rarely observed.

Day (1990) suggested that skeletal problems compromise the welfare of birds and resulted consequently in a reduced growth performance, increased mortality and increased carcass downgrading due to lesions. Avian bones are enervated and it is assumed from human analogy that avian fractures are painful (Whitehead & Fleming, 2000). It is, however, possible that pain may also arise in the absence of overt fracture from exposure of nerves, contributing to the welfare issues of battery caged laying hens.

2.8.5 Factors affecting bone strength

Bone strength is dependent on the physical, architectural and material properties thereof. Any change in shape, size, collagen fibre orientation, or molecules in bone matrix may alter bone strength (Rath *et al*., 2000). The work of Rath *et al*. (2000) suggested that nutrition, growth, age, genetics, gender, disease and physical loading affected bone breaking strength, directly or indirectly. Bone type, quantity, distribution (shape) and the proportion of collagen and mineral content are more important to bone strength than the exact form of the mineral crystal (Williams *et al*., 2000).

2.8.5.1 Nutrition

Nutritional deficiencies of calcium, phosphorus, or cholecalciferol resulted in bone losses and contribute to osteomalacia that will ultimately result in osteoporosis. Evidence to date suggested that nutritional factors during the laying period, in the absence of nutrient deficiencies, have relatively little effect on the severity of osteoporosis in end-of-lay hens (Fleming *et al*., 1998b).

It is known that providing calcium in the particulated form extends the period of calcium absorption later into the night when shell formation is occurring and it was found to have a beneficial effect on shell quality (Fleming *et al*., 1998b; Whitehead & Fleming, 2000). Rennie *et al*. (1997) found that feeding oyster shell increased the proportion of medullary bone, although it had no effect on the FTV or PTM bones. Miller & Sunde (1975) reported that hens consuming large particle size oyster shell had an improved bone weight compared to those consuming ground limestones. Cheng & Coon (1990a,b) and Guinotte & Nys (1991) reported that various calcium sources resulted in an improved tibia breaking strength, femur strength and percentage tibia ash if fed to laying hens in the coarse rather than the ground form. The maintenance of adequate calcium reserves in the GIT would reduce the needs for skeletal calcium during eggshell formation in the dark hours (Orban & Roland, 1990).

The provision of calcium with improved digestive characteristics could increase the quantity of MB without having much impact on the loss of structural bone. The report of Fleming *et al*. (1998b) suggested that cancellous bone volume, as well as MB accumulation was higher in birds consuming particulated limestone compared to ground limestone. Fleming *et al*. (1998b) concluded that diet supplementation with particulated limestone had a beneficial effect in decreasing the severity of osteoporosis. However, bones like the FTV and humerus which contains generally little MB, showed no response to particulated limestone.

Nutrition during rearing is important in maximizing bone mineralization before onset of sexual maturity. Fleming *et al*. (1998b) and Zhang *et al*. (2003) reported a significant interaction between vitamin K supplementation and bone quality that differs during the different growth phases, with a higher increase in bone quality during the starter phase (week 1 – 3) than in the finisher phase (week 6 – 7). Because the majority of bone consolidation occurs during the first three weeks of age, this period is important to improve bone quality of broiler chickens (Zhang *et al*., 2003). The results of Fleming *et al*. (2003) indicated that the combination of extra vitamin K, particulated limestone and supplemental fluoride resulted in bone quality improvements of 12 – 20%. These observations are consistent with the hypothesis that osteoporosis in hens arises as a result of cellular processes rather than nutrient supply and that osteoclastic re-absorption continued during the laying period while little formation of structural bone occurs.

Schreiweis *et al*. (2003) suggested that a hypocalcemic diet caused a dramatic decrease in body weight (BW) at 46 and 56 weeks of age and also reported an increase in mortality, lower bone mineral density (BMD), lower bone mineral content (BMC) and lower shell weights, percentage eggshell and shell thickness. The results of Clunies *et al*. (1992) indicated that the pneumatic humerus may be less sensitive to changes in dietary calcium as compared to the medullary tibia and ascribed this to the small amount of vascularization in the medullary cavity of the humerus compared to the tibia. Cheng & Coon (1990a,b) found a linear increase in bone breaking strength with an increased concentration of dietary calcium. Frost & Roland (1991) also reported that energy consumption was significantly lower with an increase in dietary calcium and reported a significant increment in tibia weight, tibia breaking strength, tibia ash and bone mineral content with increased levels of dietary calcium.

2.8.5.2 Effects of exercise and husbandry system

Nightingale *et al*. (1972) showed that induced inactivity accelerate osteoporosis in birds and suggested that the relative lack of activity in battery caged hens accounts for the severity of osteoporosis in these birds. The confinement of birds in cages with limited opportunity for exercise had undoubtedly contributed to the problem, resulting in a form of disuse osteoporosis. However, Whitehead & Wilson (1992) reported that neither housing birds in pens nor giving exercise through the use of a carousel had improved bone quality at the start of lay compare to cage rearing of pullets. Wilson *et al*. (1993) found that housing hens in pens had resulted in little change in spinal trabecular bone and suggested that merely allowing the birds more opportunity to walk does not result in bone improvement. The opportunity for flight was an important factor for improving humerus strength, suggesting that biomechanical effects on individual bones are dependent upon the degree of strain experienced by the bone (Fleming *et al*., 1994; Whitehead & Fleming, 2000). The report of Newman & Leeson (1998) suggested that exercise may involve stimulation of structural bone formation, rather than the inhibition of re-absorption.

The results of Gregory *et al*. (1990) illustrated lower incidences of new fractures during depopulation of birds from aviary or free-range systems compared to battery cages. However, the incidences of old fractures, particular in the furculum and keel, were higher with the aviary and free-range systems. It could be concluded that allowing birds more exercise in alternative systems will improve bone strength, but this does not necessarily improved bird welfare proportionately. Birds in alternative systems have a greater opportunity to experience more damaging accidents than caged birds (Whitehead & Fleming, 2000). It is likely that the designs and stocking densities of alternative husbandry systems can be established to minimize welfare problems associated with osteoporosis. However, alternative systems are also associated with other welfare problems, such as feather pecking and cannibalism.

2.8.5.3 Temperature

Hester (1994) reported that environmental factors such as the ambient temperature affected bone growth in different ways and concluded that hot temperatures influence bone growth more than cold temperatures. Bruno *et al*. (2000) reported that all long bones showed a reduced length and width growth at hot temperatures compared with thermo neutral or cold temperatures in broiler chicks at 42 days of age. They also suggested that breaking strength was affected by age but not by feed restriction or environmental temperatures.

2.8.5.4 Genetic factors

The appearance of osteoporosis in high-performance hens had been attributed to two factors, namely; (i) confining birds in cages that had weakened bones by promoting a form of disuse osteoporosis and (ii) modern selection procedures that have produced birds of low body weight and food intake but high egg output (Whitehead & Fleming, 2000). The large individual variation observed in bone characteristics in end-of-lay hens are phenotypically unrelated to egg production and suggested that osteoporosis may be alleviated by genetic selection, perhaps without serious consequences for egg productivity (Rennie *et al*., 1997). Bishop *et al*. (2000) found that the morphometric traits involving cancellous and medullary bone volumes were poorly heritable, while in contrast, the heritability of other characteristics such as bone strength and keel radiographic density were moderate to strongly inherited and respond readily to selection in both sexes of chickens. Bishop *et al*. (2000) suggested that if birds are genetically selected for increased BMD in one or two bones of the skeleton, the strength of the entire skeleton should improve, due to the high correlation in breaking strength among different skeletal bones. The results of Yalçin *et al*. (2001) indicated that strain had a significant effect on most of the anatomical properties except cortex thickness during the first 16 days of life, which is in accordance with the results of Williams *et al*. (2000) who suggested that the strain by age interaction observed during the 42 day rearing period of broilers are due to the fluctuation of the calcium and phosphorus content of bones.

Bishop *et al*. (2000) and Whitehead & Fleming (2000) suggested that selection for enhanced bone strength could be used as a long term measurement in the prevention of the serious osteoporosis problems in laying hens. However, until a genetic strategy could be expected to result in an impact on commercial laying hens, it will be important to implement nutritional and husbandry strategies to decrease the severity of osteoporosis.

2.8.5.5 Age

Yalçin *et al*. (1998a) reported that neither dietary protein nor sex affected walking ability at four weeks of age but dietary protein and sex have a significant influence on the walking ability at seven weeks of age and suggested the existence of a age x diet interaction. Although shell quality deteriorates in older hens, there is no evidence that this is related to poor bone quality and the proportion of medullary bone might be at its highest in older hens (Fleming *et al*., 1998b). It is more likely that the predisposing factor of osteoporosis is the extended production period in which hens are continuously in a reproductive condition. This increased period of structural bone re-absorption, rather than the age of the hen, resulted in a more severe type of osteoporosis at the end of the laying cycle (Whitehead & Fleming, 2000).

Al-Batshan *et al*. (1994) found that the percentage eggshell, shell thickness, percentage femur ash and intestinal calcium uptake decreased significantly with age and suggested that an induced molt would improve these traits.

2.9 Methods of determining bone quality

The methods used to determine bone quality could be categorized as either invasive or noninvasive (Rao *et al*., 1993). Invasive methods require the sacrificing of birds before the technique to determine bone quality can be applied, while noninvasive methods enable measurements of the bone mineralization process over an extended period of time without sacrificing the birds. Some of the common invasive methods are bone weight, bone volume, bone breaking strength (BBS) and percentage bone ash, while direct photon absorptiometry and ultrasound are two of the most commonly used noninvasive methods (Rao *et al*., 1993). Remodeling of bones due to changes in dietary calcium most often resulted in changes of bone mineral density (BMD) and Schreiweis *et al*. (2003) illustrated that these changes are usually measured by invasive methods.

Literature regarding the sensitivity of different measurements of bone status in experimental treatments is controversial. Cheng & Coon (1990b) concluded that a Ca-deficient diet fed to layers resulted in a significant reduction of bone ash concentration with no differences in bone size, whereas Guinotte *et al*. (1991) finds that growing broilers fed a Ca-deficient diet had smaller bones with less bone ash and a reduced growth rate. Therefore, the sensitivity of bone measurement may differ between laying hens and growing broilers in response to dietary treatments. Bishop *et al*. (2000) found that bone measurements are strongly correlated with fracture incidence, whereas body weight and fracture incidences are weakly correlated. They also observed a positive correlation between body weight and bone strength and suggested that bone measurements were all positively correlated with body weight. Bishop *et al*. (2000) suggested that selection for improved bone strength characteristics alone, without the restriction placed on the body weight, would result in heavier birds.

2.9.1 Bone ash

Historically, percentage tibia ash was used as the main method for the determination of bone mineralization. According to Hall *et al*. (2003), 89% of all the papers published in the *Journal of Poultry Science* in respect of bone mineralization in the ten years prior to 2003, used the tibia ash method, whereas 11% have used radiography to determine bone mineralization. Roberson *et al*. (2004) reported that the tibia is the most sensitive bone for bone ash determinations. Williams *et al*. (2000), reported that the mid-section of the tibia showed the greatest ash content as well as the highest calcium and phosphorus content, followed by the distal end and the proximal section.

Hall *et al*. (2003) found that the actual weight of the tibia ash may be a more sensitive indicator of bone mineralization (r^2=0.92) than the percentage (%) bone ash (r^2=0.57). They concluded that both the autoclaving and boiling/extracting methods could be used to estimate the bone ash of broiler chickens. However, although the autoclaving method minimizes time in cleaning bones, it requires more samples and therefore more birds, feed and space. The ratio of bone ash to bone weight had long been used as a predictor of bone strength. Cheng & Coon (1990a) concluded that this ratio was not related to bone strength, because both the ash and the non-ash components of bone are mobilized during bone turnover and their ratio therefore remains unchanged.

Mitchell & Edwards (1996), found a direct correlation between increased bone ash, higher amounts of available calcium and phytate phosphorus in the diet and increased bodyweight (BW) gain. Bond *et al*. (1991) also examined growth and bone mineralization data to differentiate among age, sex and method of rearing and found a curvilinear response pattern regarding tibia ash weight and weekly BW data. During calcium deficiency both mineral and organic matrix are reduced whereas the femur of hens fed a phosphorus deficient diet showed reduction in bone mineral content (ash) but not in organic matter or in bone volume (Garlich *et al*., 1982). Hurwitz (1964) found that the percentage (%) ash of the femur was neither influenced by dietary calcium nor egg production. However, the femur calcium content was significantly influenced by both dietary calcium and egg production.

Zhang & Coon (1997) found that the coefficient of variation (CV) for bone ash as a percentage of fat-free dry matter (FFDM) and bone density, were smaller than that of bone breaking strength and bone ash concentration. Their results indicated that bone ash weight was highly correlated with fat-free dry matter (FFDM), suggesting that bone ash as a percentage of FFDM (bone ash/FFDM) may not provide much information about bone. Garlich *et al*. (1982), as well as Cheng & Coon (1990b) reported that bone ash concentration is much more sensitive to different dietary Ca levels than bone ash as a percentage of FFDM (bone ash/FFDM). Zhang & Coon (1997) suggested that the relatively constant bone ash as a percentage of fat-free dry matter and the high correlation between bone ash and FFDM support the findings of Cheng and Coon (1990a) that the ash and the non-ash components of the bone are being mobilized or replenished together, in such a way that their ratio in the bone which is reflected by bone ash as a percentage of FFDM remains relatively unchanged.

In another opinion, Crenshaw *et al*. (1981a) suggested that bone strength and stress is a more sensitive indicator of bone mineralization than percentage bone ash. Orban & Roland (1990) found that the percentage tibia bone was negatively correlated with eggshell characteristics such as specific gravity, shell weight and percentage shell and suggested that an improvement in shell quality resulted in a reduction of tibia strength and percentage bone.

2.9.2 Bone breaking strength

Crenshaw *et al*. (1981b) illustrated that wet bones withstand less ultimate force than dry bones and suggested that an increase in bone mineralization resulted in the maximum stress and bending moment of bones. Certain biological factors such as dietary nutrients and age affect "bone breaking strength". The responses of mechanical properties to biological factors are different and could be used to describe changes in the bone matrix (Crenshaw *et al*., 1981b). According to Crenshaw *et al*. (1981b) does the lack of a standardized test procedure results in considerable variation of bone strength and suggested that the following definitions are to be used for the specific description of bone strength namely:

(i) Bending moment is a measure of the amount of force withstood by the bone, whereas stress is a measure of force per unit area of bone
(ii) Bone stress allows comparisons to be made between bones that differ in size and shape
(iii) Bone strain is a measure of the amount of bending per unit of length that occurs as the bone is tested.

Park *et al*. (2003) examined the effect of storage condition on bone breaking strength and bone ash during different egg production stages and suggested that breaking strength of tibias held in refrigerated storage was significantly stronger than those held in frozen storage, however the percentage bone ash was unaffected by the storage condition. Kim *et al*. (2004) found that bone breaking strength was greatly influenced by bone preparation methods and that breaking strength of fresh bones was significant higher than that of dry or fat-free bones. However, they reported no significant differences in fresh weight, bone volume, dried weight, or ash concentration among the different bone preparations, which are in accordance with the results of Park *et al*. (2003). Schreiweis *et al*. (2003) reported that the breaking strength of fresh prepared bones was positively correlated with bone ash weight. The results of Schreiweis *et al*. (2003) also indicated that BMD and BMC are well correlated with each other, as well as with other invasive parameters such as bone breaking strength and bone ash weight. However, they found no correlations between BMD, BMC and eggshell characteristics. The results of Schreiweis *et al*. (2003) suggested that bone densitometry could be used in live birds to detect bone integrity with similar precision as other tests. Fleming *et al*. (1998b) recorded little change in bone breaking strength of the tibia and humeri between 15 and 25 weeks of age, but suggested that the breaking strength declined markedly between Weeks 25 and 50. The major decrease of bone strength between Week 25 and 50 of age implies a considerable loss of structural bone, which is mainly cortical bone in the midshaft region of the tibia.

Cheng & Coon (1990b) found that measurements of bone breaking strength were good indicators of bone mineral reserves and that bone density expressed as the ratio of ash weight to bone volume are the most accurate predictor of bone strength. The results of Yalçin *et al*. (1998b) suggested a significant positive correlation between tibia strength and radiographic density. Fleming *et al*. (1994) reported a positive correlation between bone strength and bone weight, as well as significant positive correlations between body weight, bone weight, bone length and bone width. Yalçin *et al*. (1998b) reported a non-significant correlation between body weight and tibia breaking strength, which agrees with the results of Wilson (1991) who suggested that body weight, could not be used to predict bone breaking strength of caged layers. Rowland *et al*. (1968) reported significant differences in tibia breaking strength of caged layers fed rations deficient and adequate in calcium and suggested that tibia ash values were not affected by dietary Ca-levels. In another opinion, Yalçin *et al*. (1998b) suggested that tibia breaking strength is related to bone weight, length and radiographic density. The inconsistencies in literature regarding the different bone measurement procedures suggest that the correlations between these measurements need further investigation and clarification.

2.10 Egg characteristics

The avian egg is a biological container in which the organic and inorganic materials required for propagation of the specie is contained. The eggshell is a unique structure that nature had developed as package material. The eggshell must satisfy several conflicting demands. In one case, the shell must be strong enough to prevent it from being crushed, but yet must not be too strong to prevent the hatchling from breaking out of the egg at the end of the incubation period (Hamilton, 1982).

Roberts & Leary (2000) suggested that internal and external egg quality are influenced by many factors such as, strain, age, nutrition, disease, management practices, water quality, housing conditions, temperature and stress. Many other factors are associated with eggshell breakage such as the design of the cage systems and cage floors, type of material used to manufacture the cages, frequency of daily egg collection and the frequency and quality of maintenance on egg handling equipment (Hamilton, 1982). Ahmad *et al*. (2003) suggested that the level and source of dietary calcium are two of the primary macro factors involved in eggshell formation and quality. The ability of hens to produce quality shells depends largely on the availability of calcium from the diet and skeletal reserves. Eggshell breakage is related to shell quality and the term shell quality is frequently used as a synonym for shell strength. Shell strength denotes the ability of eggshells to withstand externally applied forces without cracking or breaking (Hamilton, 1982).

2.11 Egg size and composition

The size and composition of eggs are dependent upon the nutritional status, body weight, age and genotype of the hen (Etches, 1996). Etches (1996) reported that the heritability of egg weight varied between r^2=0.45 and r^2=0.85. The genetic control of egg weight is highly correlated with body weight and as such selection for increased egg weight tends to result in larger hens and *vice versa*. Egg weight increases significantly with age as a result of increased yolk, albumen and shell weight, although these increases are not proportional (Kul & Seker, 2004). Kul & Seker (2004) suggested that the yolk and shell ratios changed opposite to the albumen ratio and albumen index. The increase in $CaCO_3$ secretion as the hen ages is insufficient to overcome the simultaneous increase in egg size and consequently shell thickness decline (Etches, 1996).

2.12 Macrostructure of eggs

Rose (1997) stated that the complex structure of an egg is distinguished by four different main components namely; yolk, albumen, shell membranes and shell. The egg of the domestic fowl contains approximately 64% albumen, 27% yolk and 9% shell. The chalazae (0.25% of total egg weight) and shell membranes (0.75% of total egg weight) are usually included in the albumen and shell weight respectively (Rose, 1997).

It's been suggested that the external and internal quality traits of eggs in hens (Hurnik *et al*., 1978; Nordstrom & Ousterhout, 1982), as well as in quails (Peebles & Marks, 1991) had a significant effect on the hatchability of eggs, as well as body weight and development of the hatchling. Kul & Seker (2004) reported a positive correlation between shell weight and the internal egg qualities such as albumen height, albumen weight, yolk diameter, yolk height and yolk weight, whereas a negative correlation was found between shell weight and albumen content. Internal as well as external egg qualities influence the final product reaching the consumer and have an enormous financial implication for the commercial egg producer.

2.12.1 The yolk

Yolk consists of a suspension of particles in a protein solution and is surrounded by a transparent vitelline membrane. The yolk membrane is made up of four concentric layers, of which the outer two are derived from the oviduct and the inner two are products of the ovary (Etches, 1996). Vittelin is phosphorus containing protein which complexes with lipids to form lipovitellins (Rose, 1997). The yolk of eggs from most domestic poultry contains about 49% water, 16% protein and 33% fat. Etches (1996) stated that egg yolk contains about 25 mg Ca/kg DM, 102 mg P/kg DM and 27 IU of vitamin D. Calcium, bound with very-low-density lipoprotein (VLDL) and vitellogenin during synthesis in the liver and is absorbed by the follicles for incorporation into the yolk. Two-thirds of the fat in the yolk are present as triglycerides, while phospholipids and cholesterol represents 30% and 5% respectively (Rose, 1997).

2.12.2 The albumen

Albumen is a heterogeneous mixture of more than forty different proteins, whereas ovomucin accounts for about 75% of the total protein content (Etches, 1996; Rose, 1997). The outer- and inner thin layers of albumen contain a higher proportion of water-soluble proteins and surrounded the middle layer of thick albumen. The moisture content of albumen varies from a maximum of 89% in the outer thin layer to a minimum of 86% in the inner thin layer (Etches, 1996). The albumen contains only traces of lipids and approximately one third of the mineral content of the egg. Etches (1996) illustrated that egg albumen contain about 4 mg Ca/kg DM and 8 mg P/kg DM.

2.12.3 The shell

The shell is composed of several layers that function together as a single unit to define the boundary of embryonic growth, serve as the embryonic lung during development and impede the entry of microorganisms into the egg. Shell is composed of 98% calcium carbonate ($CaCO_3$) crystals and 2% protein (Rose, 1997). Butcher & Miles (2004) illustrated that the average eggshell contains about 0.3% phosphorus, 0.32% magnesium and traces of sodium, potassium, zinc, manganese, iron and copper. Rose (1997) reported that shell thickness varies from 0.13 mm in quail eggs to 0.45 mm in turkey eggs. The inner shell membrane is in immediate contact with the albumen of the unfertile egg, while the outer shell membrane is incorporated into the shell to form a web of intermeshing fibres, lying parallel to the surface of the egg (Etches, 1996).

Calcium mobilized from bones and supplied by the diet are sequestered by the shell gland and secreted into the lumen fluid for eggshell formation (Etches, 1996). Calcium ions (Ca^{2+}) are transported from the blood across the secretory cells of the tubular glands. The ability of the shell gland to accumulate Ca^{2+} is remarkable and a supersaturated concentration of Ca^{2+} is maintained in the lumen fluid throughout the period of calcification. Carbonic anhydrase, found in the shell gland, catalyzes the formation of carbonate ions (CO_3^{2-}) which precipitate with calcium to give calcium carbonate crystals (Klasing, 1998) to form the eggshell. In the process of forming CO_3^{2-}, a hydrogen ion (H^{1+}) is generated and must be buffered by local and systemic buffering systems. The amount of Ca^{2+} in the blood is sufficient to sustain shell accretion for less than 20 minutes, whereas the calcification process requires about 15 - 20 hours. The drain on plasma Ca^{2+} causes the release of PTH and the activation of 1,25-$(OH)_2D_3$. These hormones continue to maximize intestinal calcium absorption and cause the mobilization of MB. Mobilization of calcium also results in the mobilization of phosphorus from the skeleton that needs to be excreted.

2.13 Methods of determining shell quality

Each method used for the determination of eggshell quality has its own advantages and disadvantages. Frank *et al*. (1964) found that various measurements of shell quality are well correlated with each other and presumably with intrinsic shell quality. Strong (1989) concluded that the use of egg specific gravity is non-destructive and requires only one measurement on each egg, but is subject to environmental and operator error, whereas percentage eggshell is a destructive technique, that requires the breaking of eggs and need two measurements, but is less subject to environmental deviations. Examples of non-destructive, indirect methods are specific gravity, nondestructive deformation, beta backscatter and egg output, while resistance to impact and quasi-static compression fracture force are direct methods (Hamilton, 1982). Other variables associated with shell strength are egg weight, length, diameter, shell weight and shell thickness which are measured directly. Kul & Seker (2004) reported that it is possible to use egg weight in determining shell qualities such as the shell weight, shell thickness and the shell ratio in quail eggs.

2.14 Shell strength

Rose (1997) speculated that approximately 7% of all eggs have some degree of shell damage before they reach the consumer, whereas the work of Etches (1996) suggested that the amount of shells that might be cracked or broken could be as high as 12% in commercial practice. Egg losses due to cracked or broken shells are an economic loss for the producers and accumulate in millions of dollars each year in the U.S.A. Roland (1988) reported that the total eggs cracked or lost in the United States of America, prior to reaching their final destination ranged from 13 – 20%, with a dollar value between US. $1.32 and $2.00/bird/year. Roberts & Leary (2000), reported that losses to the Australian egg industry as the result of problems with egg and eggshell quality have been estimated to be in excess of ten million dollars (Aus $ 10 million) annually. Hamilton *et al*. (1979) reported that an average of 7 - 8% of the total eggs laid get broken from collection until they reach the consumer, compared to the 1.5 – 2.0% when all egg handling was done by hand (Gleares, 1979).

An eggshell breaks when the strength of the shell is less than the strength of the insult to which it is exposed (Carter, 1970). Although this is an obvious statement, it is important that shell breakage is dependant on both shell strength as well as the magnitude of the insult. Therefore producers need to minimize unnecessary shell breakages due to labour and equipment insults as a precautionary measurement.

2.15 Factors affecting shell strength

Etches (1996) suggested that eggshell strength is not derived equally from all shell layers. The mamillary layer contributes little to the stiffness of the shell, whereas the lower regions of the palisade layer contribute about 50% of the complete shell strength. The remaining 50% is derived from the upper regions of the palisade layer (in which the $CaCO_3$ crystals are arranged in a compact and vertical orientation) and from the cuticle.

Hamilton *et al*. (1979) suggested the existence of a curvilinear relationship between eggshell strength and breakage. The structural strength of the eggshell is dependent on the shape, size, thickness and distribution of shell over the egg (Hamilton, (1982). Egg size and shape change on a day to day basis as the hen matures and shell thickness varies within the egg, with the shell being the thickest at the small end, thinnest at the equator and intermediate at the large (blunt) end (Hamilton, 1982). It is not only shell thickness that varies within an eggshell, but also the density, crystal structure, porosity, surface roughness, hardness and chemical composition thereof. The complex interrelationship between material and structural strength hinders the determination of whether a thin shell layer with high material strength properties may be stronger than a thick shell layer of low material strength (Hamilton, 1982). However, the work of Rose (1997) suggested that thicker eggshells as well as eggs that have a large diameter are stronger than thin shell eggs. Rose (1997) suggested that shell thickness and egg shape explain only about 75% of the variation that exists in shell breaking strength.

Photoperiod as well as mineral and vitamin deficiencies and/or imbalances also influence shell strength.

