EFFECTS OF LIMESTONE SOURCE ON EGG PRODUCTION AND EGG SHELL QUALITY IN LAYING CHICKENS

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DECLARATION AND APPROVAL

This thesis is my original work and has not been presented for a degree award in any other university.

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DEDICATION

This work is dedicated to my husband Francis, and the children Wendy and Ezra. God bless them.

IN MEMORY OF

My departed parents Mr. Ayub M'Inoti and Mrs Lydia Wanja M'Inoti

May God rest their souls in Eternal Peace.

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LIST OF ABBREVIATIONS

ABNT	Brazilian Association of Technical Standards
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AR	Athi River limestone
UKC	Ukunda Kenya Coast limestone
CF	Crude fibre
CLS	Calcitic limestone
CP	Crude protein
DLS	Dolomitic limestone
DM	Dry matter
DMD	Dry matter digestibility
EE	Ether extract
FCR	Feed conversion ratio
GE	Gross Energy
KeBS	Kenya Bureau of Standards
LS	Limestone source
ME	Metabolizable energy
MOLFD	Ministry of Livestock and Fisheries Development
Mcal	Mega calories
NFE	Nitrogen free extracts
NRC	National Research Council

ABSTRACT

Laying hens require adequate calcium for bone and eggshell formation as well as other physiological functions. Limestone, dicalcium phosphate, oyster shell, bone meal, meat and bone meal are important calcium sources used in formulating diets for laying hens. Of these, limestone is widely used in Kenya because it is readily available and inexpensive. The objectives of this study were: (i) to determine the physical and chemical characteristics of limestone from two sources in Kenya. (ii) to evaluate the effects of limestone source and level of dietary Ca on layer performance and eggshell quality(iii) to determine effect of time of lay on eggshell quality. Limestone was purchased from Athi River (AR) and Ukunda in Kenyan Coast (UKC). Particle size and solubility of the limestones were determined. The specific minerals content was determined according to the AOAC procedures of 2016. One hundred and 144 birds at 25 weeks of age were selected from a layer flock at the Poultry Unit, University of Nairobi and used in this study. The birds were allocated randomly to experimental cages. Six experimental diets, the first three based on UKC and the others on AR limestone were formulated. Each limestone source was included in the diet such that calcium level was 1%, 2% or 4% in a 2X 3 factorial design. A depletion diet containing less than 1% calcium was also formulated. The birds were fed on the experimental diets for a period of 60 days. Each diet was fed to a group of six birds and was replicated four times, giving 24 birds per treatment. Prior to this the birds had been placed on a depletion diet for about 10 days until production of thin shelled eggs was observed. Egg weight (g), hen-day egg production (%), egg breakage (%), feed intake (g/bird/day), feed conversion ratio (kg/dozen), specific gravity (g/cm^3), shell weight (g), shell thickness (mm), shell percentage (%) and cost of feed (Kshs/kg or Kshs/dozen eggs) were determined. Data on time of lay was collected on the 1st, 4th and 7th week of lay at 9.00am, 12.00 noon and 3.00pm, respectively. Data analysis was done using GenStat Statistical package and significant treatment means separated using Tukey’s test. Results showed that UKC was superior in calcium concentration (42%) to AR (34%). *In vitro* solubility was 63.0 % and 29.4% for UKC and AR respectively. The mean hen-day egg production was 76 %, feed intake 118.5g, egg weight 58.03g and feed cost Kshs. 68.49. Limestone source (LS) had no effect (P > 0.05) on these parameters. However, LS had an effect (P < 0.05) on FCR, which was 1.9 and 2.0% in UKC and AR based diets, respectively. It also had an affect (P <0.05) on