2.15.1 Temperature

Temperature plays an important role in the nutrition of poultry. Environmental temperatures above the upper threshold limit will decrease feed intake, with a consequent decrease in production. Butcher & Miles (2004) stated that this inadequate feed consumption is one of the factors contributing to poorer eggshell quality in regions with high environmental temperatures. Keshavarz (2003a) reported that shell quality problems become particularly serious during the high environmental temperatures (summer months) and during the late stages of the egg production cycle. The hypothesis of Vo *et al*. (1978) that limestone solubility decreases due to the dramatic increase in water consumption associated with high temperatures are supported by the work of Van der Klis (1993) who found that an increased water to feed ratio resulted in a diluting effect on the digestive system of the bird. Marangos (2004) suggested that the increase in respiration rate resulted in hyperventilation, with a consequent decrease in blood carbon dioxide (CO_2) which could reduce shell thickness during high environmental temperatures. The laying hen could resist the rise in body temperature during short periods of heat stress by panting. However, during panting, the acid to base (acid:base) balance in the blood is changed (Butcher & Miles, 2004). Using sodium bicarbonate rather than salt as the source of sodium improved the acid:base balance as well as shell breaking strength and shell thickness (Marangos, 2004). De Andrade *et al*. (1977) and Tanor *et al*. (1984) reported that pullets were unable to maintain shell quality under high temperatures even after dietary manipulation resulted in increased calcium consumption.

2.15.2 Dietary calcium levels

It is known that inadequate dietary calcium causes demineralization of bone, low serum calcium levels and subsequently low egg production with higher thin-shelled eggs and egg breakages, while excess dietary calcium may cause reduced egg weight, egg production and extra feed consumption (Ahmad *et al*., 2003). In contrast, Keshavarz (2003b) reported that body weight, egg production, egg weight, egg output (egg weight multiplied by number of eggs), feed intake, feed conversion and egg grades were not influenced by dietary calcium levels or the source of vitamin D_3. In another study, Keshavarz & Nakajima (1993) reported that calcium retention was reduced with an increase in dietary calcium intake which is consistent with the report of Rao & Roland (1989).

Hurwitz & Bar (1967) and Grunder *et al*. (1980) suggested that certain differences in calcium metabolism contributed to the differences in shell weight and strength and that the major difference in calcium metabolism between hens laying thick-shelled (THK) and thin-shelled (THN) eggs was due to calcium retention. Clunies *et al*. (1992) reported significant differences in shell weight as well as calcium content of the eggshells between THK and THN egg laying strains. Clunies *et al*. (1992) found no difference in feed intake between the two strains of hens and concluded that differences in shell weight and shell calcium content were primarily due to the efficiency of calcium retention between the strains which agrees with the results of Hurwitz & Bar (1967) and Grunder *et al*. (1980).

It is known that the skeleton is the only major alternative source of available calcium to the hen during shell formation, suggesting that bone calcium is an important determinant of the rate of shell secretion and subsequent shell quality. It is also known that the mobilization of skeletal calcium for eggshell formation increases as the dietary supply of the calcium decreases, due to an improvement in Ca-absorption. Farmer *et al*. (1986) reported that skeletal calcium utilization is directly related to calcium intake and less calcium is deposited onto eggshells if the need for skeletal calcium increased. However, the extent to which calcium is mobilized from the skeleton when hens are fed adequate Ca-levels is still being debated, whereas some researchers indicate that adequate dietary calcium will results in no additional utilization of skeletal calcium, while others indicated that 30 to 40% of eggshell calcium is supplied by the skeleton, even if dietary calcium levels are adequate (Orban & Roland, 1990).

Clunies *et al*. (1992) found that calcium retention increased on shell forming (SF) days compared to non-shell forming (NSF) days due to an increased efficiency of calcium retention. The results of Clunies *et al*. (1992) showed that THK hens retained significantly more Ca on NSF days presumably to replenish bone mineral used in the formation of the previous eggshell and suggested that the increased repletion of bone minerals in NSF periods could be indicative of an increased utilization of Ca-reserves during shell formation. Hens in a negative Ca-balance produced heavier shells because of an increased bone mineral mobilization as indicated by the phosphorus balance, while the ability of hens in a positive Ca-balance to produce thick shells are governed by the efficiency of Ca-retention (Clunies *et al*., 1992). After Orban & Roland (1990) observed an inverse relationship between specific gravity and tibia strength, indicating that calcium was drawn from the bones during calcification of eggs, they concluded that the utilization of skeletal calcium for shell formation is a natural phenomenon of laying hens, independent of dietary calcium. However, they suggested that the magnitude to which skeletal calcium is used could be influenced by the dietary and intestinal calcium content.

2.15.3 Time of calcium intake

Mongin & Sauveur (1974) observed that higher calcium consumption occurs during the early morning hours and late in the afternoon, primarily on days concurrent with ovulation and oviposition. The time of Ca-intake is important due to the inability of hens to maintain adequate calcium reserves in the small intestine during shell formation (Scott *et al*., 1971; Lennards *et al*., 1981). The period of greatest calcium deficiency in the GIT of laying hens is between midnight and 04:00 when eggshell calcification occurs at a rapid rate (Ahmad & Balander, 2003). This phase of a daily egg cycle is characterized by the mobilization of calcium and phosphorus from bones with a consequent increase in blood phosphorus levels. Farmer *et al*. (1983a) revealed that broiler breeder hens, unlike layer hens, were unable to control calcium movement at a uniform rate from the crop into the digestive system during the shell calcification process. Farmer *et al*. (1983b) suggested that broiler breeder hens, fed in the afternoon exhibited a significant improvement in eggshell quality as measured by specific gravity and eggshell weight. Farmer *et al*. (1983b) hypothesized that the difference in pattern of calcium and dry matter flow in hens fed during the afternoon, suggested that more calcium will be available during the active stages of eggshell calcification and that Ca^{2+} may be utilized directly *via* the blood for eggshell calcification without being deposited firstly in bones. Farmer *et al*. (1983b) concluded that supplying calcium for eggshell calcification by first utilizing the skeletal storage system might not be as efficient as supplying the calcium during the active stages of calcification.

2.15.4 Mineral interactions

Ahmad & Balander (2003) illustrated that the calcium to phosphorus ratio (Ca:P) of eggshells is approximately 100:1. Bar & Hurwitz (1984) reported that the interaction between Ca and P, influence eggshell quality and that excess dietary phosphorus resulted in a detrimental effect on shell quality. It is not clear whether excess phosphorus accumulating in the blood interfered with mobilization of skeletal reserves of calcium phosphate during shell formation, or whether there is a direct antagonistic effect of blood phosphorus on the calcification process (Ahmad & Balander, 2003). Hossain & Bertechini (1998) reported that shell thickness was reduced when available phosphorus was increased from 2.5 - 4.5 g/kg DM. Keshavarz (2003b) reported that the level of dietary non-phytate phosphorus (NPP) had no effect on egg production, feed conversion and specific gravity. However, Keshavarz (2003b) found that tibia ash, percentage shell and shell weight per unit surface area (SWUSA) were significantly lower in the presence of dietary NPP. Excess dietary phosphorus levels adversely affect manganese utilization, whereas high levels of calcium have no effect on manganese utilization in chicks (Wedekind & Baker, 1990). The report of Hossain & Bertechini (1998) indicated that egg production, egg weight and eggshell thickness increased ($P<0.05$) with dietary supplements of 50 and 70 mg manganese (Mn)/kg daily feed intake, regardless the level of available phosphorus (AvP), suggesting the non-existence of an interaction between these minerals. They concluded that the NRC (1994) recommendations of 20 mg Mn/kg daily feed intake are insufficient for optimum egg production and egg weight.

2.15.5 Shell thickness

Eggshell parameters such as shell thickness, shell weight per unit surface area (SWUSA) and percentage shell are in close relationship with shell breaking strength and shell stiffness, making it possible to predict eggshell strength indirectly (Narushin *et al*., 2004). Al-Batshan *et al*. (1994) reported that shell weight increased but percentage shell and shell thickness decreased during the first production cycle but improved after an induced molting period. The results of Strong (1989) indicated that shell breaking strength, shell thickness and SWUSA were not significantly correlated with the percentage of cracks, representing unreliable indicators of egg breakage. Presenting another opinion, Narushin *et al*. (2004) suggested that non-destructive variables such as egg weight, egg volume and SWUSA are more accurate prediction techniques than shell thickness measurements.

2.15.6 Shell weight

Kul & Seker (2004) reported significant phenotypic correlations between egg weight, shell weight and average shell thickness, whereas egg weight had an indirect relationship with eggshell quality. They also reported a negative correlation between egg shape index and shell weight. Narushin *et al*. (2004) reported that maximum shell deformation was highly correlated with the shell fracture force and that shell weight had the lowest correlation with shell strength characteristics. Orban & Roland (1990) reported that shell quality criteria such as shell weight, shell strength, percentage shell and specific gravity were highly correlated with each other and suggested that one quality criterium could be used to calculate another. Orban & Roland (1990) also found that egg weight was highly correlated with shell weight and suggested that the inverse relationship between egg weight and percentage shell indicated that egg weight was influenced by shell weight (g) and not necessarily percentage (%) shell. Butcher & Miles (2004) suggested that eggshell quality could be improved by controlling the change in body weight of ageing hens and consequently controlling the egg weights at the same time.

2.15.7 Specific gravity

Holder & Bradford (1979) reported a negative curvilinear relationship between specific gravity or percentage shell and eggshell breakage. In contrast, Strong (1989) concluded that either egg specific gravity or percentage eggshell could be used with confidence to estimate the shell quality from commercial laying hens. In another opinion, Orban & Roland (1990) suggested that egg production are positively correlated with specific gravity but negatively correlated with percentage bone, suggesting that an improvement in shell quality resulted in a reduction of tibia strength and percentage bone. Orban & Roland (1990) concluded that the percentage tibia bone was negatively correlated with specific gravity, shell weight and percentage shell, whereas body weight was positively correlated with egg weight, shell weight and tibia weight.

From literature it is evident that various factors could contribute to bone and eggshell quality characteristics. It further seems that until a proven genetic solution for eggshell and bone problems are found, the best suitable way of preventing these problems would be by optimizing nutritional and management practices. It is also clear from literature that the inconsistencies regarding the ideal particle size and distribution ratio of calcium supplements still exists and needs therefore further investigation and clarification.

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CHAPTER 3

INFLUENCE OF LIMESTONE PARTICLE SIZE ON THE *IN VIVO* AND *IN VITRO* SOLUBILITY OF LIMESTONE

3.1 Introduction

Larbier & Leclercq (1994) indicated that dietary calcium is utilized by laying hens with an efficiency of between 50% and 60%. Because of the poor efficiency of Ca utilization, the importance of available Ca for absorption during bone mineralization and shell formation cannot be overstated. Jacob *et al*. (2003) stated that particle size of the calcium supplement is one of the factors that influence the availability of ionic calcium (Ca^{2+}). Various authors indicated that large particle size calcium supplements with a longer intestinal retention time and *in vivo* solubility resulted in an increased tibia breaking strength and percentage bone ash (Guinotte & Nys, 1991; Fleming *et al*., 1998; Fleming *et al*., 2003), increased egg weight (Guinotte & Nys, 1991) and an increase in eggshell quality (Scott *et al*., 1971; Brister *et al*. 1981).

Various studies have shown that many factors contribute to calcium solubility of limestone. These factors include calcium source, pH of the gastrointestinal tract, retention time in the intestines, particle size, and the influence of other nutrients (Ajakaiye *et al*., 1997). In South Africa a specific calcitic limestone source is mainly used in poultry diets. No information regarding the solubility of this limestone source is available. The techniques used in South Africa to determine the *in vitro* solubility of limestone is mostly directed at the effectiveness of limestone on soil pH and is therefore not applicable in the feed manufacturing sector. Therefore an urgent need arises to determine the *in vivo* and *in vitro* solubility of this specific calcitic limestone source.

Rao & Roland (1989) and Zhang & Coon (1997) are of the opinion that particle size and quantity of the calcium supplement have an influence on the *in vivo* solubilization by the hen. Laying hens are able to solubilize a greater percentage of intake calcium from large particles of calcium supplements than from small particles of calcium and this apparently stems from the greater retention time of the large particles in the gizzard (Rao & Roland, 1989; Zhang & Coon, 1997; Jacob *et al*., 2003). Because of the interaction between particle size and the solubility of the calcium source, the need arises in South Africa to test the solubility of different particle sizes of limestone as well as the effect of the ratio distribution of small and large particles limestone on solubility.

Cheng & Coon (1990a) clearly illustrated that the *in vitro* solubility of small (<0.15 mm) particles of limestone were higher than that of large particles (3.36 mm). By studying the *in vitro* solubility of two different sources of limestone, Cheng & Coon (1990a) reported that the *in vitro* limestone solubility varied between 17.9 to 21.3% for the small particles and 8.6 to 10.1% for the large particles. Accordingly, Ajakaiye *et al.* (1997) reported that $CaCO_3$ particles smaller than 0.50 mm and larger than 0.60 mm resulted in an *in vitro* solubility of 82.3% and 71.3% respectively. Rao & Roland (1989) and Zhang & Coon (1997) reported that large particles (2.0 – 5.0 mm) $CaCO_3$ with a high *in vivo* (84.8%) solubility were less soluble under *in vitro* (29.8%) conditions when comparisons were made within the same source. The report of Zhang & Coon (1997) suggested that the *in vivo* solubility of the limestone particles tended to be negatively correlated with the *in vitro* solubility due to the difference in retention time of the particles in the gastrointestinal tract (GIT). Because smaller limestone particles have a relatively quicker rate of passage through the GIT than larger particles of limestone, the *in vivo* solubility of small limestone particles is less than expected, compared to those results from *in vitro* tests. The opposite reasoning applies to the *in vivo* solubility of larger limestone particles.

From the literature, it is clearly illustrated that particle size and distribution of small and large particle sizes are both factors with significant effects on the solubility and the relative biological value of limestone. However, the solubility of a specific limestone source with different particle sizes and distribution of particle sizes used in South Africa is still unknown. The aim of the present study was firstly to determine the *in vivo* and *in vitro* solubility of a specific limestone source differing in particle size. Secondly, to determine the *in vivo* and *in vitro* solubility of different ratios of small and large particles from the same limestone source.

3.2 Materials and Methods

To investigate the influence of particle size and distribution ratios of particles on limestone solubility, two studies were concurrently conducted and the same experimental protocol was implemented.

3.2.1 Limestone source

The limestone used for the determination of solubility in both the studies was obtained from the same company, namely Agri Lime (Pty. Ltd.), situated between the towns of Rustenburg and Thabizimbi in the North West Province of South Africa. The geographical latitude and longitude coordinates of mine (limestone source), are respectively 24.089°S and 27.087°E. The calcium content of the limestone is according to the suppliers 360 g Ca/kg DM. The complete chemical analysis of the micro element content of this limestone source, according to the suppliers, is illustrated in Table 3.1. Limestone with different particle sizes was washed with deionized water to remove dust-size particles and then dried overnight at 105°C. After drying, the limestone was sieved to obtain the required particle sizes. The limestone was graded according to the diameter size of the particles as small (0 – 1.0 mm), medium (1.0 – 2.0 mm) and large (2.0 – 3.8 mm).

Table 3.1 The chemical analysis of the micro-element concentration of the limestone source (DM-basis)

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

3.2.1.1 Particle size

In the first study, solubility of the three different limestone particle sizes as described in paragraph 3.2 was determined. The three particle sizes used can be observed in Figure 3.1.

Figure 3.1 Different limestone particle sizes; small, medium and large

3.2.1.2 Distribution of particle sizes

Limestone with a particle size of 0 – 1.0 mm was mixed with that of a 2.0 – 3.8 mm particle size to obtain five different ratios of 0, 25, 50, 75 and 100% small or large particles (Figure 3.2).

Figure 3.2 Distribution ratios of small to large (small:large) limestone particles

The ratios of small and large particles of limestone were formulated in 1000 g batches for each treatment. The small and large particles were thoroughly mixed by hand in 10 liter plastic containers. To prevent the segregation of small and large particles in the distribution study, special care was taken in remixing the particles before each sample was taken during the *in vivo* and *in vitro* solubility studies.

3.2.2 *In vivo* solubility

The procedures described by Rao & Roland (1989) were used in both studies to determine the *in vivo* limestone solubility. Limestone was dried overnight at 105°C before representative samples for intubation was accurately weighed to the third decimal. A 10 g limestone sample per hen was used for intubation, which supplemented the calcium content (0.38% Ca) of the basal diet in Table 3.2 with 3.6 g Ca/kg, resulting in a complete diet (as is) with a 3.98% Ca-content.

Table 3.2 Physical and calculated chemical composition of the basal diet (as is %)

Physical composition (%)		**Calculated chemical composition (%)**	
Yellow maize	66.46	Protein	18.80
Prime gluten (65%)	1.52	Fat	4.60
Wheat bran	2.00	Ash	4.24
Fullfat Soya (36%)	5.53	NDF[1]	11.06
Soya oilcake (47%)	8.01	ADF[2]	5.47
Sunflower oilcake (37%)	11.06	Moisture	10.96
Fishmeal (65%)	3.61	Fibre	4.15
Limestone	0	Calcium	0.38
Mono calcium phosphate	0.82	Phosphorus	0.62
Fine Salt	0.41	AvP[3]	0.32
Phytase enzyme[6]	0.07	Ca:AvP[4]	1.19
Bicarbonate of soda	0.05	Chlorine	0.33
Choline powder[7]	0.01	Sodium	0.20
DL-Methionine[8]	0.02	Potassium	0.63
Premix E[9]	0.44	Magnesium	0.16
		AME[5] (MJ/kg)	12.71

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[1] Neutral detergent fibre

[2] Acid detergent fibre

[3] Available phosphorus

[4] Calcium: Available phosphorus ratio

[5] Apparent metabolizable energy (MJ/kg)

[6] Commercial phytase enzyme (Natuphos 500 High inclusion) with an enzyme concentration of 500 000 FTU/kg DM (FTU = Phytase Unit). Diet was formulated according to commercial laying standards to ensure a minimum inclusion level of 300 FTU in basal diet

[7] Choline chloride powder with a 60% choline and 16% chloride concentration. Diet was formulated for 0.01% choline. Choline chloride was not provided as a premix portian of diet.

[8] Methionine powder with a 99% concentration

[9] Commercial mineral/vitamin premix (Inclusion and levels of nutrients are confidential)

A plastic funnel attached to a length of polyvinyl tubing (Figure 3.3) was used to orally intubate the limestone direct into the crop. The length of the polyvinyl tube was 10 cm while the inside and outside diameter of the tube were 8 and 10 mm respectively.

Figure 3.3 Funnel and tubing used during intubation of limestone

One hundred and ninety eight, 37 weeks old Lohmann-Silver hens, individually caged in metabolic cages were randomly allocated into six treatments (n=33/treatment) for the determination of *in vivo* limestone solubility. The hens were provided with individual water nipples, feed trays, perches and excreta trays (Figure 3.4). All data were collected on an individual bird basis and each bird was considered as a replicate of the treatment.

Figure 3.4 Individual cages with hens

On the day prior to the experiment, feed was withdrawn from all the hens at 16:00 to empty the gastrointestinal tract (GIT). On the first day of the experiment, hens (n=22) that laid an egg between 07:00 and 09:00 (first oviposition) were used in the trial. At the time of first oviposition, five hens per treatment were randomly selected, sacrificed by cervical dislocation and the GIT content removed and stored individually in a cooler box at 5°C. The digesta was stored for the determination of insoluble limestone at the laboratory. At 09:00, all the hens that laid an egg were intubated with the pre-weighed 10 g limestone sample (Figure 3.5). The limestone was gradually poured into the funnel and washed down with 30 ml deionized water. The duration of intubation was approximately 2 – 4 minutes per hen, depending on the particle size of the limestone, which is in accordance with the 2 – 5 minutes time period of Rao & Roland (1989). After intubation, the hens were supplied with the basal diet as described in Table 3.2, which did not contain any supplemental limestone. Pre-weighed excreta trays were used to collect the excreta from all the intubated hens from 09:00 after intubation (first oviposition) until 09:00 the next morning (second oviposition).

Figure 3.5 Intubation of limestone

On the second day of the experiment, those hens that laid an egg between 07:00 and 09:00 (second oviposition) were identified. Five hens per treatment with two consecutive ovipositions were identified and sacrificed by cervical dislocation. Their GIT contents were collected and stored in a cooler box at 5°C for the analysis of insoluble limestone. Limestone content of the entire GIT, including the ceca and colon was determined and no allowance was made for the determination of insoluble limestone in the different parts of the small and large intestine. Excreta of the sacrificed hens with two consecutive ovipositions were weighed (wet) and homogeneously mixed before a 40 g sample per hen was taken for the analysis of insoluble limestone.

After drying a 500 ml glass beaker overnight at 105°C, it was weighed accurately to the third decimal. The digesta of the complete GIT (Figure 3.6) was placed into the dry, pre-weighed beaker and 250 ml deionized water was added. The contents in the beaker were gently stirred until all lumps or aggregates had disintegrated. After the limestone particles settled down on the bottom of the beaker, approximately two-thirds of the supernatant was removed (Figure 3.7). The beaker was filled again with deionized water (250 ml) and the stirring process repeated. This decantation process continued until all particles except the limestone were washed out of the beaker (Figure 3.8). The remaining contents in the beaker were dried overnight at 105°C, before weighing. The same procedures have been used to determine the insoluble limestone in the 40 g excreta sample. The insoluble limestone (g) from the digestive tract and excreta was used to determine the solubility of limestone.

Figure 3.6 Collection of gastrointestinal tract content

Figure 3.7 Decantation of digesta

Figure 3.8 Intestinal limestone after decantation

Rao & Roland (1989) found no limestone particles in the GIT after a 17h fasting period. However, in the present study some limestone particles still remained in the GIT after the fasting period (17h). The GIT content of the hens (n=5/treatment) slaughtered at onset of the study was used to determine the mean particle weight remained in the GIT after fasting. The equation $[(A - B) - C / (A - B)] \times 100$ used by Rao & Roland (1989) for the calculation of percentage limestone solubilized in the digestive tract was altered to $[(A - B - D) - C / (A - B - D)] \times 100$, to compensate for the limestone particles that remained in the GIT after fasting, where;

A	Limestone intubated (g DM)
B	Limestone in the digestive system after second oviposition (g DM)
C	Insoluble limestone in the excreta (g DM)
D	Limestone in the digestive system after the 17 hour fasting period (g DM)

3.2.3 *In vitro* solubility

The procedures described by Zhang & Coon (1997) were used to determine the *in vitro* solubility of limestone particles. A 400 ml Erlenmeyer glass flask was dried overnight at 105°C and weighed accurately to the third decimal. Two hundred milliliter of 0.2N hydrochloric acid (HCl) solution was poured into the pre-weighed flasks (Figure 3.9) to act as solvent for the limestone particles. The flasks with the HCl solution were warmed to 42°C for approximately 15 minutes, in a water bath (Figure 3.10), which have an internal vibrating stirrer that oscillates at 50 Hz. A pre-weighed 2.0 g limestone sample was then poured into the flask with the solution.

Figure 3.9 Rack with Erlenmeyer flasks

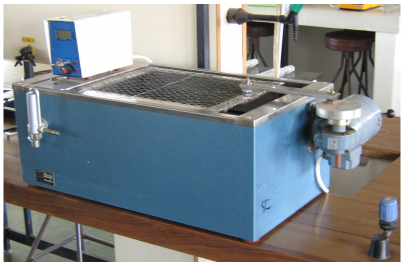

Figure 3.10 Water bath

Marked, Schleicher & Schull Nr. 589/2 ashless filter papers were dried overnight at 105°C before weighing. After allowing a 10 minute reaction time (Figure 3.11), the limestone was filtered onto the pre-weighed filter paper (Figure 3.12). The filter paper together with the undissolved (Figure 3.13) limestone was dried at 60^0C for 20 hours before weighing. Each treatment was replicated nineteen times for statistical analysis. The *in vitro* solubility of limestone particles were calculated according to Zhang & Coon (1997) as follows:

In vitro solubility (%) = (Paper and limestone sample – Paper and undisolved limestone sample after drying) x 100

Figure 3.11 Limestone before filtration

Figure 3.12 Filtration of solvent

Figure 3.13 Limestone particles after filtration

3.2.4 Statistical analysis

Significant effects in limestone solubility between particle sizes (Study 1) and distribution ratios (Study 2) of particles were estimated by means of a fully randomized one way ANOVA design. The PROC ANOVA procedures of the SAS program (SAS, 1999) were used to test for significant differences between the treatments. Tukey's studentized range (HSD) test was used to identify the differences between treatment means.

The description of the model used for PROC ANOVA analysis in the first and second study was:

$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$, Study 1: $i = 1, 2, 3; j = 1, 2, \ldots 5$

Study 2: $i = 1, 2, \ldots 5; j = 1, 2, \ldots 19$

Where: Y_{ij} = the dependant variable in the i-th trial with a j-th random error

μ = the overall population mean

τ_i = the i-th population treatment effect

ε_{ij} = the random error effect

The different, Y_{ij} dependant variables for both studies were:
In vivo limestone solubilility, *in vitro* limestone solubility, intestinal limestone content and excreta limestone content.

The i-th treatment effect (limestone particle size) during the first study was defined as:
$i_1 = <1.0$ mm, $i_2 = 1.0 - 2.0$ mm, $i_3 = 2.0 - 3.8$ mm.

The i-th treatment effect (distribution ratios of small and large particles limestone) during the second study was defined as:
i_1 = 100% small, i_2 = 75% small: 25% large, i_3 = 50% small: 50% large, i_4 = 25% small: 75% large and i_5 = 100% large.

Linear regression equations of limestone particle size on *in vivo* and *in vitro* solubility as well as intestinal and excreta limestone content were determined in both studies. A second degree polynomial regression was also fitted in both studies between the mean limestone particle size and intestinal and excreta limestone content.

The following linear regression model was used during both studies:

$Y_i = \beta_0 + \beta_1 X_i + \varepsilon_i$ Study 1: i= 1, 2, 3

Study 2: i = 1, 2, 3, 4, 5

Where: Y_i = the dependant variable in the i-th trial
β_0 = the intercept of the y-axis (where x = 0)
β_1 = the slope of the regression line
X_i = the independent variable in the i-th trial
ε_i = the random error term

The independent (X_i) variable in the i-th trial of the first study was mean limestone particle size (i_1 = 0.5 mm, i_2 = 1.5 mm, i_3 = 2.9 mm) and during the second study was the percentage (%) large particles limestone in the distribution ratio (i_1 = 0%, i_2 = 25%, i_3 = 50%, i_4 = 75%, i_5 = 100%).

The following second degree polynomial regression model was used during both studies:

$Y_i = \beta_0 + \beta_1 X_i + \beta_2 X^2_i + \varepsilon_i$ Study 1: i= 1, 2, 3

Study 2: i = 1, 2, 3, 4, 5

Where: Y_i = the dependant variable in the i-th trial
β_0 = the intercept of the y-axis (where x = 0)
β_1 = the slope of the regression line due to X_i
β_2 = the slope of the regression line due to X^2_i
X_i = the independent variable in the i-th trial
X^2_i = the independent variable in the i-th trial squared
ε_i = the random error term

The independent (X_i) variable in the i-th trial of the first study was mean limestone particle size (i_1 = 0.5 mm, i_2 = 1.5 mm, i_3 = 2.9 mm) and in the second study was the percentage (%) large limestone particles in the distribution ratio (i_1 = 0%, i_2 = 25%, i_3 = 50%, i_4 = 75%, i_5 = 100%). The same independent variables (Xi) were squared and expressed as X^2_i.

The regression equations of limestone particle size and distribution ratios of particles were used to predict *in vivo* and *in vitro* solubilities of the former. These predicted values were used for the regression of *in vitro* onto *in vivo* limestone solubility and a regression equation was recorded for *in vitro* solubility that could predict *in vivo* limestone solubility.

3.3 Results and Discussion

3.3.1 Particle size

The results of the *in vivo* and *in vitro* limestone solubility studies with different particle sizes of limestone are presented in Table 3.3 and Figure 3.14. A significant (P=0.0095) increase in the *in vivo* solubility of limestone was observed with an increase in particle size. No significant (P>0.05) solubility differences were, however, observed between the medium and small as well as between the medium and large limestone particles. In contrast, the *in vitro* solubility of the limestone decreased significantly (P<0.0001) with a proportionate increase in particle size.

Table 3.3 The *in vivo* and *in vitro* solubility of different limestone particle sizes (Mean±s.e)

	Particle size (mm)			**Significance**	
Parameters	**0 - 1.0**	**1.0 - 2.0**	**2.0 - 3.8**	**P**	**CV^3(%)**
In vivo (%)	56.13±1.17^b	59.55±1.26^{ab}	63.42±1.65^a	0.0095	5.2
In vitro (%)	25.28±0.52^a	20.88±0.46^b	16.73±0.37^c	0.0001	9.4
GIT(g)^1	1.26±0.14^b	3.37±0.16^a	3.59±0.38^a	0.0001	20.7
EXC(g)^2	3.68±0.12^a	2.24±0.08^b	1.64±0.15^c	0.0001	10.9

[abc] Figures with different superscripts in rows differ significantly (P<0.05)
[1] Intestinal limestone at the end of 2nd oviposition in gram
[2] Limestone in excreta at the end of 2nd oviposition in gram
[3] Coefficient of variation

These results were in accordance with several researchers namely Guinotte & Nys (1991), Zhang & Coon (1997) and Jacob *et al*. (2003) who found an increased *in vivo* and decreased *in vitro* limestone solubility with an increase in limestone particle size. Rao & Roland (1989) also illustrated that the *in vivo* solubility of large (2.0 - 5.0 mm) particles limestone were significantly higher (84%) than the solubility of small (0.5 - 0.8 mm) particles limestone (54%). Various other researchers (Rao & Roland, 1990; Guinotte & Nys, 1991; Zhang & Coon, 1997) also reported that large (2.36 - 5.0 mm) particles limestone compared to small (0.075 - 0.8 mm) particles resulted in a higher *in vivo* solubility (80 - 96% *vs*. 54 - 91%).

Rao & Roland (1989, 1990) and Zhang & Coon (1997) are of opinion that the quantity of Ca intake has a significant influence on the percentage of Ca solubilized by the hen and suggested that the percentage *in vivo* solubility decreases with an increase in Ca-intake. Rao & Roland (1990) concluded that Ca-deficient hens consuming a diet containing 1.50% Ca, solubilized and retained significantly more Ca than hens fed a diet of 4.50% Ca (91% *vs.* 63%). In the present study, the limestone intubated to the hens had the same calcium content of 360 g Ca/kg, while the calcium content of the basal diet was 0.38 g Ca/kg. Hence, the physical and chemical composition of calcium supplied from the basal diet (Table 3.2) was the same for all treatments. Therefore variation in calcium intake from the basal diet was expected to be negligibly small and constant across treatments. The quantity of limestone intubated (10 g/hen) was also constant for all treatments. Therefore the results illustrated in Table 3.3 could neither be influenced by the dietary calcium content nor differences in calcium intake, but only by the difference in particle size of the limestone.

The *in vivo* and *in vitro* solubility of limestone with different particle sizes are graphically illustrated in Figure 3.14. The three particle sizes used to determine the *in vivo* and *in vitro* solubility are defined in paragraph 3.2. The normal distribution of the limestone particles in the three different treatments was considered to be random and therefore the mean value of each particle size was used to determine the regression equations between particle size and *in vivo* and *in vitro* solubility in Figure 3.14. An increase in limestone particle size resulted in an increased *in vivo* solubility ($y = 3.0197x + 54.767$) and a decreased *in vitro* solubility ($y = -3.5249x + 26.719$). Zhang & Coon (1997) and Jacob *et al.* (2003) reported that the *in vivo* and *in vitro* limestone solubility is dissimilarly related with particle size insofar as *in vivo* solubility increased and *in vitro* solubility decreased with large limestone particles. In the present study, the relationship between particle size and *in vivo* limestone solubility was moderate (r^2=0.54), while that of the *in vitro* study was higher (r^2=0.76). Some of the factors that might contribute to the difference in the prediction of accuracy (r^2) between *in vivo* and *in vitro* studies could be ascribed to the replications per treatment (*in vivo* n=5 *vs.* *in vitro* n=19), experimental errors or some biological factors such as the calcium balance in the hen.

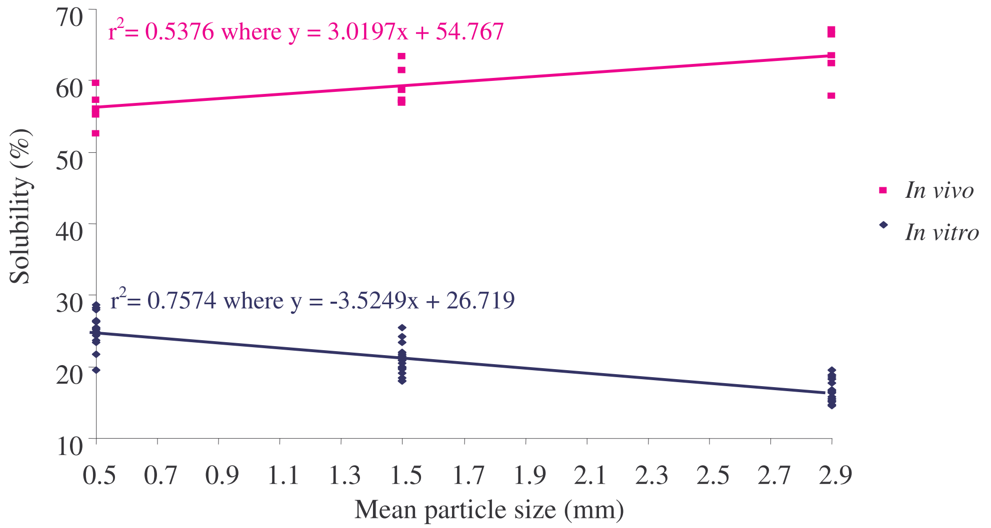

Figure 3.14 The *in vivo* and *in vitro* solubility of different limestone particle sizes

The linear regression equations in Figure 3.14 for *in vivo* limestone solubility (r^2=0.54) and *in vitro* limestone solubility (r^2=0.76) were used to predict limestone solubility of different particle sizes ranging from 0.1 to 3.8 mm. These predicted solubility values for the *in vivo* and *in vitro* techniques were used to determine the slope of the linear regression equation of *in vitro* onto *in vivo* limestone solubility in Figure 3.15.

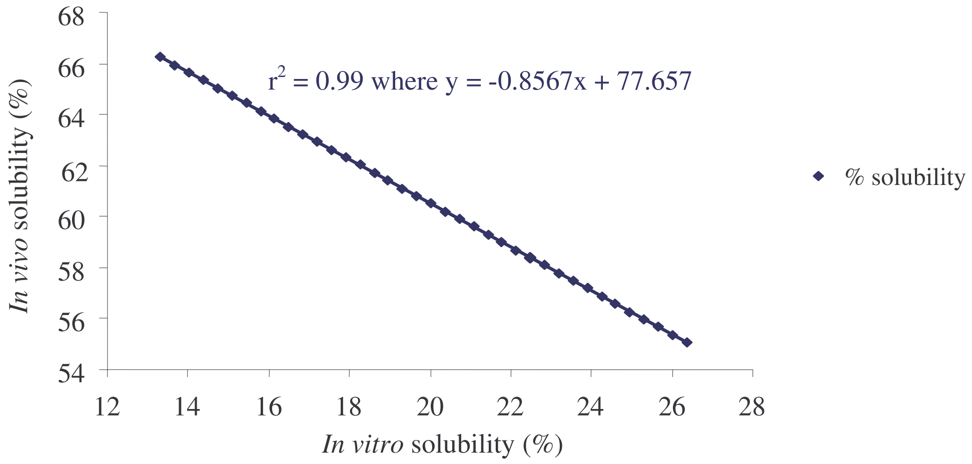

Figure 3.15 Linear regression between the predicted *in vitro* and *in vivo* solubility of limestone

The results of Figure 3.15 confirmed the findings of Zhang & Coon (1997) and Jacob *et al*. (2003) who suggested the existence of a negative correlation exists between the *in vivo* and *in vitro* solubility of limestone. The slope of the regression equation illustrated the negative relationship between the *in vivo* and *in vitro* techniques used for predicting limestone solubility. The proportion of explained variation (r^2=0.99) is extremely high as expected, due to the use of predicted solubility values using the linear regression equations in Figure 3.14. Therefore, the explained proportions of variation (r^2=0.54 and r^2=0.76) in Figure 3.14 should rather be used than that of Figure 3.15 to indicate the coefficient of determination. However, the aim was not to determine the proportion of explained variation between *in vivo* and *in vitro* solubility, but to illustrate the negative relationship between the two techniques using the slope of the regression equation. The linear regression formula in Figures 3.15 could be used to predict the *in vivo* solubility (r^2=0.54) of different particle sizes limestone if the *in vitro* solubility values are available. By substituting an *in vitro* solubility value of 20.88 in the regression equation of Figure 3.15 ($y = -0.8567x + 77.657$) a predicted *in vivo* solubility of 59.77% is calculated, which compared favourable with the mean solubility value in Table 3.3 (59.55%).

From the results in Table 3.3 and Figures 3.16 and 3.17 it further seems that larger limestone particle sizes (1.0 – 2.0 and 2.0 – 3.8 mm) resulted in a significantly ($P<0.0001$) higher intestinal limestone content. However, the results of excreta limestone content were contradictory to that of the intestinal limestone content. It therefore seems that the excreta limestone content decreased proportionally with an increase in limestone particle size. This increased excreta limestone content (3.68 g) of the birds fed small limestone particles (0 – 1.0 mm) was characterized by a decreased intestinal limestone (1.26 g), indicating that the retention time of the small particles was lower than that of large particles limestone (2.0 – 3.8 mm) (Figures 3.16 and 3.17). The results of the present study agrees with the work of Rao & Roland (1989), who found that none of the small limestone particles and 0.29 g of the large limestone particles remained in the digestive tract of the birds after the first day of intubation. The decreased retention time and/or increased passage rate of the small particle size limestone resulted in a lower *in vivo* solubility of small particles. Rao & Roland (1989) reported that total calcium in the excreta of small particle size limestone was 2.03 g Ca/kg DM while that of large limestone particles was 1.14 g Ca/kg DM, indicating that small particle size limestone had a higher rate of passage than large particles.

The greater retention time of larger particles in the gizzard allows the limestone to stay in an acidic environment for a longer period of time, increasing the opportunity to dissociate

$CaCO_3$ into Ca^{2+}, therefore resulting in a higher *in vivo* solubility of large particle size limestone and a consequent increased relative bioavailability (RBV) (Zhang & Coon, 1997; Jacob *et al*., 2003). The results of Cheng & Coon (1990a), Guinotte & Nys (1991), Guinotte *et al*. (1991) and Ajakaiye *et al*. (1997) on *in vitro* solubility of $CaCO_3$ indicated that the *in vitro* solubility was the highest (17.9 - 99.9% *vs*. 8.6 - 80.0%) when particles of the calcium supplement was small compared to large particles, irrespective of the origin of the Ca source. Guinotte *et al*. (1991) suggested that the surface area of large calcium particles was lower than that of small calcium particles, resulting in a reduced reactive surface of large calcium particles to HCl and hence the proportionate decrease *in vitro* solubility. However, the differences between *in vivo* and *in vitro* limestone solubility for the same particle size could be ascribed to the techniques used. By using a warm water bath with a oscillating stirrer, to simulate the GIT environment, no correction factors for the simulation of retention time and/or rate of passage as well as the physical contractions of the gizzard was allowed, contributing to the different results obtained by the two techniques. The physical contractions of the gizzard helped to reduce the size of limestone particles and secrete HCl in the same time and could not be simulated by the movement of the oscillating stirrer in the water bath. Therefore large limestone particles with a high *in vivo* solubility resulted in a low *in vitro* solubility.

As in Figure 3.14, the normal distribution of the limestone particle sizes in each treatment were considered to be random and therefore the mean particle size values was used to determine the regression equations between particle size and intestinal and excreta limestone content (Figures 3.16 and 3.17). The intestinal and excreta limestone content are graphically illustrated in Figure 3.16.

The linear regression equation (Figure 3.16) indicate a moderate (r^2=0.60) relationship between particle size and intestinal limestone content, while the relationship between particle size and excreta limestone content is higher (r^2=0.83). As mentioned earlier, larger particle size limestone resulted in a higher intestinal ($y = 0.9227x + 1.2314$) limestone content and a reduced excreta ($y = -0.8211x + 3.8584$) limestone content.

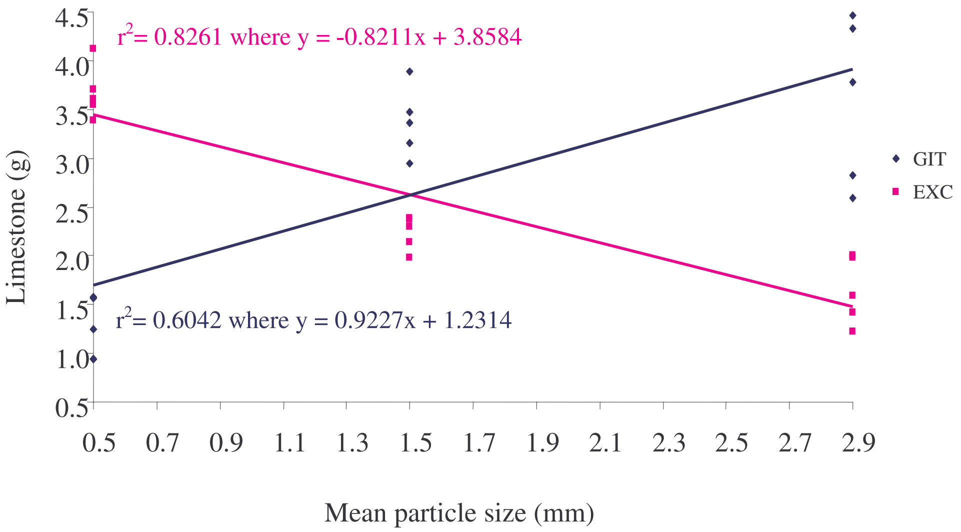

Figure 3.16 Linear regression between mean particle size and intestinal (GIT) and excreta (EXC) limestone content

In Figure 3.17 the intestinal and excreta limestone content resulted from different limestone particle sizes were fitted to a second degree polynomial regression. A second degree equation was chose due to the few replicates/treatment (n=5), only three particle sizes of limestone on the x-axis, and the variation of intestinal limestone content.

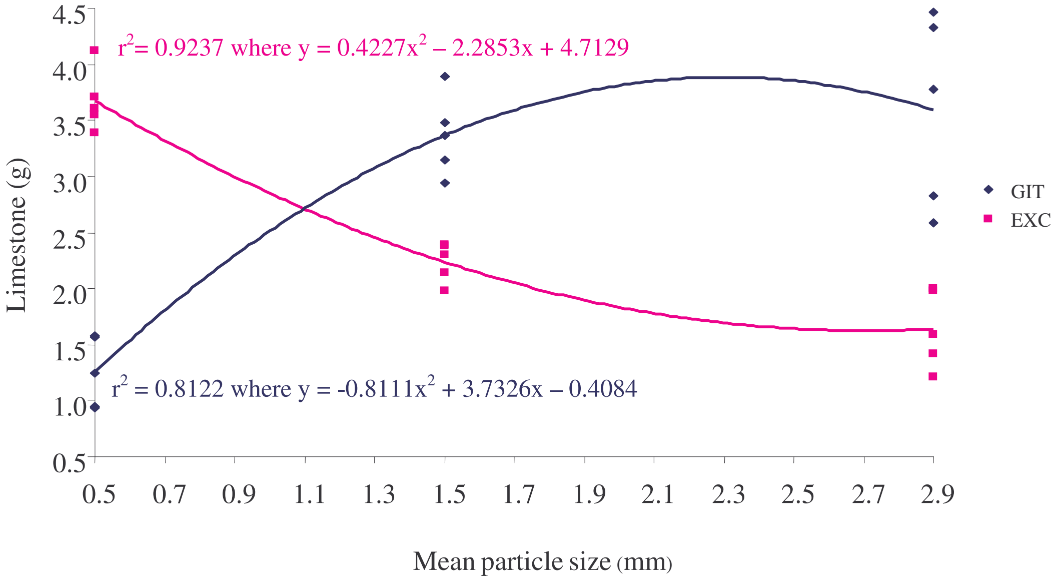

Figure 3.17 Second degree polynomial regression between the mean particle size and intestinal (GIT) and excreta (EXC) limestone content

The second degree polynomial analysis illustrated a closer relationship between limestone particle size and intestinal (r^2=0.81) as well as excreta limestone content (r^2=0.92) compared to that of the linear regression analysis in Figure 3.16. The retention of limestone particles in the intestinal tract were thus not linearly related to the particle size of the limestone alone.

Other factors such as porosity and overall *in vivo* solubility of the Ca source (Rao & Roland, 1989; Zhang & Coon, 1997) as well as the production stage of the hen (Clunies *et al*., 1992) may also affect the retention time of the limestone particles in the GIT. In accordance with the present study Cheng & Coon (1990b) reported the lowest (2.03%) *in vitro* solubility with large limestone particles (3.36 mm) and the highest (28.31%) with small particles (<0.15 mm). However, Cheng & Coon (1990b) indicated that the relationship between solubility and size is not linear and involved a lower degree polynomial equation that hinders the interpretation of the data. The results of Zhang & Coon (1997) who reported an *in vitro* solubility of 29.8% for large limestone particles (3.3 – 4.7 mm) and 49.3% for small particles (1.0 – 2.0 mm) are higher than the *in vitro* solubility values of the present study (Table 3.3 and Figure 3.14), which could be the result of different limestone sources and particle sizes used in the two studies. However, the overall effect of particle size on *in vitro* solubility of the present study was in accordance with the results of Cheng & Coon (1990a) and Zhang & Coon (1997).

3.3.2 Distribution ratios of particles

The results for the *in vivo* and *in vitro* limestone solubility studies with different distribution ratios of limestone particles are presented in Table 3.4 and Figure 3.18. It is clear that the *in vivo* solubility of limestone increased significantly ($P=0.0324$) as the proportion of large particles increased from 0 to 100% in the mixture. However, no significant ($P>0.05$) differences were observed between the treatments containing 25, 50 and 75% small or large particles limestone and that containing 100% small or large particles limestone (Table 3.4). The results of the *in vitro* study showed the opposite trend. *In vitro* limestone solubility decreased significantly ($P<0.0001$) with an increased percentage of large particles in the limestone mixture.

These results are supported by that of the first study in Table 3.3, where an increase in limestone particle size resulted in an increased *in vivo* ($P=0.0095$) and decreased *in vitro* ($P<0.0001$) solubility, as well as the results of Rao & Roland (1990), Guinotte & Nys (1991) and Zhang & Coon (1997), who reported than an increase in limestone particle size resulted in a significant ($P<0.05$) increase of *in vivo* and decrease of *in vitro* solubility.

Table 3.4 The *in vivo* and *in vitro* solubility of limestone with different particle size distribution ratios (Mean±s.e.)

			Significance	
Parameters	**Large particles (%)**	**Mean±s.e.**	**P**	**CV[3](%)**
In vivo solubility (%)	0	56.13±1.17^{a}	0.0324	6.4
	25	57.04±1.39^{ab}		
	50	59.02±1.72^{ab}		
	75	61.77±2.37^{ab}		
	100	63.42±1.65^{b}		
In vitro solubility (%)	0	25.28±0.52^{a}	0.0001	9.3
	25	23.27±0.49^{b}		
	50	21.46±0.50^{c}		
	75	19.16±0.35^{d}		
	100	16.73±0.37^{e}		
GIT[1] (g)	0	1.26±0.14^{d}	0.0001	19.9
	25	1.82±0.10^{cd}		
	50	2.23±0.13^{bc}		
	75	2.96±0.17^{ab}		
	100	3.59±0.38^{a}		
EXC[2] (g)	0	3.68±0.12^{a}	0.0001	10.3
	25	3.01±0.08^{b}		
	50	2.56±0.11^{b}		
	75	2.03±0.12^{c}		
	100	1.64±0.15^{c}		

[abcde] Parameter means±s.e. within a column with different superscripts differ significantly ($P<0.05$)

[1] Intestinal limestone at the end of 2nd oviposition (gram)

[2] Limestone in excreta at the end of 2nd oviposition (gram)

[3] Coefficient of variation

Rabon & Roland (1985) concluded that many factors influence the solubility of CaCO$_3$ and that the optimum particle size or particle size distribution to obtain optimum shell quality may therefore differ. Cheng & Coon (1990b) found that the optimum *in vitro* limestone solubility for bone breaking strength ranged between 12 – 14% and suggested that one might blend limestone of different sizes to meet this optimum solubility. The solubility of the 100% large particles treatment was the closest to the suggested *in vitro* range of Cheng & Coon (1990b). However, the origin and the particle size of the Ca source, as well as the method of *in vitro* determination, could influence this optimum *in vitro* solubility range. Cheng & Coon (1990b) indicated that the use of limestone solubility as a predictor for shell quality-related measurements resulted in a proportion of explained variation of r^2=0.91 for SWUSA, compared to the r^2=0.80 prediction from particle size, and concluded that the advantages of describing limestone in terms of solubility rather than particle size is obvious.

Gordon & Roland (1997) found that high environmental temperatures had no effect on the percentage *in vivo* limestone solubility. They stated that the cause of poor eggshell quality under high environmental temperatures is not attributable to limestone solubility. Other biological factors, such as panting, which resulted in a lower oxygen carrying capacity of the blood, could contribute to poorer eggshells. Hayes & Saunders (2002) indicated that birds start to pant at temperatures of about 29°C to 30°C. The optimal temperature for laying hens is about 21°C (Sainsbury, 2000), whereas Rose (1997) indicated that temperatures between 18 and 24°C are acceptable. During the *in vivo* study, the average house temperature was 21.79°C. If the average house temperature and the results of Gordon & Roland (1997) were brought into consideration, the effect of temperature on limestone solubility in this trial was negligible.

The *in vivo* and *in vitro* limestone solubility of different distribution ratios are graphically illustrated in Figure 3.18. The distribution ratios consists of 0, 25, 50, 75 and 100% large or small limestone particles as described in paragraph 3.2.2.

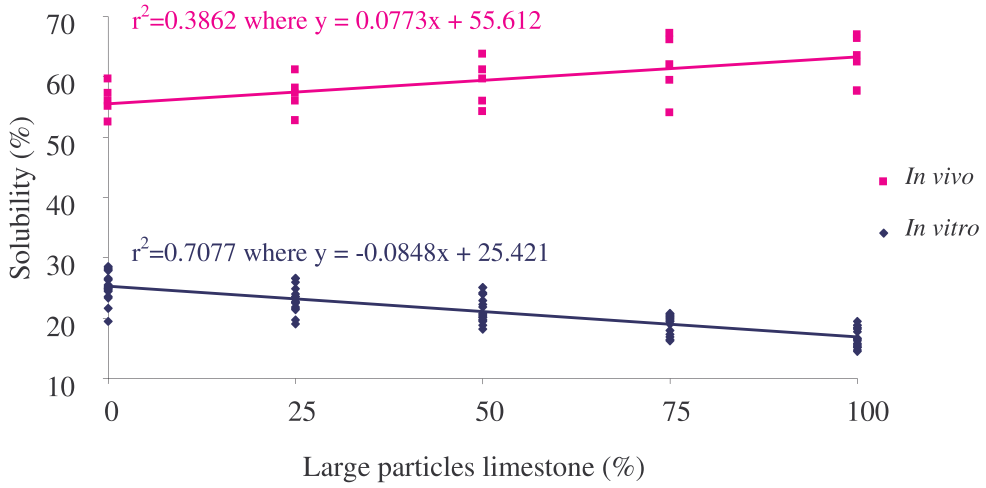

Figure 3.18 The *in vivo* and *in vitro* solubility of different percentages of large (2.0 – 3.8 mm) and small (0 – 1.0 mm) limestone particles

The inverse relationship between the *in vivo* and *in vitro* limestone solubility, as reported by Zhang & Coon (1997), Jacob *et al.* (2003) and discussed in paragraph 3.3.1 could also be observed in the linear regression equations of the present study (Figure 3.18). The relationship between the distribution ratio of different sizes limestone particles and limestone solubility in the *in vivo* study was low (r^2=0.39), while that of the *in vitro* study was considerable higher (r^2=0.71). These results are in accordance with the results of the first study (Figure 3.14), where the prediction of accuracy of the *in vitro* solubility was considerably higher than that of the *in vivo* limestone solubility (r^2=0.76 *vs.* r^2=0.54). The same factors as discussed in paragraph 3.3.1, such as replicates per treatment (n=5 *in vivo vs.* n=19 *in vitro*), experimental errors and/or biological factors could have attributed to the differences between the prediction of accuracy (r^2) of the *in vivo* and *in vitro* limestone solubility studies.

The linear regression equations in Figure 3.18 for *in vivo* solubility (y = 0.0773x + 55.612) (r^2=0.38) and *in vitro* solubility (y = -0.0848x + 25.421) (r^2=0.71) were used to predict the solubility of different mixtures ranging from 0 to 100% fine and coarse particles limestone. These predicted solubility values for the *in vivo* and *in vitro* techniques were used to determine the slope of the linear regression equation of *in vitro* onto *in vivo* solubility in Figure 3.19.