egg breakage at 5.82 and 11.72 % for UKC and AR, respectively. The effect of Ca level on performance of the layers was evaluated. Feed intake of 124 was higher (P<0.05) by 8-9g when Ca was at 2% than when it was 1 or 4%. Hen-day egg production was 66.8% at 1% Ca and increased (P< 0.05) to 81.9 and 79.1% at 2 and 4% Ca, respectively. FCR improved (P < 0.05) from 1.29 to 1.10 and 1.06 kg per dozen eggs as dietary Ca was increased to 2 and 4%, respectively. At 1% dietary Ca, egg breakage was 17.49%, which declined (P < 0.05) to 5.50 and 3.33% at 2and 4% Ca, respectively. Feed cost per dozen eggs was Kshs 74.58 at 1% Ca and declined to 63.64 and 66.78 at 2 and 4% Ca, respectively. The eggshell quality evaluated indicated that, mean egg specific gravity was 1.06 g/cm^{3,} which was not influenced by LS. The mean shell weight was (4.06g), shell thickness (0.33mm) and shell percentage (8.05%), which were higher for eggs from birds fed UKC limestone by 15.8, 12.9 and 13.8 % than those fed AR limestone. Ca level affected parameters used as eggshell quality indicators. At 1% Ca, the specific gravity of the eggs was 1.03 g/cm3, which increased significantly to 1.07 at 2% Ca. The egg shell weight increased (P < 0.05) from 3.83 to 4.68 and 5.54g as Ca level increased from 1 to 2 and 4 %, respectively. Calcium level (P< 0.05) increased eggshell thickness from 0.28 at 1% Ca to 0.33 and 0.38 mm at 2 and 4% Ca, respectively. There was a significant increase in eggshell percentage from 6.76 to 8.15 and 9.23% as Ca increased from 1 to 2and 4%, respectively. The time of lay had significant *(P<0.05)* effects on egg weight, shell weight, shell thickness and shell percentage. Eggs laid in early morning weighed 59.24g and had a shell weight of 4.93g, shell thickness 0.36 mm and shell percentage of 8.2. However, eggs laid in the afternoon were lighter by 2.7% and had a lower egg shell weight, egg shell thickness and shell percent by 9.9, 11.1 and 7.2 %, respectively. The conclusions of this study are: (i) UKC limestone had a higher proportion of coarse particles (1-2 mm) and solubility than AR limestone, (ii) the two limestone sources contained trace amounts of Fe, Cr, Cu and Zn, but UKC had a high content of Aluminium (1.07 mg /kg), (iii) limestone source had no effect on feed intake, hen-day egg production, specific gravity and egg weight but had an effect on feed conversion ratio and egg breakage. (iv) layers fed on UKC limestone laid eggs with better eggshell quality in terms shell weight, thickness and percentage than those fed on AR limestone. (v) dietary Ca level affected feed intake, hen day egg production, feed conversion ratio and egg breakage. (vi) Increasing dietary Ca improved eggshell quality characteristics i.e. shell weight, thickness and percentage. (vii) Eggs laid early in the morning had higher weights than those laid in late afternoon. In addition, they had stronger shells expressed as shell percentage and thickness than those laid in the afternoon.

Recommendation from this study are: (i) where possible UKC limestone should be used in formulating diets in Kenya because of its superior physical characteristics reflected in high egg production and good shell quality in this study. (ii) Further study be done on UKC limestone on layers growth and bone mineral content. (iii) There is also need to investigate the effect of particle size on digestibility of limestone. (iv) effect of midnight feeding on time of lay and eggshell quality.

Key words: Limestone, egg production, eggshell quality, source, dietary calcium

CHAPTER ONE

1.0.INTRODUCTION

1.1 Background information

Poultry farming is a major contributor in the Kenyan economy. Agriculture contributes 25% of GDP with the 30% being from poultry sector. The industry is important in meeting the ever-growing demand for nutritious food.

The poultry industry in Kenya is characterized by dualism in that large- and small-scale producers are involved in the enterprise (Aila *et al.,* 2012). Poultry farming in Kenya is mostly small-scale, and predominantly for domestic consumption. However, it is fast gaining popularity as a business activity especially in the urban and peri-urban areas. There are 31 million birds in Kenya consisting of indigenous chicken (75%), commercial chicken (22%), breeding stock (1 %) and other types of poultry such as water fowls, turkeys, ostriches and guinea fowls accounting for about 2 % of the total (National Census, 1999; MOLFD, 2012). The chickens are important for production of eggs for consumption, meat as well as fertile eggs for hatching into day old chicks. The key drivers of the growth of the poultry industry in Kenya are: increase in human population, increased per capita income, urbanization and improvement in technology that influence efficiency of production (Gikunju *et al*., 2018).

The level of production in terms of eggs or meat is affected by several factors such as genotype of the birds, nutrition, diseases and prevailing policies. Nutrition requires provision of those chemical substances or nutrients that will promote growth, ensure good health, improved production of egg and good quality of the food products. Therefore, to achieve the level of production desired, the laying hen must be fed a diet that provides adequate amounts of all nutrients. Adequate intake of calcium is important so as to meet the need for various functions such as formation of the skeleton, as co-factors of enzymes and for the maintenance of osmotic pressure. For the growing bird most of the calcium is used for bone formation. However, as the bird approaches sexual maturity some calcium is stored in the medullary bone for future eggshell formation. For the laying hen, most of the calcium is used for eggshell formation.

In formulating poultry diets a good source of calcium is required. Some of the common sources of calcium are limestone, oyster shell, eggshell, fish meal, bone meal, meat and bone meal and di-calcium phosphate. The use of any of the sources depends on availability and the cost and any risk associated with the raw material. In Kenya, limestone is widely used as a calcium source because of availability and relatively low cost. Different limestones vary in their Calcium contents (Aila *et al.*, 2012) which may be attributed to the locations in which they are mined. Limestone varies in the levels of Calcium and the concentration of other minerals, which can affect its utilization by the laying hen (Chambers *et al.,* 2017).

The producer is interested in producing efficiently a high number of eggs that have shells of good quality. Eggshell quality is of major concern in the industry, since eggs with shell quality that is inferior lead to economic loss to poultry producers. It has been reported that the average number of eggs cracked and lost prior to reaching the point of consumption range from 13% to 20% (Anwar *et. al.*, 2017). Therefore, better understanding of the factors that affect the quality of eggshells in layers is important (Ricke, *et al*., 2015).

In 2016, 0.287 million metric tons of poultry feed were manufactured in Kenya with a use of 20,664 kg of UKC limestone or 26,117 kg of AR limestone (MoALF annual report 2016). The limestone is mined in three main areas in Kenya, which are the Kenyan coast, Athi River plains and Fort Ternan in Western Kenya. There is no information available on chemical attributes and nutritional value of limestone from various sources.

1.2 Statement of the problem

Limestone is the main source of calcium used in making Animal feeds for the laying hens in Kenya. Approximately 95% of the eggshell is made up of calcium carbonate. The level of dietary Ca affects the eggshell quality that is essential in the egg industry. Eggs with inferior shell quality are a leading cause of economic losses to poultry producers.

It is therefore, important to identify suitable and sustainable sources of limestone, so as to have a better eggshell quality and minimise losses due to breakages. The attributes of limestone obtained from the various sources in Kenya have not been studied. Such attributes include content of calcium, other minerals, solubility and particle size and the effect of feeding this limestone on performance, eggshell quality and time of lay.

1.3 Justification

The rapid growth in poultry production has increased the demand for feed as well as raw materials. This growth is reflected in the increase in the number of eggs produced annually. There is need to improve the efficiency of egg production through reducing losses caused by egg breakage (Sultana *et al*, 2007), Eggshell thickness, influences the ability of the shell to withstand external pressure and hence resistance to breakage. This leads to the question of how eggshell quality can be improved, especially through diet manipulation. It is therefore, important to identify economically suitable and sustainable source of calcium in order to improve eggshell quality and minimize losses due to breakages. The purpose of this study was to investigate the attributes of two sources of limestone used in feeding poultry in Kenya.