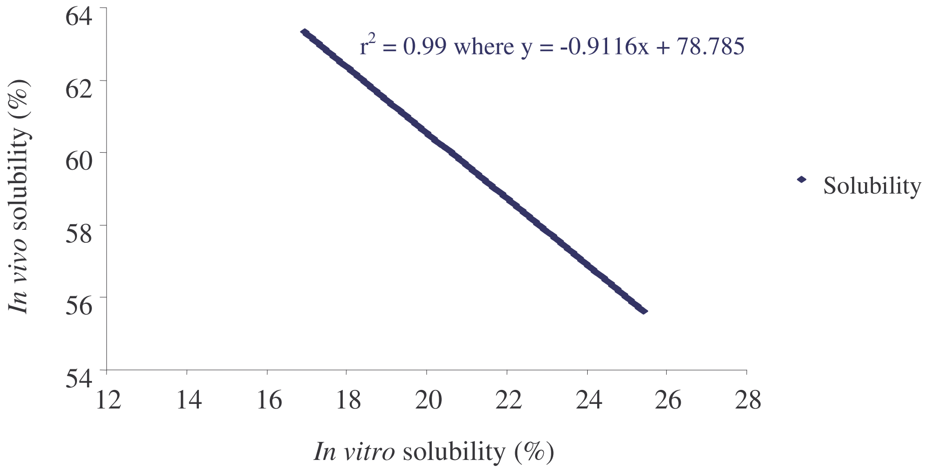

Figure 3.19 Linear regression between the predicted *in vitro* and *in vivo* solubility of limestone

The results from Figure 3.19 are consistent with that in the first study (Figure 3.15) and confirm the findings of Zhang & Coon (1997) and Jacob *et al*. (2003) which suggested the existence of a negative correlation between *in vivo* an *in vitro* limestone solubility. The slope of the regression equation illustrated the negative relationship between the *in vivo* and *in vitro* techniques used for predicting limestone solubility. The high proportion of explained variation (r^2=0.99), was as expected, due to the use of predicted solubility values obtained from the regression equations in Figure 3.18. Therefore as discussed before, the explained proportions of variation (r^2=0.38 and r^2=0.71) in Figure 3.18 should rather be used to indicate the coefficient of determination. However, the aim was not to determine the proportion of explained variation between *in vivo* and *in vitro* limestone solubility, but to illustrate the negative relationship between the two techniques using the slope of the regression equation. The linear regression equation in Figure 3.19 could be used to predict (r^2=0.71) the *in vivo* solubility of different limestone particle mixtures if the *in vitro* solubility values are known. By substituting an *in vitro* limestone solubility value of 21.50 in the linear regression equation of Figure 3.19 ($y = -0.9116x + 78.785$) a predicted *in vivo* solubility of 59.19% is calculated, which compared favourable with the real solubility value in Table 3.4 (59.02%).

The intestinal and excreta limestone content of the particle size distribution study are graphically illustrated in Figures 3.20 and 3.21.

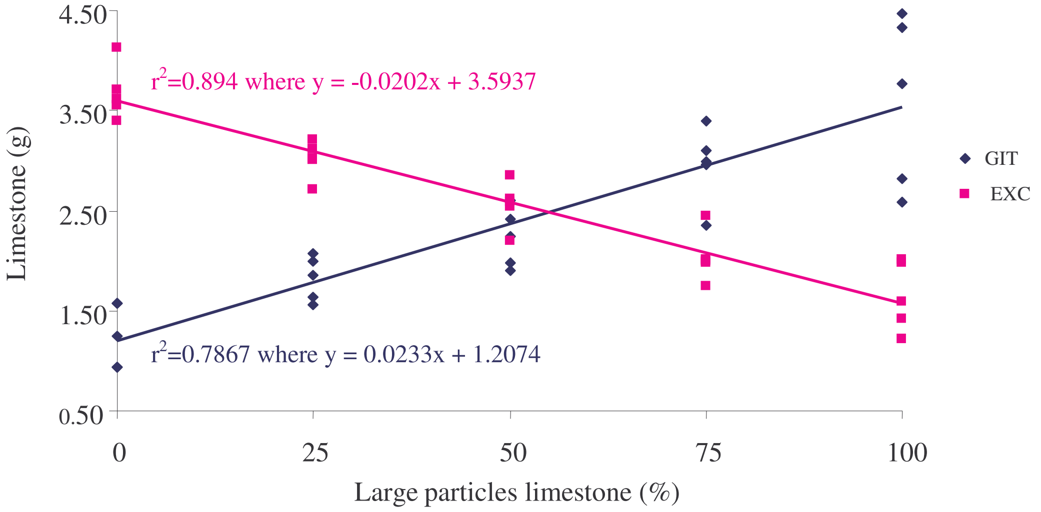

Figure 3.20 Linear regression between the percentage large limestone particles and the intestinal (GIT) and excreta (EXC) limestone content

From the results in Table 3.4 and Figures 3.20 and 3.21 it further seems that an increased percentage of large limestone particles in the distribution ratios, resulted in an increased ($P<0.0001$) intestinal and decreased ($P<0.0001$) excreta limestone content. The treatment containing 100% small particles (0 – 1.0 mm) limestone is characterized by a decreased ($P<0.0001$) intestinal (1.26 g) and increased ($P<0.0001$) excreta limestone content (3.68 g), suggesting that the retention time of small limestone particles is lower than that of large limestone particles (Figures 3.20 and 3.21). The decreased retention time and/or increased passage rate of the distribution ratios high in small particle size limestone resulted in a lower *in vivo* compared to *in vitro* solubility (Figure 3.18). These findings are in accordance with that of the first study in paragraph 3.3.1 (Figure 3.17) where large particle limestone resulted in an increased intestinal (GIT) and decreased excreta (EXC) limestone content. The findings of Rao & Roland (1989) indicated that smaller particle size limestone have a higher rate of passage than larger particles. As discussed earlier, the greater retention time of larger particles in the gizzard allows the limestone to stay in an acidic environment for a longer period of time, hence resulting in a higher *in vivo* solubility.

The linear regression analysis in Figure 3.20, indicated a close (r^2=0.79) relationship between the percentage large limestone particles in the distribution ratios and intestinal limestone content, while the relationship between the percentage large limestone particles and excreta limestone content was even closer (r^2=0.89). As mentioned earlier, an increase in the percentage of large limestone particles in the distribution ratios resulted in a higher intestinal ($y = 0.0223x + 1.2074$) and a reduced excreta ($y = -0.0202x + 3.5937$) limestone content. The increased intestinal and decreased excreta limestone content in both studies indicated that retention time in the GIT is positively affected with an increment in particle size and/or increase in the percentage large limestone particles in the mixtures.

In Figure 3.21 the intestinal and excreta limestone content resulting from different distribution ratios of limestone particles were fitted to a second degree polynomial regression. Unlike the first study (paragraph 3.3.1, Figure 3.17) regarding the particle size of limestone, the fitting of a second degree polynomial regression did not result in a higher relationship between the distribution ratio of limestone particles and intestinal (r^2=0.79) or excreta limestone content (r^2=0.90) compared to the linear regression analysis in Figure 3.20. It therefore seems that both linear and second degree polynomial analysis would explain the relationship between particle size distribution and intestinal- and excreta limestone with the same accuracy, depending on the replicates/treatment and distribution of values on the x-axis. It thus seems that a combination of different particle sizes of limestone works synergetically to establish the retention time and/or rate of passage of limestone in the GIT.

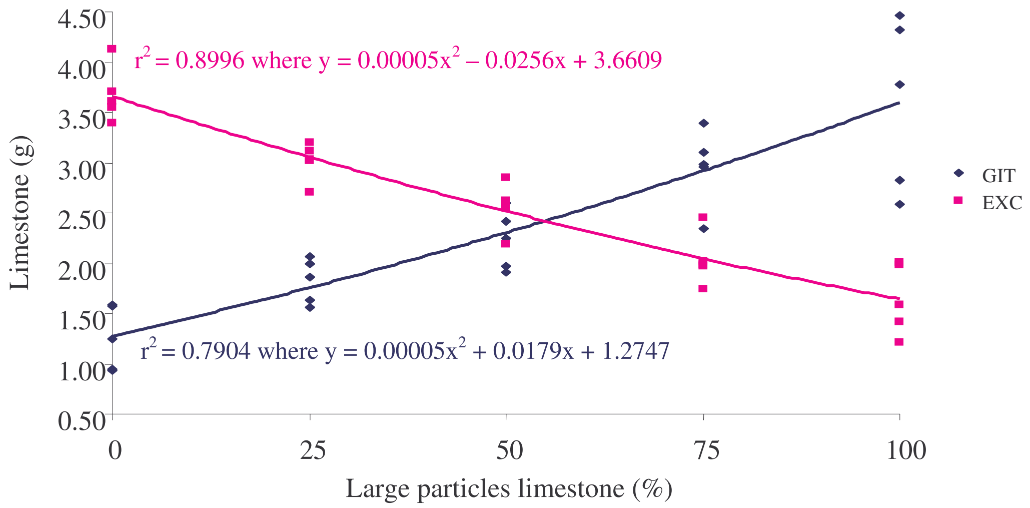

Figure 3.21 Second degree polynomial regression between the percentage large limestone particles and the intestinal (GIT) and excreta (EXC) limestone content

As described in paragraph 3.2.2, all the limestone mixtures in the second experiment were constructed by using the two extreme limestone particle sizes (0 – 1.0 mm and 2.0 – 3.8 mm) of the first experiment. The limestone in Experiment 1 with a nearly intermediate particle size of 1.0 – 2.0 mm was not used in the second study. Treatment three in the second study with a blend of 50% small and 50% large limestone particles (50/50 mixture) could be regarded as the intermediate treatment. The question therefore arises whether the two intermediates in the two different studies would result in the same solubility results.

The *in vivo* and *in vitro* limestone solubility values as shown in Tables 3.3 and 3.4 as well as the regression equations in Figures 3.14 and 3.18 were used for comparison purposes. The limestone with an intermediate particle size of 1.0 – 2.0 mm in the first study resulted in an *in vivo* and *in vitro* limestone solubility of 59.6% and 20.9% respectively. Likewise the *in vivo* and *in vitro* solubility were 59.0% and 21.5% respectively, for the intermediate 50/50 limestone mixture in the second study. It is clear that the solubility of the two intermediates (1.0 – 2.0 mm particle size and the 50/50 mixture) limestone was almost identical. These findings do not support earlier speculation that a combination of different sized particles of limestone work synergetically in determining the rate of passage and solubility. In fact, average particle size, irrespective of the distribution ratio of particles, seems to be the main determining factor of limestone solubility.

The regression equations (Figure 3.14) of y = 3.0197x + 54.767 for *in vivo* and y = -3.5249x + 26.719 for *in vitro* limestone solubility (x=1.5; mean of 1.0 – 2.0 mm particle size) were used for the prediction of *in vivo* and *in vitro* limestone solubility. The predicted values for the *in vivo* and *in vitro* limestone solubility were 59.30% and 21.43% respectively, which compare favourably with the actual values in Table 3.3. The regression equations (Figure 3.18) of y = 0.0773x + 55.612 for *in vivo* and y = -0.0848x + 25.421 for *in vitro* limestone solubility were used for predicting the *in vivo* and *in vitro* solubility of the 50/50 limestone particle mixture in the second study. The predicted values for the *in vivo* and *in vitro* limestone solubility were 59.48% and 21.18% respectively, which also compared favourably with the actual values of 59.02% and 21.46% (Table 3.4).

The regression equation for predicting *in vivo* from *in vitro* limestone solubility in the first study (different particles) in Figure 3.15 (y = -0.8567x + 77.657) as well as second study (different mixtures of limestone particles) in Figure 3.19 (y = -0.9116x + 78.785) were used to predict the *in vivo* solubility of the 1.0 – 2.0 mm limestone particle size and that of the 50/50 limestone particle mixture. The mean *in vitro* values of the 1.0 – 2.0 mm (Table 3.3) and that of the 50/50 mixture (Table 3.4) were substituted in the regression equations of y = -0.8567x + 77.657 and y = -0.9116x + 78.785 respectively. The predicted *in vivo* solubility values for the 1.0 – 2.0 mm particle size and the 50/50 mixture were 59.77% and 59.22% respectively. These predicted values compare favourable with the mean *in vivo* values of 59.55% and 59.02% in Tables 3.3 and 3.4.

The regression equations of y = 0.0773x + 55.612 for *in vivo* (r^2=0.39) and y = -0.0848x +25.3421 for *in vitro* (r^2=0.71) solubility (Figure 3.18) (x = large particles limestone in mixture) were used to predict the ideal blend of small and large particles limestone which might represent the *in vivo* and *in vitro* solubility of the 1.0 – 2.0 mm particle size used during the first study. The linear regression of y = 0.0773x + 55.612 resulted in a ratio of 51% large and 49% small particles limestone with an *in vivo* solubility of 59.55% which is the same as the *in vivo* solubility of the 1.0 – 2.0 mm particle size limestone (Table 3.3). The *in vitro* prediction with y = -0.0848x + 25.421 resulted in a ratio of 54% large particles and 46% small particles limestone with an *in vitro* solubility of 20.84%, which is close to the actual *in vitro* solubility (20.88%) of the 1.0 – 2.0 mm particle size limestone in Table 3.3. Therefore, these results suggest that the *in vivo* and *in vitro* solubility of the 50/50 limestone particle mixture used during the second study (paragraph 3.3.2) and that of the 1.0 – 2.0 mm particle size limestone used in the first study (paragraph 3.3.1) is representative of each other.

3.4 Conclusions

The results of both studies (particle size and distribution ratios of particles) are in accordance with each other regarding the *in vivo* and *in vitro* solubility of limestone. It is clear from the results in the present study that an increase in large particle size limestone promoted a higher *in vivo* solubility. This increased *in vivo* limestone solubility is related to a slower rate of passage of large particle size limestone through the alimentary tract and a subsequent longer period in the acidic environment where $CaCO_3$ could dissociate into Ca^{2+}. In contrast, *in vitro* solubility showed the opposite trend and the lower *in vitro* solubility of larger limestone particles is probably *inter alia* due to a reduced reactive surface to hydrochloric acid (HCl). Furthermore, the *in vitro* solubility method does not make provision for differences in gut retention time of various limestone particle sizes as well as physical contractions of the gizzard. These factors could contribute to the negative relationship between the *in vivo* and *in vitro* solubility techniques, as observed.

Regression analysis of different percentages large limestone particles on intestinal and excreta limestone content suggested that mixtures of different particle sizes limestone work synergistically to establish and manipulate the retention time and/or rate of passage of limestone particles through the digestive tract. However, the intermediate (1.0 – 2.0 mm) particle size and distribution ratios (50/50 small and large particle size mixture) resulted in the same limestone solubility. Therefore it seems from the intermediate limestone particle size and distribution ratios that average limestone particle size and not the variation in particle size is the main determining factor of limestone solubility.

From the results of the present studies (particle size and distribution ratios of particles), it further seemed that the regression equations of limestone particle size on *in vivo* and *in vitro* solubility could be used for the determination of limestone solubility. The use of *in vivo* technique to describe the solubility of a specific limestone source was time consuming and yielded relatively low coefficients of determination (r^2), suggesting that the *in vitro* technique should rather be used for solubility predictions. The prediction of *in vivo* solubility from *in vitro* solubility by using regression analysis however, needs further investigation with more replicates per treatment as well as more defined limestone particle sizes.

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CHAPTER 4

THE INFLUENCE OF LIMESTONE PARTICLE SIZE AND DISTRIBUTION RATIOS ON BONE CHARACTERISTICS

4.1 Introduction

The skeleton contains about 98% of a bird's calcium, of which most is in the form of calcium hydroxyapatite, with small amounts of noncrystalline calcium phosphate and calcium carbonate ($CaCO_3$) (Klasing, 1998). Compact cortical and cancellous bones are the main providers of structural integrity to the skeleton, while medullary bone provides a labile pool of calcium and phosphorus during eggshell formation (McCoy *et al*., 1996). Whitehead & Fleming (2000) defined osteoporosis in the laying hen as a decrease of the amount of fully mineralized structural bone, resulting in an increased fragility and susceptibility to fracture. The high fracture incidence (29%) recorded by Gregory & Wilkins (1989) illustrated that osteoporosis could constitute a severe welfare and economic problem in laying hens. Knott *et al*. (1995) and McCoy *et al*. (1996) concluded that medullary bone had little intrinsic strength compared to cortical and cancellous bone. However, Whitehead & Fleming (2000) and Fleming *et al*. (1998a,b) reported that large amounts of medullary bone contributed to the overall fracture resistance, particularly in the humerus which is normally pneumatized. The results of Fleming *et al*. (1998a,b; 2003) indicated that bone breaking strength increased with an increment in medullary bone.

If dietary calcium is insufficient, the mass and density of medullary bone may decline and eventually egg production would be curtailed. Orban & Roland (1990) reported that an improvement in egg production and eggshell quality resulted in a reduction of tibia strength and percentage bone ash. This inverse relationship reported by Orban & Roland (1990) suggested that hens producing good quality eggshells do it at the expense of their bones and a constant bone replenishment need arises before each shell calcification process to maintain shell quality. The results of Chapter 3 suggested that an increase in limestone particle size resulted in a significantly (P=0.0095) increased *in vivo* limestone solubility. This increased limestone solubility would result in an increased relative bioavailability (RBV) of calcium that could be used for eggshell and/or bone formation. It is therefore possible that higher *in vivo* limestone solubility could improve bone quality characteristics and this aspect needs further investigation.

It seems from literature that dietary calcium as well as particle size of the calcium source could influence bone quality. Cheng & Coon (1990), Guinotte & Nys (1991) and Fleming *et al*. (1998a; 2003) reported an increase in bone strength, tibial radiographic densities and percentage bone ash with particulated calcium sources. The use of particulated limestone resulted in a decreased loss of cancellous bone and an increased medullary bone content (Fleming *et al*., 1998a; Rennie *et al*., 1997). The results of Guinotte & Nys (1991) and Fleming *et al*. (1998b) suggested that the physical size of the calcium source rather than origin of the source is responsible for this increase in bone strength. However, it is known that dolomitic limestone, containing at least 10% magnesium, complexes and competes with calcium for absorption sites in the gastrointestinal tract (GIT) (Leeson & Summers, 1997). The consequence of feeding dolomitic limestone is an induced calcium deficiency and is usually manifested by poor skeletal growth and/or eggshell quality. The work of Rabon & Roland (1985) suggested that the solubility of limestone particles of similar size from different sources could vary by 62%, which influence the available calcium for bone and eggshell formation. It is therefore evident that not only particle size but also Ca-source had a significant effect on bone quality.

Fleming *et al*. (1998b; 2003) concluded that the provision of calcium in a particulated form decrease the severity of some of the characteristics of osteoporosis by enhancing medullary bone formation and thereby inhibiting structural bone resorption as well as contributing directly to bone strength. Particulated calcium sources that are retained longer in the gizzard than powdered sources provided a better source of calcium for eggshell formation during nighttime. The more efficient incorporation of dietary calcium from large particulated calcium sources into eggshell presumably spares the need for medullary bone resorption (Fleming *et al*., 2003).

Regarding the economic and welfare importance of bone breakages in laying hens, as well as the positive results obtained by various authors on particulated calcium supplements, the need arises in South Africa to investigate the effects of the specific calcitic limestone source described in Chapter 3 on bone quality characteristics. The aim of the present study was firstly to determine the effect of a specific limestone source differing in particle size on bone qualities at weeks 37 and 70 of age. Secondly, the effect of different ratios of small and large particles of this specific limestone source on bone quality was determined at these specific weeks.

4.2 Materials and Methods

To investigate the effect of limestone particle size and distribution ratios of particles, two studies were concurrently conducted and the same experimental protocol regarding certain bone quality characteristics was implemented. The limestone described in Chapter 3, paragraph 3.2, was used in these studies.

4.2.1 Particle size

The influence of limestone particle size on bone quality was determined in the first study. Three limestone particle sizes used during this study were classified according to size as small (0 – 1.0 mm), medium (1.0 – 2.0 mm) and large (2.0 – 3.8 mm). The physical appearance of the three limestone particle sizes is illustrated in Figure 3.1, Chapter 3.

4.2.2 Distribution of particle sizes

As in Chapter 3, the second study was conducted to determine the effects of different mixtures of small and large limestone particles on bone quality. Limestone with a particle size of 0 – 1.0 mm was mixed with that of a 2.0 – 3.8 mm particle size to obtain five different ratios of 0, 25, 50, 75 and 100% small or large particles. The physical appearance of the five distribution ratios used during the second study is illustrated in Figure 3.2, Chapter 3.

4.2.3 Diet composition

The basal diet used in both studies was the same for all treatments. The inclusion rates of 9.58% limestone (36% Ca), to result in 3.6% dietary calcium, was constant across all treatments. The paddle type mixer in Figure 4.1 was used for mixing the basal diet (containing no limestone) with the different experimental particles limestone (treatments). During the preparation of the complete layer diet, 14.37 kg limestone/treatment was mixed with 150 kg of the basal diet. To facilitate and ensure proper mixing of the limestone particles, 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 was added and mixed for the same duration of time. After adding the final quantities of basal diet and limestone into the feed mixer, the complete diet (164.37 kg) was mixed for another 10 minutes. Mixing procedures were consistent across all treatments. The commercial layer diet standards of a commercial feed supplier were used for formulation of the diet while the physical and calculated chemical composition of the complete diet is shown in Tables 4.1 and 4.2 respectively.

Figure 4.1 Paddle type feed mixer

Table 4.1 Physical composition of the complete layer diet (as is %)

Physical composition	**%**
Yellow maize	60.09
Prime gluten (65%)	1.38
Wheat bran	1.81
Full fat soya (36%)	5.00
Soya oilcake (47%)	7.24
Sunflower oilcake (37%)	10.00
Fishmeal (65%)	3.27
Limestone	9.58
Mono calcium phosphate	0.74
Fine salt	0.37
Phytase enzyme[1]	0.06
Bicarbonate of soda	0.04
Choline powder[2]	0.01
DL-Methionine[3]	0.18
Premix E[4]	0.40

[1] Commercial phytase enzyme (Natuphos 500 High inclusion) with a phytase enzyme concentration of 500 000 FTU/kg DM (FTU = Phytase Unit). Diet was formulated according to commercial laying standards to ensure a inclusion level of 300 FTU

[2] Choline chloride powder with a 60% choline and 16% chloride concentration. Diet was formulated for 0.01% choline. Choline chloride was not provided as a premix portian of diet.

[3] Methionine powder with a 99% concentration

[4] Commercial mineral/vitamin premix (Inclusion and levels of nutrients are confidential)

Table 4.2 Calculated chemical analysis of the complete layer diet (as is %)

Chemical analysis	**%**
Protein	17.00
Fat	4.16
Ash	13.37
NDF[1]	10.00
ADF[2]	4.95
Moisture	10.01
Crude fibre	3.75
Calcium	3.60
Phosphorus	0.56
AvP[3]	0.29
Ca:AvP[4]	12.41
Choline	0.30
Sodium	0.18
Potassium	0.57
Magnesium	0.24
AME[5] (MJ/kg)	11.49
Arginine	1.08
Isoleucine	0.68
Lysine	0.79
Methionine	0.36
Threonine	0.61
Tryptophane	0.18
Methionine and Cystine	0.68

[1] Neutral detergent fibre
[2] Acid detergent fibre
[3] Available phosphorus
[4] Calcium to Available phosphorus ratio
[5] Apparent metabolizable energy (MJ/kg)

4.2.4 Birds and husbandry

One hundred and ninety eight Lohmann-Silver pullets, 17 weeks of age, were obtained from a commercial egg producer. The hens were randomly divided into six treatments with 33 birds per treatment. Hens were placed into individual cages and housed in a naturally ventilated building with no climate control systems. Time switches were used to control daylight according to a prescribed photoperiod schedule and from 25 weeks of age a maximum of 15.5 hours of light/day were maintained. The cages (Figures 3.4 and 4.2) were equipped with individual feed troughs, water nipples, perches and galvanized trays for excreta collection. On reception, all birds were beak-trimmed and vaccinated according to the prescriptions of the supplier.

Figure 4.2 Two rows of cages

4.2.5 Experimental measurements

At 37 and 70 weeks of age, 10 birds per treatment (n=10) were randomly selected, weighed and sacrificed by cervical dislocation. The carcasses were stored overnight in a refrigerator at 2^{o}C. The tibia (left and right) and the right humerus from each bird were excised during the next morning. Bones were defleshed (Figure 4.3) with scalpels and scissors but without boiling, whereafter the fibula was removed from the tibia (Figure 4.4).

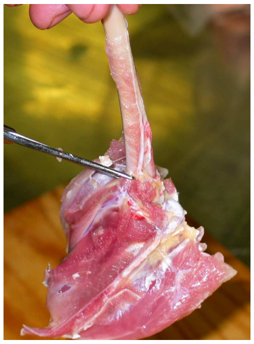

Figure 4.3 Defleshing of tibia

Figure 4.4 Removal of fibula bone

Defleshed bones were weighed (full fat) and the procedures of Zhang & Coon (1997) were used to determine the individual shaft width and length (Figures 4.5 and 4.6) of the right tibia and humerus with a vernier caliper (0.01 mm). Tibia bones (left and right) and the humerus were individually sealed in plastic bags to minimize moisture loss and stored at -18^{o}C in a freezer for later analysis of bone breaking strength and bone ash. Bone breaking strength of the right tibia and humerus were determined, while the left tibia was used for bone ash determinations.

Figure 4.5 Tibia width

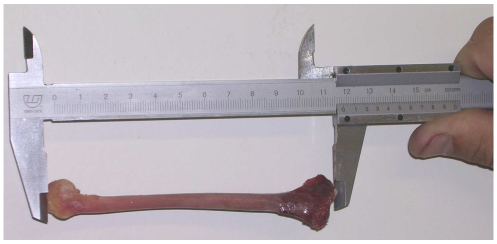

Figure 4.6 Tibia length

4.2.5.1 Breaking strength

The methods of Fleming *et al*. (1998a,b) were used for the determination of tibia and humerus breaking strength. A three-point destructive bending test was carried out with an Instron tensile/compression machine. It is computer controlled and uses a HBM MVD25010 signal conditioning and data acquisition system that take force readings every 0.02 seconds. The centre point of each bone was placed between two 10 mm diameter restraining bars which were set 30 mm apart. The 10 mm diameter crosshead probe approached the bone at 30 mm/min until the tibia or humerus was broken. The breaking strength was expressed in Newton (N/m^2). Bone breaking strength data was used to calculate bone stress according to the formula of Crenshaw *et al*. (1981):

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

4.2.5.2 Bone ash

The procedures used for the determination of fat free bone ash content are described by Al-Batshan *et al*. (1994) and Elaroussi *et al*. (1994). The left tibia was cut into three small pieces of approximately 2 – 3 cm in length (Figure 4.7) to facilitate the fitting of the complete tibia into the thimble of the Soxhlet extractor machine.