1.4 Objectives

1.4.1 Overall Objective

To determine effects of feeding limestone from two sources on layer performance and eggshell quality.

1.4.2 Specific Objectives

(1) To assess the physical and chemical characteristics of limestone used in layer feeds in Kenya

(2) To evaluate the effects of limestone source and level of dietary Ca on layer performance and eggshell quality

(3) To explore the effect of time of lay on eggshell quality.

1.4.3 Null hypotheses:

- There are no differences in the physical and chemical properties of UKC and AR limestone
- Source of calcium has no effect on layer performance and egg shell quality
- The level of dietary calcium has no effects on layer performance and eggshell quality.
- Time of lay has no effect on eggshell quality.

1.4.4 Scope and limitation

The study was limited to limestone mined in Ukunda in Kenyan Coast (UKC) and that mined at Athi River (AR). A total of 144 laying hens were used. The study encountered challenges in that only a few minerals were analysed because of the limited facilities available. It would have been desirable to determine the digestibility of calcium in the two limestone sources but this was not possible within the time and resources available

CHAPTER TWO

2. 0 LITERATURE REVIEW

2.1 Introduction

This chapter reviews literature on effect of dietary calcium (Ca) sources on overall performance and eggshell quality in chickens. It covers Ca metabolism in laying chickens, its sources in a layer diet, requirements for layers and factors affecting utilization and eggshell quality. The effects of age of the laying birds, ambient temperature and time of lay on eggshell quality are also discussed. Limestone is used widely as a supplemental calcium source in poultry feeds. The chemical and physical attributes of this feedstuff are discussed. The effect of dietary level of Ca on layer performance and eggshell quality are presented.

2.2 Calcium metabolism in laying hens

Minerals play vital functions in physiology of an animal. These include structural role, maintenance of homeostasis, ionic equilibrium, osmotic pressure, acid-base equilibrium as well in enzyme systems. The most abundant mineral in the body of an animal is Ca, most of which is found in the skeleton (Jiang, *et al*, 2013). Ca is critical in bone formation. It is also critical in formation of eggshell in laying birds since it is the main structural component of the eggshell. Laying hens requires large amounts of Ca especially during peak production. During the late stages of production Ca metabolism is under strain since hens have decreased calcium absorption efficiency (Buzała *et al.*, 2015).

Calcium for eggshell formation is obtained from the diet after absorption through the intestines and also from medullary bone reserves. Medullary bone is the primary bone Ca reserve and is observed in sexually mature, egg producing birds. At the onset of sexual maturity, the osteoblast changes from forming lamella cortico bones (structural bones) to production of woven bones the medullary bones (Prondvai and Stein, 2014 and Chinsamy *et al*., 2016).

Medullary bone is a labile supply of Ca for the formation of eggshell and is usually formed on the surface of the structural bones inside the medullary cavities in the leg bones and on the endosteal surfaces. The net impact of replacement of structural bones by medullary bones is to weaken the overall strength of hen's skeleton (Akbari-Heuthorst, *et al.,* 2018). This process of disappearance of skeletal bone and formation of medullary bone is typically reversed when the hen goes out of lay.

Eggshell formation takes place mainly at night when there is little supply of Ca from the diet (An *et al*., 2016). Dietary inclusion of limestone which has large particles reduces the phosphorus excretion and improves eggs specific gravity (Valable *at el.,* 2009). Bueno *et al*., (2016) showed that large particles of limestone release Ca slowly and uniformly all through the process of eggshell formation, permitting retention of the Ca into the medullary bone of the layers. Cufadar *et al., (*2011) in their study suggested that layers should be provided with medium limestone particle size (2–5 mm) in the diet to maintain overall performance, bone and eggshell quality.

Medullary calcium changes as the layer responds to the supply and demand of calcium during formation of the shell. Ca absorption in the intestines can reach over 70% during the process of egg formation when the shell gland is active (Allahverdi, *et al.,* 2013). Approximately 50 - 60% of dietary Ca is used for formation of eggshell. The absorption of this mineral is mainly determined by the requirement of the animal (Tumova & Gous, 2012). Consequently, its absorption is high when eggshell formation is in progress. The Ca bone reserves are replenished when the shell gland is in the inactive state (Saki *et al.,* 2019). Approximately 2.2 g of Ca is found in the egg and this is mostly present in the eggshell (Rodriguez, 2013). Eggshell quality is greatly dependent on the skeletal condition of the layer. Hens with soft bones will, though not always, produce eggs with thin shells (Bingfan Zhang *et al*., 2017).

Low Ca in layer diets will result in production of thin shells after four days of feeding (An *et al.,* 2016). The quality of the eggshell is major challenge to many contributors in the poultry production chain (Samiullah *et al*., 2016). Good quality eggshell ensures proper development and hatchability of the chicken embryo (Bueno *et al.*, 2016 and Buzała *et al*., 2015). It protects the embryo from adverse environmental conditions, infection and water loss (Kingori, *et al,* 2014). A thick eggshell withstands breakage during handling better than a thin one. In addition, the shell is the first barrier towards protecting the egg from bacterial entry and as such should be free of any form of defects. Shell breakage is a big challenge to egg producers due to the fact that about 80 to 90% of eggs problems are related to eggshell (Geleta *et al.,* 2013).

In most cases Ca deficiency occurs due to shortcomings during formulating, preparation, storage, transportation and distribution of feeds to poultry or due to decreased intake by layers. The reduced intake could be due to a number of reasons such as inadequate feeding space and heat stress (Geleta *et al,* 2013). In most cases egg defects are of two types: (i) those laid without shell and often are not observed or counted among the fresh laid eggs, (ii) the eggs with defects in frame before oviposition which may be partially and completely eliminated. The egg deformities include irregular shells, partially thin shell and rugged eggshell (Pavlovski *et al.*, 2012). Egg formation takes approximately 20 hours in layers, which suggests the necessities for continuous supply of required quantities of Ca. In case the supply of Ca is interrupted during this process, there is decline in the quality of the eggshell.

Pavlovski *et al.*, (2012) reported that about 6-8% of the total eggs produced are not always marketable because of low quality shells, while Buzała, *et al*., (2015) reported that between 14.3 and 21.3% of all the eggs laid internationally, are either broken or cracked before they reach the end user. Due to the economic losses and the risks associated with cracked shells, the quality of the eggshell therefore remains one of the primary worries to this enterprise (Mazzuco & Bertechini, 2014). The potential of the eggshell to resist the effect of the external pressure depends on the quality of shell (Keta and Tumova, 2018). There are many factors that impact on the quality of the shell and its strength, these includes age of the layer, nutrition, genetics, health of the layers and environmental situation (Pavlovski *et al*, 2012).

2.3 Calcium sources and utilization by laying hens

2.3.1 Calcium sources

In many countries feed manufacturers use two supplemental dietary Ca sources, which are oyster shell and limestone (Tunc & Cudafar 2015). Both of them provide Ca in the form of calcium carbonate, and each contains about 38% calcium (Saunders-Blades *et al*, 2009). However, limestone is cheaper than oyster shell, which has been in use for more than 100 years (Saunders-Blades *et al*, 2009). Nonetheless, at best only 50 – 60% of Ca in feeds are retained. However, to make sure the retention of 2.5 g of Ca on daily basis, 4.0 - 4.5 g must be included in the diet.

Limestone, the other important Ca source, often has the problem of high level of magnesium or sand and silica.

Limestone is a sedimentary rock. It is naturally occurring and high levels of Ca and/or magnesium carbonate, and/or dolomite (calcium and magnesium carbonate), together with trace amounts of other minerals (Lakhundi,2012). It is extracted from quarries and underground mines

Ketta and Tůmová (2016) reported that different quarries produce different types of limestones with varying physical and chemical attributes, particularly with respect to solubility and Mg levels.