The three pieces of an individual bone were wrapped in marked Schleicher & Schull Nr. 589/2 ashless filter paper before putting it into a marked extractor thimble. One hundred and fifty milliliters of hexane (98% concentration) were used as solvent for fat extraction. After four hours of extraction, the tibia pieces were removed from the filter paper and placed into a pre-weighed ashing crucible. Crucibles with their tibia content were dried at 105°C for 24 hours before the weight of the dry, fat free tibia was recorded. Thereafter, the fat free tibia was ashed according to the methods of McCoy *et al.* (1996) for 24 hours at 550°C in a muffle furnace to determine bone ash (g). The percentage (%) of bone ash from the fat free left tibia was calculated as follows (Al-Batshan *et al*., 1994):

%Tibia ash = (Tibia ash weight/Fat free tibia weight) x100

The percentage of tibia and humerus bone (full fat) was calculated as a ratio of tibia or humerus to body weight as described by Orban & Roland (1990):

%Right tibia bone = (Right tibia weight/Body weight) x 100

%Right humerus bone = (Right humerus weight/Body weight) x 100

Figure 4.7 Tibia pieces after ashing

4.2.6 Statistical analysis

The effect of limestone particle size (Study 1) and distribution ratios of different particle sizes (Study 2) on bone quality characteristics was analyzed using a fully randomized one way ANOVA design. The PROC ANOVA procedures of the SAS program (SAS, 1999) were used to test for significant differences between treatments. When significant differences were found ($P<0.05$) further multiple comparisons using Tukey's studentized range (HSD) test was used to identify these differences.

The description of the model used for PROC ANOVA analysis in the first and second study was:

$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$, Study 1: $i = 1, 2, 3; j = 1, 2, \ldots 10$

Study 2: $i = 1, 2, \ldots 5; j = 1, 2, \ldots 10$

Where: Y_{ij} = the dependant variable in the i-th trial with a j-th random error

μ = the overall population mean

τ_i = the i-th population treatment effect

ε_{ij} = the random error effect

The different, Y_{ij} dependant variables for both studies were:
Tibia length, tibia width, tibia weight, tibia breaking strength, tibia stress, percentage tibia bone, percentage tibia ash, humerus length, humerus width, humerus weight, humerus breaking strength, humerus stress and percentage humerus bone.

The i-th treatment effect (limestone particle size) during the first study was defined as:
$i_1 = <1.0$ mm, $i_2 = 1.0 - 2.0$ mm, $i_3 = 2.0 - 3.8$ mm.

The i-th treatment effect (distribution ratios of small and large particles limestone) during the second study was defined as:
i_1 = 100% small, i_2 = 75% small: 25% large, i_3 = 50% small: 50% large, i_4 = 25% small: 75% large and i_5 = 100% large.

The correlation method of Pearson was used for the determination of correlations between bone breaking strength and percentage bone ash.

4.3 Results and Discussion

4.3.1 Particle size

4.3.1.1 Bone dimensions

The effect of limestone particle size on tibia and humerus bone dimensions such as length, width and weight for weeks 37 and 70 of age are illustrated in Table 4.3. Large particles limestone (2.0 – 3.8 mm) resulted in a significantly decreased tibia length (P=0.0317), tibia weight (P=0.0265) and humerus length (P=0.0060) at week 37 of age. Limestone particle size had no significant (P>0.05) effect on humerus width as well as -weight at week 37 of age. At 70 weeks of age, the effect of limestone particle size on tibia and humerus length, -width and -weight was not significant (P>0.05) for all treatments. Guinotte *et al.* (1991) reported that coarse (1.18 – 4.75 mm) limestone particles resulted in a significant (P<0.01) decrease in tibia thickness (1.20 *vs*. 1.24 mm) compared to ground (<0.15 mm) particles in broiler chicks. The report of Guinotte *et al*. (1991) also suggested that coarse compared to ground limestone particles resulted in a significant (P<0.05) decreased tibia length (719 *vs.* 726 mm). These results of Guinotte *et al*. (1991) are supportive to the results of the present study, where large limestone particles resulted in a significant decrease in bone length and width at 37 weeks of age (Table 4.3). Guinotte *et al*. (1991) ascribed the significant (P<0.05) decrease in tibia length with an increase in limestone particle size to the slower transit time of coarse limestone particles compared to ground particles, due to gizzard retention and their lower solubility in combination with the shorter GIT transit time of digesta in growing chickens. However, in the present study, the large limestone particles described in Chapter 3, which resulted in a slower transit time and high *in vivo* solubility (63.42%) was accompanied by a decreased tibia length and weight as well as humerus length (Table. 4.3). Therefore, the decrease in tibia length and weight as well as humerus length could not be ascribed to the lower solubility values of the limestone particles in this study. The decrease in tibia length and weight and humerus length as observed at week 37 of the present study was, however, not perceptible at week 70 of age. The same number of birds (n=10/treatment) was sacrificed for the determination of bone qualities at week 37 and 70 of age. The different effects of limestone particle size on bone quality can probably not be ascribed to a variation in sample size and should be interpreted with caution.

A significant (P<0.05) decrease in humerus weight was observed with an increase in age from week 37 to 70. This was in accordance with the work of Al-Batshan *et al.* (1994) who found that the femur weight of DeKalb XL-Link and Hy-Line W-36 hens decreased significantly (P<0.05) from week 37 to 72 of age. These researchers ascribed the decrease in

Table 4.3 The effect of limestone particle size on bone dimensions (Mean±s.e)

Parameters	Age (weeks)	Particle size (mm)			Significance	
		0-1.0	**1.0-2.0**	**2.0-3.8**	**P**	**CV[1](%)**
Right tibia						
Length (mm)	37	119.72±1.06[a]	119.06±0.72[ab]	116.17±1.02[b]	0.0317	2.5
	70	116.75±1.50	118.86±1.12	114.30±4.09	0.4767	7.1
Width (mm)	37	6.55±0.11	6.38±0.11	6.40±0.13	0.5513	5.8
	70	6.44±0.06	6.45±0.08	6.48±0.08	0.9130	3.6
Weight[2] (g)	37	11.16±0.27[a]	10.77±0.18[ab]	10.29±0.18[b]	0.0265	6.3
	70	10.30±0.35	10.36±0.35	10.90±0.28	0.3746	9.7
Right humerus						
Length (mm)	37	79.21±0.73[a]	77.70±0.28[ab]	76.61±0.44[b]	0.0060	2.1
	70	78.07±0.65	78.31±0.72	78.44±0.73	0.9311	2.8
Width (mm)	37	6.13±0.08	6.29±0.14	5.97±0.09	0.1129	5.3
	70	6.14±0.08	6.09±0.12	6.18±0.09	0.7967	5.1
Weight[2] (g)	37	4.07±0.11	4.16±0.16	4.05±0.30	0.9259	15.9
	70	3.94±0.17	3.73±0.21	3.82±0.17	0.7392	15.2

[a,b] Figures with different superscripts within the same row differ significantly (P<0.05)

[1] Coefficient of variation

[2] Freshly defleshed, full fat bone

femur weight to the decrease in intestinal calcium absorption and suggested that molt might improve the intestinal calcium uptake of laying hens. On the other hand, Fleming *et al*. (1998a) reported a significant increase (P<0.001) in humerus weight and radiographic density with an increase in medullary bone score at week 70 of age. They suggested that the increase in the humerus weight is presumably reflecting the increased amounts of medullary bone in the pneumatised diaphysis of the humerus. Therefore, the decrease in humerus weight observed between week 37 and 70 of age during the present study (Table 4.3) could be ascribed to either the decreased intestinal absorption of calcium in older laying hens, or due to the loss of medullary bone from the humerus.

4.3.1.2 Bone mechanical properties

Bone stress allowed comparisons to be made between the breaking strength of bones that differ in length, size and shape and are normally expressed as force per unit area (Crenshaw *et al.*, 1981) and would therefore be affected by bone breaking strength values. The effect of limestone particle size on bone breaking strength and bone stress are presented in Table 4.4. No significant effect (P>0.05) of limestone particle size on tibia breaking strength and stress could be detected at 37 weeks of age. Similarly, limestone particle size had no significant (P>0.05) effect on humerus breaking strength and stress at weeks 37 and 70 of age. However, large particles limestone (2.0 – 3.8 mm) resulted in a significant (P=0.0150) increase in tibia breaking strength and tibia stress (P=0.0419) at week 70. In the case of the humerus, the increase in breaking strength and stress at week 70 was not statistically significant (P=0.7749). These results are in accordance with Guinotte & Nys (1991) who reported that particulated limestone resulted in a significant (P<0.05) higher bone breaking strength (18.93 N *vs.* 17.26 N) than ground limestone in ISA-Brown laying hens at 77 weeks of age. Fleming *et al.* (1998b) reported that particulated limestone resulted in a non-significant (P>0.05) increase in tibia (23.6 kg) and humerus (12.1 kg) breaking strength compared to tibia (19.5 kg) and humerus (11.8 kg) breaking strengths of ground limestone in 70 week old ISA-Brown layers. Although Fleming *et al.* (1998b) failed to illustrate significant differences between the effects of limestone particle size and tibia breaking strength, it seems that large particles limestone resulted in higher tibia breaking strengths.

The report of Guinotte *et al*. (1991) illustrated that the tibia breaking strength (99.6 *vs*. 92.4 N) of 4 weeks old broilers was significantly (P<0.01) higher in coarse (1.18 – 4.75 mm) than in ground (<0.15 mm) limestone particles. Cheng & Coon (1990) suggested that an increase in limestone particle size resulted in a significant (P<0.05) increase in bone breaking strength

Table 4.4 The effect of limestone particle size on bone mechanical properties (Mean±s.e)

Parameters	Age (weeks)	Particle size (mm)			Significance	
		0-1.0	**1.0-2.0**	**2.0-3.8**	**P**	**CV^1(%)**
Right tibia						
Breaking strength (N/m^2)	37	268.38±1.92	276.51±2.26	292.78±1.89	0.6803	22.5
	70	276.30±21.13^a	331.96±26.79^{ab}	395.01±29.68^b	0.0150	24.5
Stress (kg/cm^2)	37	95.81±0.97	104.26±0.95	111.18±1.00	0.5333	29.2
	70	101.21±8.01^a	121.21±10.50^{ab}	143.58±13.48^b	0.0419	28.1
Right humerus						
Breaking strength (N/m^2)	37	209.83±0.98	238.56±1.45	209.83±1.27	0.1955	17.5
	70	236.65±21.46	252.24±18.53	256.54±20.88	0.7749	25.3
Stress (kg/cm^2)	37	89.48±0.55	95.46±0.76	97.45±0.74	0.6902	22.7
	70	98.55±7.64	110.70±10.01	108.94±12.76	0.6910	30.8

[a,b] Figures with different superscripts within the same row differ significantly ($P<0.05$)

[1] Coefficient of variation

and indicated that large (2.38 mm) and small (<0.15 mm) particles limestone resulted in respectively the highest (13.28 kg) and lowest (10.70 kg) bone breaking strength. These results are supportive to the findings of the present study (Table 4.4) where an increase in particle size resulted in an increased tibia breaking strength and consequently an increased in tibia stress.

Bone breaking strength and bone stress (tibia and humerus bone) increased significantly ($P<0.01$) with age from week 37 to 70. McCoy *et al.* (1996) also reported that bone breaking strength increased significantly ($P<0.05$) from 14.5 kg in week 35 to 19 kg in week 70, suggesting that an increase in medullary bone could contribute to the increase in bone breaking strength with age. Rath *et al*. (1999) also reported that bone stress in broiler breeder hens, increased from 1.97 kg/mm^2 to 7.55 kg/mm^2 between 7 and 72 weeks of age. However, the work of Moreki (2005) suggested that bone stress in broiler breeder hens decreased significantly with age ($P<0.0081$), from 32.33 N/mm^2 (week 35) to 19.07 N/mm^2 (week 60), which is in contrast with the results of the present study. The different effect of age on bone stress observed between the present study and that of Moreki (2005) could be attributed to the differences in calcium utilization between laying hens and broiler breeders, as well as the differences in age (60 *vs*. 70 weeks).

The report of Fleming *et al*. (1998a) suggested that humerus breaking strength of laying hens (70 weeks of age) increased significantly ($P<0.001$) with an increase in medullary bone content, confirming the suggestion of McCoy *et al.* (1996) that medullary bone contributed to bone breaking strength. The significant ($P<0.05$) decrease in humerus weight from week 37 to 70 (Table 4.3) was in contrast to a significant ($P<0.05$) increase in humerus breaking strength. The results of the present study are in disagreement with the work of Fleming *et al*. (1998a) who suggested that the amount of medullary bone in the humerus had a direct relationship with humerus breaking strength and that increased humerus weights reflected the increased amounts of medullary bone in the humerus of older laying hens. The increase in humerus breaking strength between week 37 and 70 of age in the present study could rather be ascribed to the changes in morphometric measurements (increase/decrease in bone length and/or width) that would result in a more completely mineralized bone matrix and stronger cortical bone.

A highly significant ($P<0.01$) correlation between tibia breaking strength and tibia ash ($r^2=0.75$), as well as tibia breaking strength and humerus breaking strength ($r^2=0.87$) were recorded. The relationship between tibia ash and tibia stress ($r^2=0.75$) was also significant

(P<0.01) and was as expected, of the same magnitude than the correlation between tibia breaking strength and tibia ash. The correlations between tibia breaking strength and tibia ash (r^2=0.75) were considerably higher than the significant (P<0.01) positive correlation (r^2=0.57) recorded by Guinotte *et al.* (1991) in broiler chicks. The significant (P<0.01) correlation between tibia breaking strength and tibia ash (r^2=0.75) observed in the present study is in contrast with the work of Moreki (2005) who was unable (P>0.05) to report a positive relationship between tibia breaking strength and percentage tibia ash. Moreki (2005) reported that while tibia breaking strength (P=0.011) increased with dietary calcium levels, no significant (P=0.160) effect could be recognized on tibia ash. The differences observed in tibia breaking strength and consequently the percentage tibia ash could be responsible for the differences in the relation between tibia breaking strength and percentage tibia ash, observed between the two studies.

4.3.1.3 Bone ash

In Table 4.5, the effect of limestone particle size on bone ash and percentage bone are illustrated. At week 37 of age, limestone particle size resulted in a significant (P=0.0318) increase in percentage tibia ash. However, the only statistical significant differences were observed between medium (1.0 – 2.0 mm) and large (2.0 – 3.8 mm) limestone particles and no clear tendency regarding the effect of limestone particle size on tibia ash could be observed. Accordingly, the work of Guinotte *et al*. (1991) showed that coarse limestone particles resulted in a significant (P<0.01) higher tibia ash content (47.2% *vs*. 44.7%) than ground particles. In another study on broiler chickens, Anderson *et al.* (1984) suggested that fine limestone particles (<0.074 mm) resulted in a significant (P<0.01) lower tibia ash content than medium particles (0.074 – 1.0 mm). Cheng & Coon (1990) reported that femur breaking strength and ash content are significantly (P<0.05) affected by limestone particle size, suggesting that medium (1.68 mm) limestone particles result in the highest (57.46%) percentage bone ash. During the present study, medium (1.0 – 2.0 mm) particles limestone resulted in the lowest (53.19%) percentage tibia ash at week 37. At 37 weeks of age, medium size limestone particles resulted in an intermediary tibia breaking strength (276.51 N/m^2), which are in accordance with the intermediary femur breaking strength (13.18 kg) of medium (1.68 mm) limestone particles used by Cheng & Coon (1990). Therefore, differences between the bone ash results of the present study and that of Cheng & Coon (1990) could probably be ascribed to the differences in bone mineralization between the femur and tibia bones.

Table 4.5 The effect of limestone particle size on bone ash and percentage bone (Mean±s.e)

Parameters	Age (weeks)	Particle size (mm)			Significance	
		0-1.0	**1.0-2.0**	**2.0-3.8**	**P**	**CV[1](%)**
Tibia						
Percentage bone[2] (%)	37	0.57±0.01	0.54±0.01	0.55±0.01	0.1374	5.7
	70	0.52±0.01[a]	0.51±0.01[a]	0.55±0.01[b]	0.0056	5.6
Ash (%)	37	53.99±0.74[ab]	53.19±0.77[a]	56.43±0.99[b]	0.0318	4.8
	70	59.90±1.22	61.40±0.77	61.27±0.73	0.4575	4.6
Humerus						
Percentage bone[3] (%)	37	0.21±0.01	0.21±0.01	0.21±0.01	0.8614	14.8
	70	0.20±0.00	0.18±0.01	0.19±0.01	0.3498	11.9

[a,b] Figures with different superscripts within the same row differ significantly ($P<0.05$)

[1] Coefficient of variation

[2] Percentage right tibia bone = (Right tibia weight/Body weight) x 100

[3] Percentage right humerus bone = (Right humerus weight/Body weight) x 100

Large particle size limestone resulted in a significant (P=0.0056) higher percentage tibia bone (0.55%) at week 70 of age. The effect of limestone particle size on percentage tibia bone was however insignificant (P=0.1374) at week 37 of age. No significant (P>0.05) effect of limestone particle size on percentage right humerus bone was observed at either weeks 37 or 70 of age.

Al-Batshan *et al.* (1994) concluded that the percentage femur ash of DeKalb XL-Link and Hy-Line W-36 hens decreased significantly (P<0.05) from week 37 to 72 of age. They ascribed the significant (P<0.05) decrease in percentage ash to the decrease in intestinal calcium absorption and suggested that a forced molting could improve the percentage femur ash. In contrast, the results of the present study (Table 4.5) illustrated a significant increase (P<0.05) in percentage tibia ash from 37 to 70 weeks of age. The differences in results could be ascribed by the genetic differences between the laying hens as well as the different bones (tibia and femur) used for comparisons in the two studies. The structural functions of tibia- and femur bone differ and these differences could contribute to the differences noticed between the present study and that of Al-Batshan *et al.* (1994).

4.3.2 Distribution ratios of particles

4.3.2.1 Bone dimension

The effect of different distribution ratios of large and small limestone particles on bone dimensions such as bone length, width and weight are presented in Table 4.6. It seems that the effect of different percentages of large particles limestone on tibia and humerus length, width and weight was not significant (P>0.05) at weeks 37 and 70 of age. These results differ from that of the first study (Table 4.3) where large particles limestone resulted in a significant decreased tibia length (P=0.0317), tibia weight (P=0.0265) and humerus length (P=0.006) at week 37 of age. However the non-significant (P>0.05) effect of different distribution ratios of limestone particles on tibia and humerus length, -width and -weight are in accordance with the results at week 70 of the first study (Table 4.3). The results of the present study are however in contrast with that of Guinotte *et al.* (1991) who reported a significant (P<0.01) decrease in tibia thickness with coarse (1.18 – 4.75 mm) particles limestone in broiler chicks. The difference in results between the present study and that of Guinotte *et al.* (1991) could be ascribed by the differences between growing chickens and laying hens in the digestive utilization of various particle sizes. Coarse particles had an increased transit time in the GIT due to a prolonged gizzard retention time, while the

Table 4.6 The effect of different percentages large limestone particles on bone dimensions (Mean±s.e)

		Large particles (%)					Significance	
Parameters	**Age (weeks)**	**0**	**25**	**50**	**75**	**100**	**P[1]**	**CV[2](%)**
Right tibia								
Length (mm)	37	119.72±1.06	119.56±1.04	119.67±0.77	119.20±1.06	116.17±1.02	0.0699	2.6
	70	116.75±1.50	118.83±1.16	120.27±1.03	117.89±1.30	114.30±4.09	0.3704	5.8
Width (mm)	37	6.55±0.11	6.68±0.13	6.40±0.08	6.43±0.11	6.40±0.13	0.3511	5.6
	70	6.44±0.06	6.47±0.17	6.51±0.08	6.59±0.09	6.48±0.08	0.8796	5.0
Weight[3] (g)	37	11.16±0.27	11.22±0.36	10.81±0.25	10.42±0.24	10.29±0.18	0.0534	7.8
	70	10.30±0.35	10.61±0.32	10.81±0.16	10.96±0.24	10.90±0.28	0.4775	8.1
Right humerus								
Length (mm)	37	79.21±0.73	79.18±1.24	78.61±0.57	77.86±0.43	76.61±0.44	0.0917	3.0
	70	78.07±0.65	78.14±0.67	78.44±0.67	78.11±0.69	78.44±0.73	0.9896	2.7
Width[3] (mm)	37	6.13±0.08	6.24±0.06	6.13±0.05	6.05±0.05	5.97±0.09	0.0780	3.5
	70	6.14±0.08	6.07±0.10	6.19±0.09	6.21±0.07	6.18±0.09	0.7895	4.3
Weight (g)	37	4.07±0.11	4.03±0.18	3.96±0.09	3.90±0.18	4.05±0.30	0.9651	14.8
	70	3.94±0.17	3.76±0.18	3.80±0.13	3.98±0.18	3.82±0.17	0.8542	13.8

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[1] (P>0.05) = non significant

[2] Coefficient of variation

[3] Freshly defleshed, full fat bone

physiological transit time in the growing chicken are relatively short (±3 – 5 hours). When the upper limit of the gizzard is exceeded, un-dissolved limestone particles are pushed into the small intestine where the acidity is inadequate for further limestone solubilization. The physical limitations of the gizzard as well as a combination of increased/decreased transit time of limestone particles and digesta through the GIT of growing chickens resulted in an inadequate limestone solubility and consequently a decrease in bone quality as observed by Guinotte *et al.* (1991).

The femur weight of DeKalb XL-Link and Hy-Line W-36 hens decreased significantly ($P<0.05$) from week 37 to 72 of age due to a decreased intestinal calcium absorption (Al-Batshan *et al.*, 1994). The differences in results observed between the present study and that of Guinotte *et al.* (1991) and Al-Batshan *et al.* (1994) could probably be ascribed to the different physiological limitations of the gizzard, in laying hens and growing chickens, that affected the calcium solubilization process as discussed earlier. The general significant ($P<0.05$) decrease in tibia length from week 37 to 70 of age noticed in the first study (Table 4.3) was not observed ($P>0.05$) during the second study. The effect of age on bone width and weight are also less clear during this study and the tendencies of increased/decreased bone width and/or weight are inconsistent between treatments which hinder the interpretation of data.

4.3.2.2 Bone mechanical properties

The effect of different distribution ratios of limestone particles on bone mechanical properties are presented in Table 4.7. Different percentages of large particles limestone had no significant ($P>0.05$) effect on tibia breaking strength and tibia stress at week 37 as well as humerus breaking strength and stress at weeks 37 and 70 of age. An increase in the percentage large particles limestone resulted in a significantly increased tibia breaking strength ($P=0.0123$) and tibia stress ($P=0.0304$) at week 70 of age. These results are in accordance with the first study (Table 4.4) where an increase in limestone particle size had resulted in a significant increase in tibia breaking strength ($P=0.0150$) and stress ($P=0.0419$). These results supported the findings of Guinotte & Nys (1991) that particulated limestone resulted in a significant ($P<0.05$) higher bone breaking strength (18.93 N *vs.* 17.26 N) than ground limestone in ISA-Brown laying hens at 77 weeks of age. Fleming *et al.* (1998b) reported that particulated limestone compared to ground limestone resulted in a non-significant ($P>0.05$) increased tibia (19.5 to 23.6 kg) and humerus (11.8 to 12.1 kg) breaking strength in 70 weeks old laying hens.

Table 4.7 The effect of different percentages large limestone particles on bone mechanical properties (Mean±s.e)

		Large particles (%)					Significance	
Parameters	**Age (weeks)**	**0**	**25**	**50**	**75**	**100**	**P**	**CV[1](%)**
Right tibia								
Breaking strength	37	268.37±1.92	248.86±1.02	276.32±0.63	285.46±1.93	292.78±1.89	0.3411	18.2
(N/m^2)	70	276.30±21.13^{a}	290.44±21.22^{ab}	265.89±31.16^{a}	309.52±30.03^{ab}	395.01±29.60^{b}	0.0123	27.7
Stress (kg/cm^2)	37	95.81±0.97	82.60±0.55	103.55±0.42	106.88±0.96	111.18±1.00	0.1282	25.8
	70	101.21±8.01^{a}	107.30±9.59^{ab}	96.37±12.22^{a}	106.34±10.22^{ab}	143.58±13.48^{b}	0.0304	31.0
Right humerus								
Breaking strength	37	209.83±0.98	273.26±2.81	234.22±1.86	207.39±1.32	209.83±1.27	0.0540	24.7
(N/m^2)	70	236.65±21.46	218.25±25.25	225.49±23.87	271.79±25.81	256.54±20.88	0.4750	30.6
Stress (kg/cm^2)	37	89.48±0.55	109.96±1.18	100.23±1.01	91.53±0.62	97.45±0.74	0.4717	27.6
	70	98.55±7.64	97.57±12.19	94.54±11.70	113.28±13.37	108.94±12.76	0.7572	36.2

[a,b] Figures with different superscripts within the same row differ significantly ($P<0.05$)

[1] Coefficient of variation

It was also recorded that coarse limestone particles (1.18 – 4.75 mm) resulted in a significantly ($P<0.01$) higher tibia breaking strength (99.6 N *vs.* 92.4 N) than ground (<0.15 mm) limestone particles (Guinotte *et al.*, 1991). In another opinion, Cheng & Coon (1990) suggested that the use of large (2.38 mm) limestone particles resulted in a significant ($P<0.05$) higher bone breaking strength (13.28 kg *vs.* 10.70 kg) than small limestone particles. Results of these authors are supportive to the findings of the present study at week 70 of age (Table 4.7), where the distribution ratio containing 100% large (2.0 – 3.8 mm) limestone particles resulted in a significantly ($P=0.0123$) higher tibia breaking strength (395.01 N/m^2) than that (276.30 N/m^2) of the 100% small (0 – 1.0 mm) limestone particles. However, although significant differences ($P<0.05$) were observed between treatments containing 100% large or small limestone particles, the effect of different distribution ratios on bone strength were not clearly illustrated by the data in Table 4.7. At week 70 of age, both tibia breaking strength and tibia stress were the highest (395.01 N/m^2 and 143.58 kg/cm^2) for the ratio containing 100% large particles and lowest (265.89 N/m^2 and 96.37 kg/cm^2) for the ratio containing 50% large and 50% small particles limestone. Accordingly humerus breaking strength at neither week 37 or 70 of age seems to be related to the effect of different distribution ratios of limestone particles.

The generally significant ($P<0.01$) increase in bone breaking strength and bone stress observed from 37 to 70 weeks of age during the first study (Table, 4.4) was not noticeable in the present study. The results of the present study indicated that some of the tibia and humerus breaking strengths increased significantly ($P<0.01$) while others decreased significantly ($P<0.01$) with an increase in age.

The correlation between tibia breaking strength and tibia ash ($r^2=0.66$) and tibia and humerus breaking strength ($r^2=0.82$) were statistically significant ($P<0.01$). A significant ($P<0.01$) correlation between tibia ash and tibia stress ($r^2=0.65$) was observed as expected. Although the correlation between tibia breaking strength and tibia ash ($r^2=0.66$) was higher than that of Guinotte *et al.* (1991) ($r^2=0.57$), it remained lower than the correlation in the first study ($r^2=0.75$). No clear tendency could be recognized regarding the effect of distribution ratios of limestone particles on bone breaking strength, bone stress (Table 4.7) and percentage bone ash (Table 4.8) and these inconsistencies of results could be responsible for the lower correlations between bone mechanical properties noticed in the present study and will be discussed later.

4.3.2.3 Bone ash

Table 4.8 illustrates the effect of different distribution ratios of limestone particles on the percentage tibia and humerus bone as well as percentage tibia ash. It is clear that at weeks 37 and 70 of age, no significant ($P>0.05$) effect of different distribution ratios of particles on percentage right tibia and right humerus bone occurred. The effect of different distribution ratios of limestone particles on percentage tibia ash at week 70 was also not significant ($P=0.1342$).

However, different percentages of large limestone particles resulted in a significant ($P=0.0007$) effect on the percentage tibia ash at week 37 of age. No clear trend could, however, be detected regarding the effect of distribution ratios of particles on percentage tibia ash (Table 4.8). These results are in accordance with the first study (Table 4.5) where the significant ($P=0.0318$) effect of limestone particles size on percentage bone ash in week 37 of age was not characterised by a clearly illustrated tendency.

In accordance with the first study, the percentage tibia ash increased significantly ($P<0.05$) from 37 to 70 weeks of age (Table 4.4). Results of the first study suggested that large limestone particles (2.0 – 3.8 mm) resulted in the highest tibia breaking strength (292.78 N/m^2), although not significantly ($P=0.6803$) different from that of medium or small limestone particles. The percentage tibia ash in the first study was also the highest (56.43%) for large limestone (2.0 – 3.8 mm) particles, although no statistically significant ($P>0.05$) differences occurred between the largest and smallest limestone particle sizes (Table 4.5). It therefore seems that the effect of limestone particle size on tibia breaking strength and percentage ash was generally similar in the first study. During the present study, the highest tibia breaking strength (292.78 N/m^2) at week 37 of age, not significant ($P=0.3411$), was recorded for the 100% large particles limestone treatment (Table 4.7). However, the highest percentage tibia ash (59.70%) was observed in the treatment containing 25% large and 75% small limestone particles (Table 4.8) at week 37 of age. It is therefore evident that the effect of different distribution ratios on tibia breaking strength and percentage tibia ash was not similar in the second study. These differences in the effect of limestone distribution ratios on tibia breaking strength and percentage bone ash could have contributed to the lower correlations between tibia breaking strength and tibia ash ($r^2=0.66$) in the second study.

Table 4.8 The effect of different percentages of large limestone particles on percentage bone and bone ash (Mean±s.e)

Parameters	Age (weeks)	Large particles (%)					Significance	
		0	25	50	75	100	P	CV[1](%)
Tibia								
Tibia bone[2] (%)	37	0.57±0.01	0.55±0.01	0.55±0.01	0.55±0.01	0.55±0.01	0.4974	5.7
	70	0.52±0.01	0.53±0.01	0.55±0.01	0.54±0.01	0.55±0.01	0.0508	6.0
Ash (%)	37	53.99±0.74^{b}	59.70±1.08^{a}	55.91±0.89^{b}	54.43±0.90^{b}	56.43±0.99^{ab}	0.0007	5.2
	70	59.90±1.22	62.83±0.48	61.59±0.63	61.47±0.60	61.27±0.73	0.1342	3.8
Humerus								
Humerus bone[3] (%)	37	0.21±0.01	0.20±0.01	0.20±0.00	0.21±0.01	0.21±0.01	0.7155	13.6
	70	0.20±0.00	0.19±0.01	0.19±0.01	0.20±0.01	0.19±0.01	0.9119	13.6

[a,b] Figures with different superscripts within the same row differ significantly ($P<0.05$)

[1] Coefficient of variation

[2] Percentage right tibia bone = (Right tibia weight/Body weight) x 100

[3] Percentage right humerus bone = (Right humerus weight/Body weight) x 100

4.4 Conclusions

It is clearly noticeable that an increase in limestone particle size as well as an increase in the percentage large limestone particles only resulted in a significantly ($P<0.05$) higher tibia breaking strength, tibia stress and in some instances bone ash at 70 weeks of age. From the present studies, it is clear that the use of bone dimensions to predict responses to the effect of limestone particle size and/or distribution ratios yielded inexplicable results. The use of bone mechanical properties seems to be more reliable response variables for predicting the influence of limestone particle size on bone quality characteristics. The effect of limestone particle size on bone quality characteristics during the first study was more evident and well defined than the influence of distribution ratios of limestone particles during the second study. These inconsistencies regarding the effect of different distribution ratios of limestone particles necessitate further investigation. Accordingly, the effect of limestone particle size and different distribution ratios of limestone particles on egg production and eggshell quality characteristics needs investigation. It is important to determine if the increased *in vivo* limestone solubility recorded for large particles limestone, as well as the increased tibia strength and stress recorded in the present study are reflected in eggshell quality.

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CHAPTER 5

THE INFLUENCE OF LIMESTONE PARTICLE SIZE AND DISTRIBUTION RATIOS ON EGG PRODUCTION AND EGGSHELL CHARACTERISTICS

5.1 Introduction

Butcher & Miles (2005) stated that eggshell breakage is directly related to the quality of the eggshell. It is estimated that between 14.3 and 21.3% of the total number of eggs laid worldwide, are cracked (Chrystal, 2000) and due to the financial implications, eggshell quality still remains one of the primary concerns to the poultry industry. Eggshell formation normally happens during nighttime when no feed intake occurs. Scott *et al*. (1971) suggested that the prolonged retention time of large particles limestone in the gizzard provides a pool of more available calcium during night time, which could be utilized for shell formation. The results of Farmer *et al*. (1986) indicate that the maintenance of adequate calcium reserves in the gastrointestinal tract (GIT) would reduce the dependency on skeletal calcium for eggshell formation. Accordingly Scott *et al*. (1971) and Brister *et al*. (1981) indicated that the retention time of Ca particles in the digestive tract is an important factor in improving eggshell quality. Zhang & Coon (1997), Jacob *et al*. (2003) and the results in Chapter 3 indicated that an increase in limestone particle size resulted in an increased amount of limestone retained in the gizzard, allowing the limestone to stay for a longer period of time in the acidic environment of the gastrointestinal tract (GIT). This resulted in a higher *in vivo* solubility of limestone. In Chapters 3 and 4, large limestone particles resulted in a increased *in vivo* limestone solubility and increased bone breaking strength. However, a production study is needed to verify the effects of large particles limestone (high *in vivo* solubility) on egg production and eggshell quality.

Various authors reported an increase in eggshell quality (Scott *et al*., 1971; Brister *et al*., 1981; Roland, 1986; Keshavarz & McCormick, 1991) and an increased egg weight (Guinotte & Nys, 1991) by replacing a part of pulverized limestone with particulated sources of $CaCO_3$. In contrast, Cheng & Coon (1990) reported that an increase in $CaCO_3$ particle size had no influence on egg weight and egg production. However, their findings indicated that large particles $CaCO_3$ resulted in an increased shell weight, shell thickness and specific gravity of eggs. The ideal particle size of limestone for optimum eggshell quality is under continued investigation. Bristol (2006) reported that limestone with a particle size of between 0.005 and 1.18 mm are equally effective in supplying ionic calcium (Ca^{2+}) to most poultry, but suggested that the ideal limestone particle size for laying hens ranged between 1.40 and 5.60 mm.

Roland (1986) suggested that larger limestone particles must contribute 33 – 66% to the limestone mixture in diets, while Marangos (2004) is of the opinion that limestone with a particle size of 2.0 – 4.0 mm must make out 60 – 70% of limestone mixtures used as calcium supplements. In another opinion, Scheideler (2004) suggested that a 65/35 blend of fine/large particles $CaCO_3$ must be used during peak production, while a 50/50 blend of fine/large particle size limestone would result in optimum eggshell quality after the peak production period. It seems that no clear guidelines exist in literature regarding the ideal limestone particle size in layer diets, indicating a need for further investigation and clarification.

The largest supplier of limestone to feed manufacturing companies in South Africa produce approximately 8000 to 10 000 ton limestone/month of various particle sizes (G. Maritz & F.P. Viljoen, 2006, Pers. Comm., Agri Lime, P.O. Box 20366, Protea Park 0305, R.S.A.). Because of conflicting reports in literature regarding the ideal particle size for optimum eggshell quality, the need arises in South Africa to determine the effect of different particle sizes of this specific calcitic limestone source on eggshell qualities and production parameters.

The aim of the study was firstly to determine the effect of a specific South African limestone source, differing in particle size, on egg production and eggshell qualities. Secondly, the effect of different ratios of small and large particles of this specific limestone source on egg production and eggshell quality was determined.

5.2 Materials and Methods

To investigate the effect of limestone particle size and distribution ratios of particles on egg production and eggshell quality characteristics, two studies were simultaneously conducted and the same experimental protocol was implemented for both. The limestone described in Chapter 3, paragraph 3.2 was used in these studies.

5.2.1 Particle size

The limestone particles used during this study were classified as small (0 – 1.0 mm), medium (1.0 – 2.0 mm) and large (2.0 – 3.8 mm), representing the three treatments. The physical appearance of the limestone particles is illustrated in Figure 3.1, Chapter 3.

5.2.2 Distribution of particle sizes

Limestone with a particle size of 0 – 1.0 mm was mixed with that of a 2.0 – 3.8 mm particle size to obtain five ratios of 0, 25, 50, 75 and 100% small or large particles, representing the five treatments. The physical appearance of the five distribution ratios used during the second study are illustrated in Figure 3.2, Chapter 3.

5.2.3 Diet composition

The diet described in Chapter 4, paragraph 4.2.3, was used in both studies and was isocaloric and isonitrogenous for all treatments. The inclusion rate of 9.58% limestone (36% Ca), were constant across all treatments. The diets were received and fed in a mash form, to facilitate the inclusion and mixture of limestone particles. The physical composition of the complete diet with the inclusion of limestone used during the entire trial is indicated in Table 4.1, while the calculated chemical analysis is indicated in Table 4.2.

5.2.4 Birds and husbandry

The same birds as well as husbandry procedures described in Chapter 4, paragraph 4.2.2, were implemented during this study. One hundred and ninety eight Lohmann-Silver pullets, 17 weeks of age, were randomly divided into six treatments with 33 birds per treatment. Hens were placed in individual cages and housed in a naturally ventilated building with no climate control systems. Time switches were used to control daylight according to a prescribed photoperiod schedule and from 25 weeks of age a maximum of 15.5 hours of light/day were maintained.

5.2.5 Experimental measurements

5.2.5.1 Production parameters

Egg numbers were recorded daily and hen-day egg production was calculated according to Ahmad & Balander (2003) where the number of eggs produced was divided by the number of live birds in each replicate. Hen-day production were summarized on a weekly basis and then expressed as percentage weekly egg production. Shell-less eggs as well as eggs with cracks and deformations were recorded for production measurements, but were not considered for egg weight and eggshell quality data.

Individual egg weights were recorded for all eggs produced by individual hens at the age of 24, 28, 32 and 70 weeks. The total weekly egg weight was used to calculate mean egg weight for individual weeks. Egg output was determined in the former weeks according to Rose

(1997) where number of eggs produced by an individual hen is multiplied by her weekly mean egg weight. The mean egg content was calculated by deducting the mean shell weight from the mean egg weight for each week and is expressed in gram (g) (Narushin, 1977).

Feed intake was individually determined on a weekly basis for the entire experimental period. Weekly feed intake was used to calculate the average daily feed intake of individual hens. Feed efficiency was determined during weeks 24, 28, 32 and 70 of age by dividing the mean weekly feed intake by the mean weekly egg weight and expressed as gram feed per gram egg (g/g). Individual body weight (Figure 5.1) was respectively determined at 10% and 50% egg production and thereafter at the age of 24, 28, 32 and 70 weeks.

Figure 5.1 Weighing of hens

5.2.5.2 Eggshell quality

All the eggs collected at weeks 24, 28, 32 and 70 were used for the determination of certain eggshell quality parameters. After recording egg weight, the individually marked eggs were broken in two halves at the equator. The procedures described by Strong (1989) and Kul & Seker (2004) were used for washing the eggshells under slightly flowing water to remove the adhering albumen. The shells were allowed to dry for an hour at room temperature, before thickness measurements were conducted. A shell thickness meter (micrometer) (Figure 5.2), accurate to 0.01 mm were used to make three thickness measurements per measurement point (Figure 5.3), on the blunt end (BE), equator (EQ) and sharp end (SE) of each individual egg

(De Ketelaere *et al*., 2002; Ehtesham & Chowdhury, 2002; Ahmad & Balander, 2003; Kul & Seker, 2004). In total, nine shell thickness (ST) readings were recorded per egg. Shell thickness data for each hen at a specific collection period were pooled to calculate the mean ST at the BE, EQ and SE.

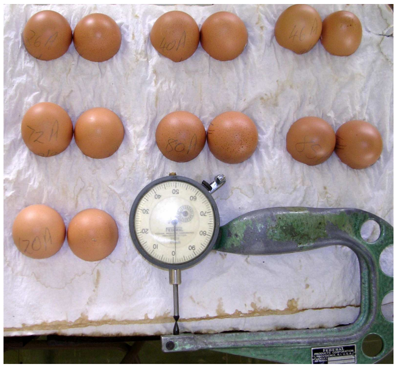

Figure 5.2 Thickness (micro-) meter with shells

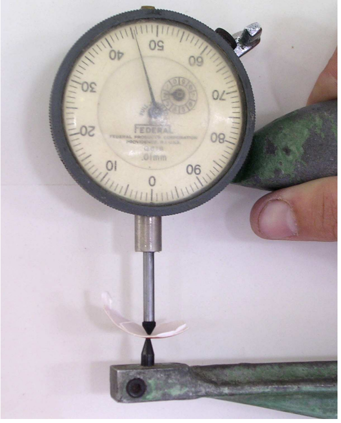

Figure 5.3 Measuring shell thickness

After recording eggshell thickness, all shells were stored individually for the determination of dry shell weight and shell ash content. The procedures of Clunies *et al*. (1992) with some alterations were used to determine dry eggshell weight and shell ash content. Individually marked crucibles were dried overnight at 105^{o}C and weighed. Individual eggshells (shell and adhering membranes) were carefully crushed into a pre-weighed crucible and dried for 12 hours at 105^{o}C. After recording dry eggshell weight, shells were ashed in a muffle furnace at 550^{o}C for 16 hours to determine the eggshell ash content.

Eggshell quality variables such as egg surface area, shell weight per unit surface area (SWUSA), percentage eggshell and calculated eggshell Ca content (g) were determined by using the following formulae:

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

5.2.6 Statistical analysis

The effect of limestone particle size (Study 1) and distribution ratios of different particle sizes (Study 2) on egg production and eggshell quality characteristics were analyzed using a fully randomized one way ANOVA design. The PROC ANOVA procedures of the SAS program (SAS, 1999) were used to test for significant differences between treatments. When significant differences were found ($P<0.05$) further multiple comparison tests, using Tukey's studentized range (HSD) test was used to identify these differences. Fisher's least-significant-difference test (LSD) was used to explain statistically significant differences with low magnitude that could not be explained by Tukey's studentized range test (HSD) in Table 5.11.

The description of the model used for PROC ANOVA analysis in the first and second study was:

$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$ Study 1: $i = 1, 2, 3; j = 1, 2, \ldots 33$
Study 2: $i = 1, 2, \ldots 5; j = 1, 2, \ldots 33$

Where: Y_{ij} = the dependant variable in the i-th trial with a j-th random error
μ = the overall population mean
τ_i = the i-th population treatment effect
ε_{ij} = the random error effect

The different, Y_{ij} dependant variables for both studies were:
Feed intake, body weight, egg production, egg output, feed efficiency, egg weight, egg content, shell weight, percentage eggshell, eggshell calcium content, egg surface area, shell weight per unit surface area (SWUSA), shell thickness at the blunt end, equator and sharp end of the egg.

The i-th treatment effect (limestone particle size) during the first study was defined as:
i_1 = <1.0 mm, i_2 = 1.0 – 2.0 mm, i_3 = 2.0 – 3.8 mm.

The i-th treatment effect (distribution ratios of small and large particles limestone) during the second study was defined as:
i_1 = 100% small, i_2 = 75% small: 25% large, i_3 = 50% small: 50% large, i_4 = 25% small: 75% large and i_5 = 100% large.

The method of Pearson was used to determine the correlation between eggshell thicknesses at the different regions of the egg.

5.3 Results and Discussion

5.3.1 Particle size

5.3.1.1 Production parameters

The results of limestone particle size on daily feed intake and body weight of hens are presented in Table 5.1. The effect of limestone particle size on both feed intake and body weight were non-significant (P>0.05) during all the weeks. These results are in accordance with the work of Guinotte & Nys (1991) and Scheideler *et al*. (2005), who reported that limestone particle size had no significant (P>0.05) influence on feed intake of laying hens. Guinotte & Nys (1991) reported that large limestone particles had no significant (P>0.05) effect on body weight of laying hens, which are supportive to the results of the present study (Table 5.1). As expected, body weight increases significantly (P<0.001) with an increase in age.

On the other hand, an inverse relationship between laying hens and broiler chicks in the utilization of different particle size Ca-supplements are clearly illustrated by Guinotte *et al*. (1991). By using ground (<0.15 mm), medium (0.30 – 1.18 mm) and large (1.18 – 4.75 mm) particles limestone in broiler diets, Guinotte *et al*. (1991) indicated that ground (<0.15 mm) limestone resulted in an increased (P<0.01) weight gain and improved (P<0.001) feed conversion. Guinotte *et al*. (1991) suggested that calcium supply at intestinal level is mainly dependent on the particle size of the calcium source, which affected its availability during the gastrointestinal transit in growing chicks. Factors affecting the GIT transit (crop and gizzard retention) and solubilization of calcium in the digestive tract might contribute to the differences in utilization of limestone particles between laying hens and broiler chicks (Scott *et al*., 1971; Rao & Roland, 1989; Guinotte *et al*., 1991).

Table 5.1 The effect of limestone particle size on daily feed intake and body weight (Mean±s.e)

		Particle size (mm)			**Significance**	
Parameter	**Age (weeks)**	**0-1.0**	**1.0-2.0**	**2.0-3.8**	**P[1]**	**CV[2](%)**
FI[3]	19	112.16±3.61	106.29±4.63	114.35±2.88	0.3043	19.2
(g/hen/day)	20	107.00±2.52	103.94±2.73	106.77±2.35	0.6372	13.5
	21	103.07±1.87	105.23±2.82	104.50±2.09	0.7943	12.4
	22	106.65±1.50	107.76±1.71	107.11±1.75	0.8908	8.7
	23	109.39±1.49	108.20±1.84	107.59±1.68	0.7441	8.7
	24	111.12±1.39	107.12±2.55	106.89±1.75	0.2335	10.2
	25	110.49±1.52	107.34±2.26	110.51±1.52	0.3640	9.3
	26	112.23±1.38	111.33±1.58	114.49±1.80	0.3562	7.9
	27	106.17±1.24	103.29±1.20	104.41±1.50	0.2977	7.1
	28	105.92±1.24	105.65±1.52	105.05±1.32	0.9023	7.3
	29	107.21±1.53	107.34±1.26	106.92±1.25	0.9758	7.1
	30	108.59±1.47	109.77±1.73	107.30±1.34	0.5220	7.9
	31	109.89±1.20	110.39±1.68	109.34±1.53	0.8834	7.6
	32	110.03±1.34	107.93±1.67	106.26±1.41	0.2047	7.7
	70	104.42±1.88	103.54±1.68	101.89±2.13	0.6389	8.6
BW[4]	20	1797.63±31.36	1781.16±34.77	1826.58±20.81	0.5548	9.3
(g/hen)	21	1859.69±30.49	1844.38±32.58	1872.77±20.62	0.7815	8.6
	24	1875.44±21.62	1882.34±25.06	1859.23±17.65	0.7449	6.5
	28	1930.16±21.68	1932.88±27.20	1901.42±17.82	0.5588	6.6
	32	1953.41±24.57	1961.16±27.86	1940.10±16.57	0.8175	6.8
	70	2000.55±42.14	1983.14±37.90	2002.48±26.52	0.9176	8.5

[1] (P>0.05) = non significant
[2] Coefficient of variation
[3] Daily feed intake
[4] Body weight

The results for egg production, egg output and feed efficiency on a weekly basis are shown in Table 5.2. Limestone particle size had generally no significant (P>0.05) effect on production except at week 26 when a significant (P=0.0367) difference between treatments was observed. The effect of limestone particle size on egg production (Table 5.2) was however not clearly illustrated by any tendency and therefore, the results should be interpreted with caution.

The results in Table 5.2 are supported by the work of Brister *et al*. (1981), Cheng & Coon (1990) and Guinotte & Nys (1991) who concluded that neither calcium source nor particle size had a significant effect on egg production of either Babcock B-300V (24 weeks of age), DeKalb-DK hens (36 weeks of age) or ISA-Brown hens (77 weeks of age). The only significant ($P<0.05$) decrease in egg production, recorded by Cheng & Coon (1990), was found when fine (<0.50 mm) particles limestone was used in the diets. Although the fine particles limestone resulted in the highest *in vitro* solubility (16.4 – 21.3%), the decreased egg production was characterized by a decrease in eggshell quality, suggesting that the retention time of the fine limestone particles was insufficient for optimum calcium solubility (Cheng & Coon, 1990).

The variation in egg production at 70 weeks of age was between 72.79% and 81.16% ($P=0.1164$). Egg output is a production parameter calculated by multiplying the number of eggs produced in a specific week by the mean egg weight (Rose, 1997). Egg output decreased significantly with an increase in limestone particle size (Table 5.2) at weeks 24 ($P=0.0519$) and 70 ($P=0.0292$) of age. During weeks 28 and 32 of age it only tended ($P>0.05$) to decrease with larger limestone particles.

Limestone particle size had no significant effect ($P>0.05$) on feed efficiency during the experimental period, which is in accordance with the results of Brister *et al*. (1981). Guinotte & Nys (1991) also reported that limestone particle size had no significant ($P>0.05$) effect on the efficiency of feed utilization by laying hens. In contrast, Guinotte *et al*. (1991) indicated that small (<0.15 mm) calcium particles resulted in a significantly ($P<0.001$) decreased feed conversion in male broiler chicks.

Table 5.2 The effect of limestone particle size on egg production, egg output and feed efficiency (Mean±s.e)

		Particle size (mm)			**Significance**	
Parameter	**Age (week)**	**0-1.0**	**1.0-2.0**	**2.0-3.8**	**P**	**CV^{4}(%)**
EP^{1} (%)	19	2.23±1.83	0.89±0.62	1.38±1.02	0.7487	472.9
	20	33.93±5.87	38.39±6.03	38.71±5.22	0.8041	87.2
	21	83.48±4.36	74.11±6.36	81.11±5.40	0.4475	38.5
	22	95.98±2.74	87.95±4.89	95.39±2.24	0.1911	21.2
	23	96.43±1.28	93.75±3.14	94.47±1.27	0.6468	12.5
	24	98.66±0.75	95.98±1.15	96.31±1.14	0.1375	6.0
	25	95.98±1.15	95.98±1.15	92.62±1.30	0.0826	7.1
	26	97.32±1.00^{ab}	94.64±1.40^{b}	98.62±0.77^{a}	0.0367	6.4
	27	96.87±1.24	97.32±1.00	95.39±1.39	0.4731	6.7
	28	95.98±1.32	95.98±1.15	97.23±1.23	0.7130	7.2
	29	97.32±1.00	97.77±1.13	94.93±1.25	0.1698	6.6
	30	96.43±1.43	95.98±1.15	94.93±1.41	0.7217	7.9
	31	95.09±1.22	94.64±1.24	93.55±1.86	0.7472	8.7
	32	95.53±1.35	94.64±1.40	92.63±2.47	0.5086	10.7
	70	79.22±1.81	81.16±1.97	72.79±4.40	0.1164	17.5
EO^{2} (g)	24	364.75±4.02^{a}	355.95±6.38^{ab}	345.10±6.17^{b}	0.0519	8.9
	28	369.19±6.89	367.45±6.09	362.59±6.46	0.7598	10.0
	32	376.81±6.19	375.94±7.46	355.89±10.21	0.1254	12.3
	70	351.05±9.80^{a}	346.83±7.68^{ab}	308.61±17.00^{b}	0.0292	16.6
FE^{3} (g/g)	24	2.11±0.03	2.06±0.03	2.09±0.04	0.5203	8.6
	28	1.93±0.03	1.93±0.02	1.98±0.02	0.3799	6.9
	32	1.95±0.02	1.91±0.02	1.94±0.02	0.3747	7.2
	70	1.66±0.04	1.70±0.03	1.67±0.04	0.7458	10.7

[a,b] Figures with different superscripts within the same row differ significantly ($P<0.05$)

[1] Egg production (%)

[2] Egg output (g)

[3] Feed efficiency (g feed/g egg)

[4] Coefficient of variation

Production data were further investigated by pooling the data from weeks 19 to 32 of age and analyzing it on a mean basis. These mean values for daily feed intake, body weight, egg production, egg output and feed efficiency are shown in Table 5.3. With the exception of egg output, these results support the findings as discussed earlier in Table 5.2. No significant (P>0.05) differences occurred in feed intake, body weight, egg production and feed efficiency when data of the individual weeks were combined. Limestone particle size had a significant effect on egg output during weeks 24 (P=0.0519) and 70 (P=0.0292) (Table 5.2). However the effect of limestone particle size on the mean egg output (weeks 19 to 32) was not significant (P=0.0928) (Table 5.3).

Table 5.3 The influence of limestone particle size on the mean values of feed intake, body weight, egg production, egg output and feed efficiency during weeks 19 to 32 of age (Mean±s.e)

			Significance	
Parameter	**Particle size (mm)**	**Mean±s.e.**	**P[1]**	**CV[2](%)**
Feed intake (g/hen/day)	0-1.0	108.56±1.00		
	1.0-2.0	107.26±1.36	0.7429	6.3
	2.0-3.8	107.96±1.23		
Body weight (g/hen)	0-1.0	1883.26±23.49		
	1.0-2.0	1880.38±27.96	0.9942	7.0
	2.0-3.8	1880.02±16.50		
Egg production (%)	0-1.0	84.37±0.88		
	1.0-2.0	82.72±1.39	0.5846	7.7
	2.0-3.8	83.38±1.08		
Egg output (g)	0-1.0	370.25±4.54		
	1.0-2.0	366.45±4.90	0.0928	8.1
	2.0-3.8	354.52±6.16		
Feed efficiency (g feed/g egg)	0-1.0	2.00±0.02		
	1.0-2.0	1.97±0.02	0.4164	6.0
	2.0-3.8	2.00±0.02		

[1] (P>0.05) = non significant

[2] Coefficient of variation

5.3.1.2 Egg weight and egg content

The effect of limestone particle size on egg weight and egg content is illustrated in Table 5.4. Particle size had no significant (P>0.05) effect on egg weight and egg content, during the specific collection weeks of this study. There was only a tendency (P>0.05) for these two egg parameters to be lower when large (2.0 – 3.8 mm) limestone particles were used in the diet. The results of the present study are in accordance with that of Brister *et al*. (1981), Hamilton *et al*. (1985) and Cheng & Coon (1990) who reported that particle size had no significant (P>0.05) influence on egg weight. However, the results of the present study are in contrast with the work of Guinotte & Nys (1991) who reported that limestone particle size had a significant (P<0.05) effect on egg weight. They found that particulated limestone resulted in heavier eggs (68.54±6.7 g *vs*. 66.02±4.7 g) compared to ground limestone. However, this increase in egg weight as a result of an increased particle size was only observed when Guinotte & Nys (1991) used limestone. Neither particulated seashells nor oyster shells had result in significant differences of egg weights. Guinotte & Nys (1991) recorded a significant (P<0.001) interaction between calcium source x particle size on egg weight, suggesting that the influence of limestone particle size on egg weight differs between calcium sources. The effect (P=0.0530) of limestone particle size on egg weight during week 24 (Table 5.4) could contribute to the differences observed in egg output (Table 5.2).