Table 1 shows the classification of pure limestone based on calcium and magnesium levels. The type of limestone depends on the Ca to Mg ratio and the Mg content. Dolomitic limestone (DLS) consists of 5 to 46 % magnesium carbonate and more than 20 percent magnesium oxide.

Table 1: Classification of limestone based on calcium and magnesium levels (%)

Classification[1]		Calcite	Dolomite	$CaCO_3$	$MgCO_3$
Calcite limestone	High Ca limestone	95-100	5 - 0	98 - 100	2 - 0
	Mg limestone	90 - 95	10 - 5	95-98	5-2
Dolomite limestone		50 - 90	50 - 10	77 - 95	23 - 5
Calcitic dolomite		11 - 50	89 - 50	59 - 77	40 - 23
Dolomite		0 - 10	100 - 90	54 - 59	46 - 41

Source: https://dnr.mo.gov>geology>docs>mineralChem.limestone;

On the other hand, calcitic limestone (CLS) contains less than 5% magnesium carbonate and is generally considered to contain more than 95 percent Ca carbonate, but most, commercial high-calcium limestones contain more than 97 or 98 percent Ca carbonate (Willman, 1943). Table 2 shows other sources of Ca. In Kenya, limestone quarries are found in the coastal region, Athi River near Nairobi and Fort Ternan in Western Kenya

2.3.2 Calcium requirements

The commercial laying hen requires approximately 3g/day of calcium for maximum egg production. To ensure maximum shell thickness the Ca levels are increased to 3.8g/day (Ferguson,2015). This means that the requirement is dependent on the physiological function under consideration (Buzała, 2015). During the last phase of production when the Ca intake is under strain, it is recommended at 3.8% of the diet (Nzioka *et al.*, 2017). The source of Ca at this stage should contain two-thirds large particles, which are of diameter of 1.0 to 1.4mm (Skrivan *et al*. 2010 and Guo and Kim, 2012). However, if the layer is provided with a different source of calcium, like grit, then it is able to adjust intake to its requirements (Ferguson *et al.*, 2015). The Ca requirement for the layers varies during the day. During this time, between 12–14 hours in which eggshell is forming, the layers requirement exceeds the rate of calcium absorption and this results into the use of calcium reserves.

Table 2: Sources of calcium and their mineral content

Supplement	DM, %	CP, %	Ca, %	Cl, %	Mg, %	P, %	K, %	Na, %
Bone meal (Steamed)	97	13.2	30.7	-	0.33	12.8	0.19	5.7
Calcium carbonate	100	-	39.4	-	0.05	0.04	0.01	0.01
Dicalcium phosphate	97	-	22	-	0.6	19.3	0.1	0.1
Eggshell waste	-	5.2	0.12	-	0.41	37.3	-	-
Limestone	100	-	34	0.03	2.06	0.02	0.12	0.06
Oyster shell	99	-	38	0.01	0.3	0.07	0.1	0.21
Fish Meal	98	60	7.9	-	14	3.6	0.5	0.5
Meat & Bone meal 50%	100	50	10.6	0.75	19	5.1	1.43	0.7

Source: (Veum, 2010)

Laying hens should be given Ca rich feed not only for eggshell formation, but also for thick eggshell that will withstand breakage during handling (Rodriguez, 2013). A well-formed eggshell is necessary for high hatchability. In case of inadequate dietary supply of Ca, deficiency symptoms may be observed (Ferguson, 2015). These will include thin eggshells, lower production of egg, inappetance, leg weakness, osteoporosis and cage layer fatigue (An *et al.,* 2016). Mild deficiency Ca results in decreased egg production but does not stop it completely. However, hens sometimes could lay at a higher rate, even though they are fed on a low Ca diet (Gongruttananun, 2011). In such cases Ca is removed from the bones resulting in hens becoming lame and crippled and sometimes death (An *et al*., 2016). As a rule, diets containing Ca levels that are below the nutritional requirement of layers impairs performance and egg quality (An *et al*., 2016). On the other had those diets with excessive dietary calcium have been found to lower feed intake, cause soft faeces and increase white deposit on the eggshell (Pilicia *et al*., 2011).