Table 5.4 The effect of limestone particle size on egg weight and egg content of laying hens (Mean±s.e)

		Particle size (mm)			**Significance**	
Parameter	**Age (week)**	**0-1.0**	**1.0-2.0**	**2.0-3.8**	**P^1**	**CV^2(%)**
Egg weight (g)	24	52.82±0.44	52.93±0.59	51.17±0.65	0.0530	6.1
	28	54.93±0.65	54.69±0.60	53.21±0.53	0.0954	6.2
	32	56.38±0.56	56.75±0.75	54.94±0.60	0.1182	6.2
	70	63.27±0.92	61.26±0.98	61.42±1.19	0.3133	7.7
Egg content (g)	24	47.48±0.41	47.59±0.11	45.90±0.59	0.0447	6.3
	28	49.54±0.61	49.31±0.56	47.90±0.49	0.0855	6.4
	32	50.87±0.52	51.17±0.72	49.51±0.55	0.1236	6.7
	70	57.41±0.86	55.46±0.89	55.52±1.11	0.2647	7.9

[1] (P>0.05) = non significant

[2] Coefficient of variation

5.3.1.3 Eggshell quality characteristics

The effect of limestone particle size on shell weight, percentage eggshell, eggshell calcium content, eggshell ash, egg surface area and SWUSA is presented in Table 5.5. The effect of limestone particle size on these eggshell characteristics was not statistically significant (P>0.05).

Table 5.5 The effect of limestone particle size on eggshell characteristics of laying hens (Mean±s.e)

Parameter	Age (week)	Particle size (mm) 0-1.0	1.0-2.0	2.0-3.8	Significance P[1]	CV[2](%)
Shell weight (g)	24	5.34±0.06	5.34±0.05	5.27±0.06	0.6426	6.1
	28	5.39±0.05	5.38±0.06	5.32±0.06	0.6013	5.9
	32	5.51±0.06	5.58±0.06	5.43±0.05	0.2153	5.8
	70	5.86±0.11	5.80±0.15	5.91±0.12	0.8438	10.2
Eggshell (%)	24	10.12±0.08	10.11±0.11	10.31±0.07	0.1680	4.8
	28	9.83±0.07	9.85±0.09	10.00±0.07	0.2744	4.5
	32	9.77±0.07	9.85±0.10	9.90±0.07	0.5414	4.6
	70	9.30±0.14	9.47±0.20	9.63±0.16	0.3152	8.2
Eggshell Ca (g)	24	1.99±0.02	1.99±0.02	1.97±0.02	0.6423	6.1
	28	2.01±0.02	2.01±0.02	1.98±0.02	0.6014	5.9
	32	2.05±0.02	2.08±0.02	2.03±0.02	0.2154	5.8
	70	2.19±0.04	2.16±0.06	2.20±0.05	0.8437	10.2
Eggshell ash (g)	24	0.25±0.00	0.25±0.00	0.25±0.00	0.7846	9.2
	28	0.26±0.00	0.25±0.00	0.25±0.00	0.6481	7.9
	32	0.26±0.00	0.26±0.00	0.26±0.00	0.6890	7.4
	70	0.25±0.00	0.25±0.00	0.25±0.01	0.6711	10.0
ESA[3] (cm^2)	24	65.34±0.39	65.43±0.51	63.88±0.57	0.0507	4.3
	28	67.15±0.56	66.95±0.52	65.68±0.46	0.0982	4.4
	32	68.41±0.48	68.71±0.65	67.17±0.51	0.1214	4.6
	70	74.20±0.77	72.52±0.82	72.65±0.98	0.3069	5.4
SWUSA[4] (mg/cm^2)	24	81.74±0.64	81.65±0.74	82.53±0.53	0.5767	4.4
	28	80.28±0.50	80.38±0.67	80.93±0.57	0.7004	4.1
	32	80.49±0.54	81.22±0.69	80.90±0.51	0.6747	4.1
	70	78.95±1.16	79.88±1.70	81.28±1.26	0.5007	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

The effect of limestone particle size on eggshell thickness (blunt end, equator and sharp end) is shown in Table 5.6. The general effect of limestone particle size on shell thickness was not significant ($P>0.05$) during the individual weeks. The only significant ($P<0.0001$) effect of particle size on shell thickness (sharp end) was observed at week 32 of age when large limestone particles resulted in thinner eggshells at the sharp end of the egg. These results are inexplicable and difficult to interpret. The results of the present study are in contrast with that of Brister *et al*. (1981), Cheng & Coon (1990) and Guinotte & Nys (1991) who concluded that an increase in calcium particle size resulted in a significant ($P<0.05$) increase in eggshell weight, specific gravity, shell thickness, SWUSA and shell breaking strength. In the present study non-significant ($P>0.05$) differences were observed by comparing more extreme particles sizes than these researchers. The ground limestone used by Guinotte & Nys (1991) was smaller than 0.6 mm (0.075 – 0.60 mm) while the size of the particulated limestone varied from 1.18 – 2.36 mm. The smallest limestone particles used in the present study varied between 0 – 1.0 mm (mean 0.5 mm), while the largest particles varied between 2.0 – 3.8 mm (mean 2.9 mm).

Table 5.6 The effect of limestone particle size on eggshell thickness (Mean±s.e)

		Particle size (mm)			Significance	
Parameter	**Age (week)**	**0-1.0**	**1.0-2.0**	**2.0-3.8**	**P**	**CV^1(%)**
Blunt end (mm)	24	0.43±0.00	0.43±0.00	0.43±0.00	0.7790	4.5
	28	0.41±0.00	0.41±0.00	0.41±0.00	0.8863	4.5
	32	0.40±0.00	0.41±0.00	0.40±0.00	0.7012	4.4
	70	0.36±0.01	0.36±0.01	0.36±0.01	0.9493	10.3
Equator (mm)	24	0.44±0.00	0.44±0.00	0.44±0.00	0.3693	3.9
	28	0.42±0.00	0.42±0.00	0.42±0.00	0.8674	4.1
	32	0.41±0.00	0.43±0.00	0.42±0.00	0.0888	4.0
	70	0.38±0.01	0.37±0.01	0.39±0.01	0.7872	9.2
Sharp end (mm)	24	0.43±0.00	0.43±0.00	0.43±0.00	0.8950	4.8
	28	0.42±0.00	0.41±0.00	0.41±0.00	0.5265	4.1
	32	0.42^a±0.00	0.42^a±0.00	0.36^b±0.00	0.0001	5.7
	70	0.36±0.01	0.37±0.01	0.38±0.01	0.6355	10.4

[a,b] Figures with different superscripts within the same row differ significantly ($P<0.05$)

[1] Coefficient of variation

The prolonged retention time of larger calcium particles in the GIT of laying hens, as suggested by Scott *et al*. (1971), Rao & Roland (1989), Rao & Roland (1990) and Zhang &

Coon (1997), allowed the large particles calcium to stay a prolonged period of time in the acidic environment of the gizzard, resulting in a constant metering of Ca^{2+} into the blood. However, the physical particle sizes used by various authors differ and therefore inconsistencies regarding the optimum particle size for prolonged gizzard retention exist. The optimum particle size for eggshell quality is also not specified in literature for different calcium sources and researchers tend to use the terms particulated, coarse, large particles, ground or small particles, without referring to the specific particle size in millimeter. The literature review of Roland (1986) clearly illustrated that the various responses of hens fed different sources and sizes of $CaCO_3$ are mainly because the $CaCO_3$ sources used for comparison are not always within the same particle size or solubility range

The results of Cheng & Coon (1990) suggested, as already discussed, that a significant difference in eggshell weight, specific gravity, shell thickness and SWUSA exists between the two smallest calcium particles (0.15 and 0.50 mm) and the larger particles (1.02, 1.68, 2.38 and 3.36 mm). The study of Cheng & Coon (1990) failed to illustrate any significant ($P>0.05$) differences between the larger calcium particles 1.02, 1.68, 2.38 and 3.36 mm. The limestone particles used during the present study (0 – 1.0, 1.0 – 2.0 and 2.0 – 3.8 mm) were in the same range as the larger particles used by Cheng & Coon (1990). Therefore the non-significant differences between particle size and eggshell quality characteristics observed in the present study compared favorable with that of Cheng & Coon (1990) when the same particle sizes are compared.

Because particle size resulted in no significant differences in egg weight, egg content and eggshell quality characteristics, data from week 24 to 32 of age (peak period) were pooled together and analysed on a mean basis. The mean values for these characteristics are shown in Table 5.7. It is clear that limestone particle size had no significant ($P>0.05$) influence on egg weight, egg content as well as the eggshell quality characteristics when compared on an mean value for week 24 to 32 of age.

The influence of particle size on egg weight, egg content, eggshell characteristics and eggshell thickness as illustrated in Tables 5.4, 5.5, 5.6 and 5.7 are consistent and indicate that limestone particle size had a non-significant ($P>0.05$) influence at either individual weeks or on the means of the production period (24 to 32 weeks of age). Although it seem that an increase in particle size resulted in a decrease of mean egg weight, egg content and egg surface area (Table 5.7), these observations was not supported by Tukey's (HSD) test and

Table 5.7 The influence of limestone particle size on the mean values of egg weight, egg content and eggshell quality characteristics during weeks 24 to 32 of age (Mean±s.e)

Parameter	Particle size (mm)	Mean±s.e.	Significance	
			P	CV[2](%)
Egg weight (g)	0-1.0	54.71±0.49		
	1.0-2.0	54.79±0.59	0.0557	5.7
	2.0-3.8	53.11±0.55		
Shell weight (g)	0-1.0	5.41±0.05		
	1.0-2.0	5.43±0.05	0.4235	5.3
	2.0-3.8	5.34±0.05		
Egg content (g)	0-1.0	49.30±0.46		
	1.0-2.0	49.36±0.56	0.0517	5.9
	2.0-3.8	47.77±0.51		
Eggshell (%)	0-1.0	9.91±0.07		
	1.0-2.0	9.94±0.09	0.2707	4.3
	2.0-3.8	10.07±0.06		
Eggshell Ca content (g)	0-1.0	2.02±0.02		
	1.0-2.0	2.03±0.02	0.4236	5.3
	2.0-3.8	1.99±0.02		
Eggshell ash (g)	0-1.0	0.25±0.00		
	1.0-2.0	0.25±0.00	0.9616	7.2
	2.0-3.8	0.25±0.00		
Egg surface area (cm^2)	0-1.0	66.97±0.43		
	1.0-2.0	67.03±0.51	0.0560	4.0
	2.0-3.8	65.58±0.48		
SWUSA (mg/cm^2)[1]	0-1.0	80.84±0.51		
	1.0-2.0	81.08±0.65	0.7301	3.8
	2.0-3.8	81.45±0.48		
Shell thickness				
Blunt end (mm)	0-1.0	0.41±0.00		
	1.0-2.0	0.41±0.00	0.9404	4.2
	2.0-3.8	0.41±0.00		
Equator (mm)	0-1.0	0.42±0.00		
	1.0-2.0	0.43±0.00	0.3157	3.6
	2.0-3.8	0.42±0.00		
Sharp end (mm)	0-1.0	0.42±0.00[a]		
	1.0-2.0	0.42±0.00[a]	0.0001	4.3
	2.0-3.8	0.40±0.00[b]		

[a, b] Parameter means±s.e. within a column with different superscripts differ significantly ($P<0.05$)

[1] SWUSA - Shell weight per unit surface area

[2] Coefficient of variation

therefore regarded as non-significant ($P>0.05$). As in Table 5.6, the only significant ($P<0.0001$) effect of limestone particle size was observed on shell thickness at the sharp end of the egg. As discussed earlier, these significant differences occurred only at the sharp end of the egg and not at the blunt end ($P=0.9404$) or equator ($P=0.3157$) and are therefore difficult to explain and/or interpret.

A significant ($P<0.0001$) moderate correlation ($r^2=0.67$) between eggshell thickness at the blunt end and equator of the eggshell were recorded. The significant ($P<0.0001$) correlations between the blunt end and sharp end ($r^2=0.45$) and equator and sharp end ($r^2=0.38$) was, however, low. From the correlation coefficients it is noticeable that shell thickness measurements at the sharp end of the egg were not as well related to the other measurement areas BE and EQ. These results are in accordance with the differences observed in Tables 5.6 and 5.7 between shell thicknesses at the sharp end, compared to the other two measurement areas.

5.3.2 Distribution ratios of particles

5.3.2.1 Production parameters

The effect of different distribution ratios of limestone particles on daily feed intake and body weight of hens are presented in Table 5.8. The influence of different distribution ratios of limestone particles on feed intake and body weights was not significant ($P>0.05$) when data was analyzed for individual weeks. The results of the present study are in accordance with the work of Brister *et al.* (1981), Bryant & Roland (1999), Scheideler (2004) and Scheideler *et al*. (2005) who reported that different mixtures of $CaCO_3$ particles had a not significant ($P>0.05$) effect on feed intake. The work of Guinotte & Nys (1991) and the data of the first study (Table 5.1) also suggested that limestone particle size had non-significant ($P>0.05$) influence on feed intake and body weight. It is thus supportive to the results of the present study.

Table 5.8 The effect of different percentages of large limestone particles on daily feed intake and body weight (Mean±s.e.)

		Large particles limestone (%)					**Significance**	
Parameter	**Age (week)**	**0**	**25**	**50**	**75**	**100**	**P^1**	**CV^2(%)**
Feed intake (g/hen/day)	19	112.16±3.61	112.11±2.75	109.52±3.14	106.62±3.00	114.35±2.88	0.4444	15.8
	20	107.00±2.52	104.79±2.18	103.54±2.97	103.54±2.81	106.77±2.35	0.7924	14.0
	21	103.07±1.87	106.02±1.86	104.11±2.48	103.95±2.23	104.50±2.09	0.9841	11.6
	22	106.65±1.50	107.41±2.21	108.73±1.56	112.06±1.86	107.11±1.75	0.1899	9.3
	23	109.39±1.49	107.65±1.86	106.83±1.75	110.34±1.73	107.59±1.68	0.5641	8.9
	24	111.12±1.39	108.66±1.74	109.55±1.74	109.84±1.58	106.89±1.75	0.4658	8.5
	25	110.49±1.52	108.63±2.13	111.88±1.88	113.90±1.81	110.51±1.52	0.3049	9.1
	26	112.23±1.38	112.77±1.58	114.26±2.03	115.90±1.76	114.49±1.80	0.5710	8.6
	27	106.17±1.24	102.85±1.29	100.92±1.17	101.86±1.29	104.41±1.50	0.0767	7.8
	28	105.92±1.24	104.63±1.37	105.47±1.69	105.15±1.18	105.06±1.32	0.9744	7.4
	29	107.21±1.53	105.19±1.54	107.67±1.74	106.65±1.32	106.92±1.25	0.8142	7.9
	30	108.58±1.47	107.43±1.46	109.19±2.27	108.63±1.73	107.29±1.34	0.9187	8.9
	31	109.89±1.20	109.93±1.64	110.47±2.17	110.88±1.76	109.34±1.53	0.9744	8.7
	32	110.03±1.34	107.92±1.51	109.80±1.81	109.20±1.72	106.26±1.41	0.4284	8.2
	70	104.42±1.88	101.39±1.69	101.44±1.26	104.57±1.53	101.89±2.13	0.4693	7.8
Body weight (g) (g/hen)	20	1797.63±31.36	1806.19±28.19	1786.19±27.44	1828.12±43.42	1826.58±20.81	0.8541	9.9
	21	1859.69±30.49	1860.48±27.81	1853.19±24.09	1850.24±25.80	1872.77±20.62	0.9784	7.9
	24	1875.44±21.62	1883.00±25.99	1891.34±24.77	1871.18±24.27	1859.23±17.65	0.8960	7.0
	28	1930.16±21.68	1932.39±25.44	1933.28±25.35	1915.53±26.40	1901.42±17.82	0.8541	7.0
	32	1953.41±24.57	1964.77±29.14	1961.28±27.02	1948.79±27.25	1940.10±16.57	0.9638	7.4
	70	2000.55±42.14	2011.05±40.47	1999.81±34.49	2006.58±43.42	2002.48±26.52	0.9996	8.9

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[1] (P>0.05) = non significant

[2] Coefficient of variation

The results of the different distribution ratios of limestone particles on egg production, egg output and feed efficiency can be observed in Table 5.9. With the exception of week 25, no significant (P>0.05) effect could be recorded on egg production, egg output and feed efficiency. The significant difference in egg production, observed during week 25 of the present study was, however, not supported during any other week and therefore the effect of different distribution ratios of limestone particles, as well as limestone particle size on egg production (Table 5.2) could generally be described as non-significant. In the first study (Table 5.2) the effect of limestone particle size on egg production was generally also not significant, except during week 26, when limestone of a 1.0 – 2.0 mm particle size resulted in a significant (P=0.0367) lower egg production than that of the 2.0 – 3.8 mm particle size. Bryant & Roland (1999), Scheideler (2004) and Scheideler *et al* (2005) concluded that different ratios of large and small particles limestone had no significant (P>0.05) effect on egg production.

The results of the first study (Table 5.2) did not support the non-significant (P>0.05) influence of particle size on egg output of the second study (Table 5.9). On the other hand, the results of the first study (Table 5.2) are in support of the present study, where distribution ratios of limestone particle size had no significant (P>0.05) effect on the efficiency of feed utilization by laying hens. Brister *et al*. (1981), Guinotte & Nys (1991) and Bryant & Roland (1999) also reported that limestone particle size had no significant (P>0.05) effect on feed efficiency, illustrating that neither limestone particle size nor mixtures of different limestone particles had a significant effect on the efficiency of feed utilization by laying hens. Guinotte & Nys (1991) also indicated that the calcium origin x particle size interaction on feed efficiency was not significant (P>0.05) and indicated that different calcium sources had no effect of the efficiency of feed utilization. Feed efficiency is calculated by using the weekly feed intake and the weekly egg weight. As limestone particle size (Tables 5.1 and 5.4) and distribution ratios of limestone particles (Tables 5.8 and 5.11) had no significant (P>0.05) effect on feed intake and egg weight, it could be accepted that feed efficiency would also be unaffected by particle size and mixtures of limestone particle sizes.

Table 5.9 The effect of different percentages of large limestone particles on egg production, egg output and feed efficiency (Mean±s.e.)

		Large particles limestone (%)					Significance	
Parameter	**Age (week)**	**0**	**25**	**50**	**75**	**100**	**P**	**CV^1(%)**
Egg production (%)	19	2.23±1.83	2.76±1.92	0.00±0.00	2.94±2.54	1.38±1.02	0.7467	524.0
	20	33.93±5.87	36.87±4.90	30.36±5.66	39.08±5.30	38.71±5.22	0.7652	85.5
	21	83.48±4.36	80.64±4.80	79.02±5.37	89.50±3.32	81.11±5.40	0.5284	31.9
	22	95.98±2.74	96.77±1.44	93.75±2.72	97.90±0.88	95.39±2.24	0.7000	12.5
	23	96.43±1.28	96.77±1.09	97.77±1.13	97.06±1.01	94.47±1.27	0.3484	6.8
	24	98.66±0.75	95.39±1.54	95.54±1.50	97.06±1.17	96.31±1.14	0.3404	7.3
	25	95.98±1.15^{ab}	96.31±1.48^{ab}	93.30±1.57^{a}	98.74±0.71^{b}	92.62±1.30^{a}	0.0049	7.5
	26	97.32±1.00	94.23±1.41	95.98±1.47	96.64±1.05	98.62±0.77	0.2470	6.8
	27	96.90±1.06	96.77±1.09	95.98±1.60	98.32±0.80	95.39±1.39	0.4949	7.1
	28	95.98±1.32	96.77±1.44	93.75±1.92	96.64±1.05	97.24±1.23	0.4431	8.3
	29	97.32±1.00	95.85±1.51	96.43±1.70	97.48±0.95	94.93±1.25	0.6229	7.6
	30	96.43±1.43	95.39±1.39	91.96±2.56	97.48±1.12	94.93±1.41	0.1766	9.8
	31	95.09±1.22	95.85±1.18	94.64±2.00	96.64±1.05	93.55±1.85	0.6511	8.9
	32	95.54±1.35	94.93±1.56	96.43±1.57	97.48±0.95	92.63±2.47	0.2914	9.7
	70	79.22±1.81	79.59±2.53	75.51±3.44	82.14±2.62	72.79±4.40	0.2138	18.2
Egg output (g)	24	364.75±4.02	349.18±8.33	352.97±6.89	355.06±6.16	345.10±6.17	0.2716	10.3
	28	369.19±6.89	367.68±6.86	357.58±8.98	366.41±6.14	362.59±6.46	0.7887	11.1
	32	376.81±6.19	368.74±7.62	381.91±8.53	376.41±5.10	355.89±10.21	0.1472	11.6
	70	351.05±9.80	339.03±11.33	323.17±15.80	346.00±10.14	308.61±17.00	0.1345	18.1
Feed efficiency (g feed/g egg)	24	2.11±0.03	2.09±0.03	2.08±0.03	2.11±0.03	2.09±0.04	0.9693	8.6
	28	1.93±0.03	1.93±0.02	1.94±0.03	1.95±0.02	1.98±0.02	0.6579	6.7
	32	1.95±0.02	1.95±0.02	1.95±0.03	1.98±0.02	1.94±0.02	0.8095	7.3
	70	1.66±0.04	1.67±0.04	1.67±0.03	1.74±0.03	1.67±0.04	0.5629	10.4

[a, b] Figures with different superscripts within the same row differ significantly ($p<0.05$)

[1] Coefficient of variation

As described before, data from week 19 to 32 were pooled together and analysed on a mean basis. The effect of different distribution ratios of limestone particles on the mean values for daily feed intake, body weight, egg production, egg output and feed efficiency are shown in Table 5.10. This method of comparison revealed no significant (P>0.05) effect of different

Table 5.10 The effect of different percentages of large limestone particles on the mean feed intake, body weight, egg production, egg output and feed efficiency during weeks 19 to 32 of age (Mean±s.e)

			Significance	
Parameter	**Large particles (%)**	**Mean±s.e.**	**P[1]**	**CV[2](%)**
Feed intake	0	108.56±1.00		
(g/hen/day)	25	107.36±1.47		
	50	108.00±1.57	0.9740	7.1500
	75	108.47±1.47		
	100	107.96±1.23		
Body weight	0	1883.26±23.49		
(g/hen)	25	1889.37±25.75		
	50	1885.06±23.67	0.9992	7.1032
	75	1882.77±26.75		
	100	1880.02±16.50		
Egg production (%)	0	84.37±0.88		
	25	84.00±0.82		
	50	82.49±1.20	0.1424	6.5911
	75	85.92±0.86		
	100	83.38±1.08		
Egg output (g)	0	370.25±4.54		
	25	361.87±6.18		
	50	364.15±7.27	0.4221	9.0841
	75	365.96±4.74		
	100	354.52±6.16		
Feed efficiency	0	2.00±0.02		
(g feed/g egg)	25	1.99±0.02		
	50	1.99±0.03	0.9570	6.5194
	75	2.01±0.02		
	100	2.00±0.02		

[1] (P>0.05) = non significant

[2] Coefficient of variation

mixtures of large and small particles limestone on feed intake, body weight, egg production, egg output and feed efficiency.

Data in Tables 5.8, 5.9 and 5.10 clearly illustrate that different distribution ratios of limestone particles had no significant ($P>0.05$) effect on the measured production parameters in either individual weeks, or during the average of weeks 19 to 32. These results are in accordance with that of the first study (Tables 5.1, 5.2 and 5.3) where the effect of limestone particle size was also not significant ($P>0.05$) on feed intake, body weight, egg production and feed efficiency. Particle size had a significant ($P<0.05$) influence on egg output at only two individual weeks during the first study. Therefore the effect of limestone particles as well as different distribution ratios of limestone particles on any of the parameters illustrated in Tables 5.3 and 5.10 could generally be described as non-significant ($P>0.05$).

5.3.2.2 Egg weight and egg content

The effect of different distribution ratios of limestone particles on egg weight and egg content are illustrated in Table 5.11. The effect of different mixtures of large and small limestone particles on egg weight and egg content was not significant ($P>0.05$) during the various collection weeks. These results are in accordance with that of Bryant & Roland (1999), Scheideler (2004) and Scheideler *et al.* (2005) who reported that different distribution ratios of limestone particles had no significant influence on egg weight in Hy-Line W-36 and Hy-Line W-98 hens. The results of the first study (Table 5.4) and that of Brister *et al*. (1981), Hamilton *et al*. (1985) and Cheng & Coon (1990) indicated that limestone particle size had no significant ($P>0.05$) effect on egg weight are supportive to the results of the present study.

During the present study, the effect of different distribution ratios of limestone particles on egg content was statistically non-significant ($P>0.05$). Egg weight and shell weight are the two factors affecting the calculation of egg content. In the present study, no significant differences were recorded between egg weight and shell weight, explaining the non significant ($P>0.05$) differences observed for egg content. The results of the first study (Table 5.4) where the influence of limestone particle size on egg content was non-significant ($P>0.05$) are supportive to the results of the present study.

Table 5.11 The effect of different percentages of large limestone particles on egg weight and egg content (Mean±s.e.)

		Large particle limestone (%)					**Significance**	
Parameter	**Age (week)**	**0**	**25**	**50**	**75**	**100**	**P[1]**	**CV[2](%)**
Egg weight (g)	24	52.82±0.44	52.14±0.65	52.78±0.58	52.22±0.56	51.17±0.65	0.2703	6.3
	28	54.93±0.65	54.31±0.67	54.45±0.70	54.12±0.59	53.21±0.53	0.4216	6.6
	32	56.38±0.56	55.50±0.68	56.54±0.75	55.19±0.57	54.94±0.60	0.2876	6.4
	70	63.27±0.92	61.05±1.21	61.01±1.07	60.48±0.84	61.42±1.19	0.3698	7.9
Egg content (g)	24	47.48±0.41	46.92±0.61	47.49±0.54	46.86±0.51	45.90±0.59	0.2220	6.5
	28	49.54±0.61	48.99±0.63	49.10±0.65	48.75±0.54	47.90±0.49	0.3797	6.8
	32	50.87±0.52	50.07±0.64	51.00±0.70	49.69±0.54	49.51±0.55	0.2682	6.7
	70	57.41±0.86	55.32±1.15	55.31±0.98	54.70±0.79	55.52±1.11	0.3419	8.2

[1] (P>0.05) = non significant

[2] Coefficient of variation

5.3.2.3 Eggshell quality characteristics

Data regarding the effect of different distribution ratios of large and small limestone particles on eggshell quality characteristics are presented in Table 5.12. No statistical significant (P>0.05) influence of different mixtures of limestone particles on eggshell weight, percentage eggshell, eggshell calcium content, eggshell ash, egg surface area and SWUSA (Table 5.12) was observed. These results of the present study regarding eggshell quality characteristics are in accordance with that of the first study (Table 5.5) where different limestone particle sizes had no significant (P>0.05) effect on eggshell quality characteristics.

The only significant (P=0.0476) effect of different mixtures of limestone particles was detected for percentage eggshell at week 24. However, the magnitude of this difference between treatments was not adequate for explanation by Tukey's studentized range honest significant difference test (HSD). Therefore, Fisher's least-significant-difference (LSD) test was then used for multiple comparisons between treatments. Although the LSD test revealed that limestone ratios with 25% and 50% large particles resulted in the lowest (P<0.05) percentage eggshell at week 24, no clear trend could be detected. The significant results of Fisher's least-significant-difference test (LSD) was expected because it had a lower degree of accuracy and revealed significant differences easier than the more comprehensive HSD test of Tukey. However, to ensure higher accuracy, it was decided to interpret all data in Table 5.12 according to Tukey's studentized range test (HSD) which did not support the observed significant differences (P=0.0476) of the analysis of variance.

Scheideler (2004) and Scheideler *et al*. (2005) reported that percentage eggshell and specific gravity were significantly (P<0.05) lower when Hy-Line W-36 and W-98 hens consume diets with 100% fine particles limestone from week 21 to 40 of age. However, the results of Scheideler *et al*. (2005) indicated that as soon a hens were switched from a 100% fine particle size limestone diet to a diet containing 50% small and 50% large particles, all eggshell quality measurements recovered to normal. By switching the fine:large limestone particles in the mixture from 75:25 (18 – 20 weeks) to 65:35 (21 – 40 weeks) to 40:60 (51 – 60 weeks) and to 30:70 (61 – 70 weeks of age), no significant (P>0.05) effect on eggshell quality was found, suggesting that a 50:50 mixture will meet the needs of the laying hen for optimal egg production and shell quality. These observations were, however, not supported by the results of the present study.

Table 5.12 The effect of different percentages large limestone particles on eggshell quality characteristics (Mean±s.e.)

		Large particles limestone (%)					**Significance**	
Parameter	**Age (week)**	**0**	**25**	**50**	**75**	**100**	**P**	**CV[3](%)**
Shell weight (g)	24	5.34±0.06	5.21±0.06	5.29±0.06	5.36±0.07	5.27±0.06	0.4868	6.6
	28	5.39±0.05	5.32±0.06	5.35±0.07	5.37±0.06	5.32±0.06	0.8981	6.4
	32	5.51±0.06	5.43±0.06	5.54±0.06	5.50±0.07	5.43±0.05	0.6361	6.2
	70	5.86±0.11	5.73±0.13	5.70±0.15	5.78±0.13	5.91±0.12	0.7742	10.2
Eggshell (%)	24	10.12±0.08^{ab}	10.02±0.10^{b}	10.03±0.08^{b}	10.17±0.09^{a}	10.31±0.07^{a}	0.0476	4.8
	28	9.83±0.07	9.82±0.07	9.84±0.08	9.94±0.08	10.00±0.07	0.3570	4.3
	32	9.77±0.07	9.79±0.07	9.81±0.07	9.98±0.12	9.90±0.07	0.3659	4.9
	70	9.30±0.14	9.42±0.19	9.35±0.19	9.57±0.19	9.63±0.16	0.5728	8.8
Eggshell Ca (g)	24	1.99±0.02	1.95±0.02	1.97±0.02	2.00±0.03	1.97±0.02	0.4869	6.6
	28	2.01±0.02	1.99±0.02	2.00±0.03	2.00±0.02	1.98±0.02	0.8981	6.4
	32	2.05±0.02	2.02±0.02	2.07±0.02	2.05±0.03	2.03±0.02	0.6364	6.2
	70	2.19±0.04	2.14±0.05	2.13±0.05	2.16±0.05	2.20±0.05	0.7742	10.2
Eggshell ash (g)	24	0.25±0.00	0.24±0.00	0.24±0.00	0.24±0.00	0.25±0.00	0.3716	9.0
	28	0.26±0.00	0.25±0.00	0.25±0.00	0.25±0.00	0.25±0.00	0.6078	8.3
	32	0.26±0.00	0.25±0.00	0.25±0.00	0.25±0.00	0.26±0.00	0.1564	8.3
	70	0.25±0.00	0.24±0.00	0.24±0.00	0.25±0.01	0.25±0.01	0.5249	10.9
Egg surface area (cm^2)	24	65.34±0.39	64.73±0.57	65.30±0.50	64.80±0.49	63.88±0.57	0.2616	4.4
	28	67.15±0.56	66.62±0.59	66.74±0.61	66.46±0.52	65.68±0.46	0.4325	4.7
	32	68.41±0.48	67.65±0.59	68.53±0.64	67.38±0.50	67.17±0.51	0.2922	4.6
	70	74.20±0.77	72.33±1.02	72.31±0.90	71.88±0.70	72.65±0.98	0.3696	5.6
SWUSA (mg/cm^2)[1]	24	81.74±0.64	80.59±0.72	80.95±0.61	82.63±0.75	82.53±0.53	0.1085	4.6
	28	80.28±0.50	79.92±0.50	80.20±0.59	80.83±0.64	80.93±0.57	0.6815	4.0
	32	80.49±0.54	80.24±0.48	80.81±0.49	81.64±0.91	80.90±0.51	0.5559	4.3
	70	78.95±1.16	79.29±1.49	78.75±1.64	80.40±1.57	81.28±1.26	0.7017	8.4

[a,b] Figures with different superscripts within the same row differ significantly ($P<0.05$)

[1] SWUSA = shell weight per unit surface area

[2] Coefficient of variation

The effect of different distribution ratios of small and large limestone particles on eggshell thickness measured at the blunt end, equator and sharp end of the egg are shown in Table 5.13. With the exception of week 32, different distribution ratios of limestone particles resulted in an insignificant ($P>0.05$) effect on eggshell thickness. The significant ($P<0.0001$) effect of distribution ratios of limestone particles on eggshell thickness at the sharp end of the shell during week 32 was not perceptible in other weeks or regions of the eggshell and is inexplicable.

Eggshell thickness data of the blunt end, equator and sharp end of the egg were pooled to result in one average value for each egg and analyzed during the individual weeks (24, 28 and 32). Again no significant ($P>0.05$) differences were observed for average eggshell thickness, except at week 32 of age, when the treatment with 100% large limestone particles (2.0 – 3.8 mm) resulted in a significant ($P<0.0001$) lower (0.3917 mm) average shell thickness. The average eggshell thickness of the ratios consisting of 100%, 75%, 50% and 25% small limestone particles (0 – 1.0 mm) during week 32 was respectively 0.4101, 0.4081, 0.4109 and 0.4151 mm. These results on the average eggshell thickness are in accordance with that of shell thickness (sharp end of the egg) observed in Table 5.13 illustrating an inexplicable significant ($P<0.05$) decrease in eggshell thickness with 100% large particles limestone in the distribution ratio.

As done before, all data for egg weight, egg content and eggshell quality characteristics measured during the individual weeks set out in Tables 5.11, 5.12 and 5.13 were combined to calculate the mean values over the peak production period (week 24 to 32 of age). The effect of different distribution ratios of limestone particles on the mean egg weight, egg content and eggshell quality characteristics during the peak production period are shown in Table 5.14. Different mixtures of large and small limestone particles had no significant ($P>0.05$) effect on egg weight, egg content and eggshell quality characteristics when the means for the peak production period were statistically compared. The only significant ($P<0.0001$) effect of different distribution ratios of limestone particles was again recorded for shell thickness at the sharp end of the egg. However, as discussed in the first study (Tables 5.6, 5.7 and 5.12), the significant ($P<0.0001$) decrease in shell thickness at the sharp end of the egg, was not characterized by a similar decrease in shell thickness at the blunt end or equator regions, making the data inexplicable. Although shell thickness measurements at the sharp end of the egg in Table 5.14 were the lowest for hens consuming a ratio of 100% large limestone particles, no obvious relationship between distribution ratio of limestone particles and shell

Table 5.13 The effect of different percentages of large limestone particles on eggshell thickness (Mean±s.e.)

Parameter	**Age (week)**	**Large particles limestone (%)**					**Significance**	
		0	**25**	**50**	**75**	**100**	**P**	**CV[1](%)**
Blunt end (mm)	24	0.43±0.00	0.42±0.00	0.43±0.00	0.43±0.00	0.43±0.00	0.2733	4.7
	28	0.41±0.00	0.40±0.00	0.41±0.00	0.41±0.00	0.41±0.00	0.4131	4.6
	32	0.40±0.00	0.40±0.00	0.40±0.00	0.41±0.00	0.40±0.00	0.7313	4.3
	70	0.36±0.01	0.36±0.01	0.36±0.01	0.37±0.01	0.36±0.01	0.6869	10.7
Equator (mm)	24	0.44±0.00	0.43±0.00	0.43±0.00	0.43±0.00	0.44±0.00	0.1242	4.3
	28	0.42±0.00	0.41±0.00	0.41±0.00	0.41±0.00	0.42±0.00	0.3278	4.1
	32	0.41±0.00	0.41±0.00	0.41±0.00	0.42±0.00	0.42±0.00	0.0591	3.9
	70	0.38±0.01	0.37±0.01	0.37±0.01	0.38±0.01	0.39±0.01	0.2897	9.0
Sharp end (mm)	24	0.43±0.00	0.42±0.00	0.43±0.00	0.43±0.00	0.43±0.00	0.1509	4.5
	28	0.42±0.00	0.41±0.00	0.42±0.00	0.42±0.00	0.41±0.00	0.2959	4.1
	32	0.42±0.00[a]	0.41±0.00[a]	0.42±0.00[a]	0.42±0.00[a]	0.36±0.00[b]	0.0001	5.0
	70	0.36±0.01	0.35±0.01	0.37±0.01	0.37±0.01	0.38±0.01	0.5121	10.9

[a,b] Figures with different superscripts within the same row differ significantly ($P<0.05$)

[1] Coefficient of variation

Table 5.14 The effect of different percentages of large limestone particles on the mean egg weight, egg content and eggshell quality characteristics during week 24 to 32 of age (Mean±s.e.)

	Large particles limestone (%)					Significance	
Parameter	**0**	**25**	**50**	**75**	**100**	**P**	**CV[2](%)**
Egg weight (g)	54.71±0.49	53.98±0.64	54.59±0.64	53.84±0.54	53.11±0.55	0.2991	6.0
Shell weight (g)	5.41±0.05	5.32±0.05	5.39±0.06	5.41±0.06	5.34±0.05	0.6892	5.8
Egg content (g)	49.30±0.46	48.66±0.60	49.20±0.60	48.43±0.50	47.77±0.51	0.2624	6.2
Percentage shell (%)	9.91±0.07	9.88±0.07	9.89±0.07	10.06±0.09	10.07±0.06	0.1480	4.1
Eggshell Ca content (g)	2.02±0.02	1.99±0.02	2.01±0.02	2.02±0.02	1.99±0.02	0.6891	5.8
Eggshell ash (g)	0.25±0.00	0.25±0.00	0.25±0.00	0.25±0.00	0.25±0.00	0.3880	7.8
Egg surface area (cm^2)	66.97±0.43	66.33±0.56	66.86±0.56	66.22±0.47	65.58±0.48	0.3012	4.3
SWUSA (mg/cm^2)[1]	80.84±0.51	80.25±0.47	80.65±0.53	81.70±0.67	81.45±0.48	0.3099	3.8
Shell thickness							
Blunt end (mm)	0.41±0.00	0.41±0.00	0.41±0.00	0.42±0.00	0.41±0.00	0.5132	4.2
Equator (mm)	0.42±0.00	0.42±0.00	0.42±0.00	0.42±0.00	0.42±0.00	0.2663	3.7
Sharp end (mm)	0.42±0.00^{a}	0.41±0.00^{a}	0.42±0.00^{a}	0.42±0.00^{a}	0.40±0.00^{b}	0.0001	4.0

[a, b] Figures with different superscripts within the same row differ significantly ($p<0.05$)

[1] Shell weight per unit surface area (mg/cm^2)

[2] Coefficient of variation

thickness at the sharp end of the egg exists. Therefore the shell thickness data of the sharp end should be interpreted with caution.

A significant ($P<0.001$) moderate correlation ($r^2=0.69$) between eggshell thickness at the blunt end and equator of the shell was recorded. However, the significant ($P<0.001$) correlations between the blunt end and sharp end ($r^2=0.51$) and equator and sharp end ($r^2=0.35$) were moderate to low. These data are in accordance with that of the first study (paragraph 5.3.1.3) where the correlation between the blunt end and equator was significantly ($P<0.001$) higher than the correlations between the blunt end and sharp end as well as between the equator and sharp end. From the correlation coefficients it is noticeable that shell thickness measurements at the sharp end of the egg are not representative of the other measurement areas. As discussed in the first study, these results are in accordance with the differences observed in Tables 5.13 and 5.14 regarding shell thickness at the sharp end of the egg as well as the other two measurement areas.

5.4 Conclusions

The results of both the studies (particle size and distribution ratios of particles) are in accordance with each other regarding the egg production and eggshell quality characteristics. It seems that the influence of limestone particle size as well as different distribution ratios of small and large particles had generally a non-significant ($P>0.05$) effect on egg production parameters as well as eggshell quality characteristics. Although the results in Chapter 3 suggested that larger limestone particles (2.0 – 3.8 mm) and distribution ratios high in large particles limestone would result in the highest *in vivo* solubility and relative bioavailability, it was not supported by the results of the present study. The results in Chapter 4 suggested that an increase in limestone particle size would result in an increased bone breaking strength, stress and percentage ash. However, the positive effect of large particles limestone on certain bone parameters as illustrated in Chapter 4 were not observed in the present study. From the results of the present studies it seems that irrespective of the limestone particle size or particle size distribution, adequate calcium for optimum egg production and eggshell thickness for Lohmann-Silver laying hens between 24 and 32 weeks of age was supplied.

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CHAPTER 6

GENERAL CONCLUSIONS

The vital role of calcium during the formation of cortical and medullary bone as well as eggshell is well known and cannot be overstated. However, the variations in chemical and physical composition of calcium supplements have a significant influence on the solubility and consequent bioavailability thereof. Therefore, the need arises to determine the effect of a specific calcitic limestone source with varying particle sizes on *in vivo* and *in vitro* limestone solubility as well as bone quality, egg production and eggshell quality characteristics during the peak production as well as end-of-lay period.

From the results of the present study it seems that an increase in limestone particle size has an inverse related effect on *in vivo* and *in vitro* limestone solubility. An increment in limestone particle size resulted in a significantly increased ($P<0.05$) *in vivo* and decreased ($P<0.0001$) *in vitro* limestone solubility. An increase in limestone particle size also resulted in a significant increased ($P<0.0001$) intestinal limestone content and a decreased ($P<0.0001$) faecal limestone content. The increased *in vivo* limestone solubility of larger limestone particles could be ascribed to the increased retention time and/or slower rate of passage of the large limestone particles, which were manifested in the increased intestinal and decreased faecal limestone results.

The use of limestone particle size to express the bioavailability of a specific limestone source is subject to a large degree of error. It would therefore be the ideal to use *in vivo* limestone solubility for expressing the bioavailability of a source. It was found during the present study that the techniques used for the determination of *in vivo* limestone solubility are time consuming and yielded relatively low coefficients of determination ($r^2=0.54$ and $r^2=0.39$). However, the relatively few replications per treatment for *in vivo* limestone solubility ($n=5$) could be responsible for the low coefficients of determination. It is therefore suggested that future studies should include more replicates per treatment to ensure that more variation can be explained for *in vivo* solubility. During the present study, an inverse relation between *in vivo* and *in vitro* limestone solubility was consistently illustrated, suggesting that regression equations could be used to determine *in vivo* from *in vitro* limestone solubility. The welfare and financial implications during sacrificing of birds for the determination of *in vivo* limestone solubility therefore necessitate an alternative procedure that will predict the relative bioavailability of different particle size with a fair degree of accuracy. The use of *in vitro* limestone solubility to predict the *in vivo* solubility thereof would be more time efficient and could assist the feed manufacturers to class/categorise limestone before purchasing. Therefore, the development of equation models that could be used by nutritionist to predict *in vivo* limestone solubility by the use of *in vitro* solubility needs further investigation.

The results of the present study suggested that an increase in limestone particle size as well as an increased percentage large particles limestone, resulted in a significant ($P<0.05$) increase in bone breaking strength and bone stress in older laying hens (70 weeks of age). The effect of different distribution ratios of limestone particles on certain bone dimensions such as bone length, width and weight as well as percentage bone ash were, however, not clearly illustrated and poorly defined during the second study. In contrast, the effect of limestone particle size on bone mechanical properties during the first study resulted in more consistent and clearly defined results with significantly ($P<0.01$) higher correlations. Data of the present study as mentioned above, illustrated that large particles limestone resulted in higher tibia breaking strength and bone stress. In accordance, *in vivo* limestone solubility data suggested that an increase in limestone particle size would result in higher *in vivo* limestone solubility (63.42%). With a dietary inclusion level of 9.58% limestone ($CaCO_3$) and an *in vivo* limestone solubility of 63.42% (2.0 – 3.8 mm particle size), the soluble dietary $CaCO_3$ level derived from large particles limestone in the diet and needed for maximum bone strength could be calculated as 6.08%. *In vivo* solubility determination of dietary soluble $CaCO_3$ could therefore be of utmost importance to the nutritionist during diet formulation.

The effect of limestone particle size on egg production and eggshell quality characteristics seems to be non-significant ($P>0.05$) during the present study. Limestone particle size revealed some significant ($P<0.05$) effects on certain eggshell quality parameters (eggshell thickness at the sharp end). However, these effects were not clearly defined and did not allow reliable conclusions. The non-significant ($P>0.05$) effects of limestone particle size on egg production and eggshell quality observed during the present study are in contrast with that of some other authors and could be ascribed to the differences in genetic strains as well as the variation in limestone particle sizes. During the present study, limestone particles were classified as small (0 – 1.0 mm), medium (1.0 – 2.0 mm) and large (2.0 – 3.8 mm) and the distribution of particles was considered to be proportionally representative. Therefore, the mean particle size (0.5, 1.5 and 2.9 mm) of each treatment was used in the regression equations. However, the range (1.0 to 1.8 mm) in the different classification groups of limestone particle sizes could have influenced the proportional distribution and thus average particle size. It is therefore suggested that limestone particle sizes should be better categorized in certain defined particle sizes before future research are conducted.

Although an increase in limestone particle size resulted in a significantly ($P<0.05$) increased *in vivo* limestone solubility, bone breaking strength and bone stress, the same tendency was not observed for egg production and eggshell quality characteristics. From the results of the present studies it seem that irrespective of the limestone particle size or particle size distribution, adequate calcium for optimum egg production and eggshell thickness for Lohmann-Silver laying hens between 24 and 32 weeks of age was supplied. However, the effect of different, more defined limestone particle sizes on eggshell quality characteristics, as well as correlations between *in vivo* limestone solubility, bone quality and eggshell quality needs further investigation.

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 *in vivo* and *in vitro* solubility, bone quality, egg production and eggshell quality was determined. Limestone was classified according to particle size 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 *in vivo* and *in vitro* solubility, bone quality, egg production and eggshell quality was determined. Small (0 – 1.0 mm) and large (2.0 – 3.8 mm) particles limestone from the first study was 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. One hundred and ninety eight, 17 weeks old Lohmann-Silver pullets, were obtained from a commercial egg producer and randomly allocated to six treatments (n=33/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 19 to 32 as well as week 70 of age. During weeks 24, 28, 32 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. The *in vivo* and *in vitro* limestone solubility of different limestone particles and the different distribution ratios of particles were determined during week 37 of age. At 37 and 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 first study clearly illustrated that an increase in limestone particle size resulted in a significantly ($P=0.0095$) increased *in vivo* and decreased ($P<0.0001$) *in vitro* limestone solubility. The inverse relation between *in vivo* and *in vitro* limestone solubility could be calculated from the following regression equation of: $y = -0.8567x + 77.657$ ($r^2=0.54$), where x is *in vitro* limestone solubility. An increased ($P<0.0001$) excreta limestone content of small limestone particles was characterized by a decreased intestinal limestone content, indicating a lower retention time. Large limestone particles resulted in a significant

decreased tibia length (P=0.0317), tibia weight (P=0.0265) and humerus length (P=0.0060) at week 37 of age. A significant positive effect of large limestone particles on tibia breaking strength (P=0.0150) and tibia stress (P=0.0419) was observed at week 70 of age. No clear tendency regarding the effect of particle size on tibia ash could be recorded. Large particle size limestone resulted in a significantly (P=0.0056) higher percentage tibia bone at week 70 of age. Egg output decrease significantly with an increase in limestone particle size at weeks 24 (P=0.0519) and 70 (P=0.0292) of age. Large limestone particles resulted in thinner eggshells (P<0.0001) at the sharp end of the egg during week 32 of age.

Results of the second study regarding the *in vivo* and *in vitro* limestone solubility are generally in accordance with that of the first study. During the second study, a significant increased *in vivo* (P=0.0324) and decreased *in vitro* solubility (P<0.0001) of limestone was observed with an increase in the percentage large limestone particles in the distribution ratios. The inverse relation between *in vivo* and *in vitro* limestone solubility in this study could be calculated from the following regression equation of $y = -0.9116x + 78.785$ ($r^2=0.39$), where x is *in vitro* limestone solubility. An increase in the percentage large particles limestone resulted in a significant higher tibia breaking strength (P=0.0123) and tibia stress (P=0.0304) at week 70 of age. However, no clearly defined tendency of different distribution ratios of limestone particles on bone mechanical properties was observed. Higher percentages of large particles limestone resulted in a significant (P=0.0007) higher percentage tibia ash at week 37 of age. However, the effect of different percentages of large particles limestone on percentage tibia ash was not clearly defined and no obvious tendency could be recorded. The effect of different distribution ratios of limestone particles on eggshell quality was generally not significant (P>0.05).

The general conclusion of these studies were that irrespective of the limestone particle size and/or particle size distribution, adequate calcium for optimum egg production and eggshell quality for Lohmann-Silver laying hens between 24 and 32 weeks of age was supplied.

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 die *in vivo*- en *in vitro*-kalksteenoplosbaarheid, 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 *in vivo*- en *in vitro*-oplosbaarheid, 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 negentig, Lohmann-Silver lêhenne (17 weke oud), is vanaf ‘n kommersiële eierprodusent verkry en ewekansig in ses behandelings ingedeel (n=33/behandeling). Die invloed van partikelgrootte en partikelgrootte-verspreiding op voerinhame, liggaamsgewig en eierproduksie vanaf week 19 tot 32 ouderdom asook gedurende week 70 is bepaal. Gedurende die ouderdom van week 24, 28, 32 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. *In vivo* en *in vitro*-kalksteenoplosbaarheid van die verskillende partikelgroottes en partikelgrootte-verspreidings is gedurende week 37 bepaal. Gedurende week 37 en 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 eerste studie toon dat ‘n toename in partikelgrootte van kalksteen met ‘n betekenisvolle toename (P=0.0095) in *in vivo* en afname (P<0.0001) in *in vitro*-oplosbaarheid gepaard gegaan het. Die negatiewe verwantskap tussen *in vivo*- en *in vitro*-kalksteenoplosbaarheid kan deur die volgende regressievergelyking bereken word: $y = -0.8567x + 77.657$ ($r^2=0.54$), waar x die *in vitro* oplosbaarheid van kalksteen verteenwoordig. Growwe partikels kalksteen het ‘n betekenisvolle afname in tibia-lengte (P=0.0317), tibia-gewig (P=0.0265) en humerus-lengte (P=0.0060) in week 37 teweeggebring. ‘n Betekenisvolle positiewe invloed van growwe partikels op tibia-breeksterkte (P=0.0150) en tibia-spanning (P=0.0419) is in week 70 waargeneem. Geen duidelike invloed (P>0.05) op

tibia-as kon waargeneem word nie. Growwe partikels kalksteen het ‘n betekenisvolle (P=0.0056) hoër persentasie tibia-been in week 70 tot gevolg gehad. Eiermassa het betekenisvol afgeneem met ‘n toename in kalksteenpartikelgrootte in weke 24 (P=0.0519) en 70 (P=0.0292). Growwe kalksteen het ‘n dunner eierdop (P<0.0001) by die skerp punt van die eier gedurende week 32 teweeggebring.

Die resultate van die tweede studie aangaande die *in vivo*- en *in vitro*-kalksteenoplosbaarheid was algemeen in ooreenstemming met die eerste studie. Gedurende die tweede studie is ‘n betekenisvolle toename in *in vivo*- (P=0.0324) en afname in *in vitro*-oplosbaarheid (P<0.0001) met ‘n toename in die persentasie growwe kaklsteenpartikels in die mengsels verkry. Die negatiewe verwantskap tussen *in vivo*- en *in vitro*-kalksteenoplosbaarheid kan deur die volgende regressievergelyking bereken word: $y = -0.916x + 78.785$ ($r^2=0.39$), waar x die *in vitro*-oplosbaarheid van die kalksteen verteenwoordig. ‘n Toename in die persentasie growwe partikels kalksteen het ‘n betekenisvolle hoër tibia-breeksterkte (P=0.0123) en tibia-spanning (P=0.0304) in week 70 tot gevolg gehad. Geen duidelike invloed van verskillende verspreidingsverhoudings van kalksteen-partikelgroottes op die meganiese eienskappe van been kon waargeneem word nie. Hoër persentasies growwe kalksteenpartikels het ‘n betekenisvolle (P=0.0007) hoër persentasie tibia-as in week 37 tot gevolg gehad. Die invloed van verskillende persentasies growwe kalksteenpartikels op persentasie tibia-as was egter moeilik waarneembaar. Die invloed van verskillende verspreidingverhoudings van kalksteenpartikels op eierdopkwaliteit was oor die algemeen nie betekenisvol (P>0.05) nie.

Die algemene gevolgtrekking van hierdie studies is dat, ongeag die kalksteenpartikelgrootte of verskillende partikelgrootte-verspreiding, voldoende kalsium vir optimale eierproduksie en eierdopkwaliteit vir Lohmann-Silver lêhenne gedurende die ouderdom van 24 to 32 weke beskikbaar was.