Calcium, phosphorous and vitamin D_3, are vital for eggshell quality. The consequence of feeding DLS is calcium deficiency, generally manifested by poor skeletal growth or eggshell quality (Leeson, 2005). Optimum eggshell high-quality and bone development in younger birds are based upon a consistent pattern of calcium solubility and availability (Bingfan Zhang *et al.*,2017)

The mature bird's skeleton incorporates around 1g of medullary Ca that is available for shell calcification on any one day (Prondvai and Stern, 2014). This Ca is always replenished among successive ovulations, and in instances of insufficient Ca repletion, the medullary reserve may be maintained on the expense of structural cortical bone (Prondvai and Stern, 2014). Around 60-70% of the medullary calcium reserves are positioned in the lengthy bones, and so long-time period troubles of calcium deficiency can cause lameness and cage layer fatigue (Leeson, 2005).

2.3.3 Factors affecting calcium utilization and eggshell quality

It is the desire of every producer to have an egg with a high quality shell. The shells from large sized eggs and those that are collected immediately after lay are more prone to cracking. Genetically, layers influence the levels of calcium deposited for eggshell formation. This suggests that the amount of calcium in the diet is not directly proportional to the quality of the eggs but there are others factors to be considered (Tůmová, 2016). The size of the egg increases as the layer advances in age. This implies that, as the layers ages, the egg weight changes and this has an influence on eggshell quality. Proper nutrition is important for a better quality eggshell. The availability of Ca to the layers varies and this depends on its sources. The best organic source of calcium is ground shells from marine animals followed by ground eggshells and then by common limestone. In case of Ca deficiency, the most sensitive response can be seen when the layers are at the age of 150 to 180 days.

Ca utilization decreases at the end of the production period. Ca utilization can be improved by changing its source and size of the particles in which it is supplied. Difference in Ca utilization is affected by the fineness and coarseness of the grains (Świątkiewicz *et al.*, 2015). The coarser the particles, the longer they are retained in the upper gastrointestinal tract (De Witt *et al*., 2009). The Ca release from coarser particles is lower which is suitable since shell formation is continuous process and occurs even at night when the birds are not feeding (Harms *et al.*, 2016). This has been shown by the fact that eggs that are laid in the afternoon have thicker eggshells (Saki *et al.*, 2019). The rate of change of solubility is affected by size of the particle and porosity (Zhang *et al*., 2017). Consistent pattern of calcium solubility influences optimum bones development in growing birds and eggshell high quality (Leeson *et al*, 2015).

Vitamin D_3 is important during shell formation and normal medullary bone reserves (Londero *et al*, 2016). It enables absorption of both calcium and phosphorus from the GIT. Deficiencies of vitamin D_3 can result into shells that are light in weight, while excess dietary levels of vitamin D_3 may cause pimpling of the shell (AkbariMoghaddamKakhki *et al*., 2018). However, higher levels of vitamin D_3 do not affect the quality eggshell. Vitamin D_3 deficiency leads to soft and rubbery beaks and bones, retarded growth and weak legs (AkbariMoghaddamKakhki *et al*., 2018). The egg production and eggshell quality decreases (Allahverdi *et al.*, 2013).

Egg production

Egg production increases with age from age 20 weeks up to age 36 weeks then declines up to the end of production cycle (Negoita *et al*., 2017). The evolution of the egg production curve for the birds that were fed on 2.56% dietary calcium was higher than for the laying hens fed on 3.12%, 3.86% and 4.22% dietary calcium but showed no significance difference. When the birds were fed 2.6% Ca, between start of laying and at 68 weeks, the eggshell weight increased significantly by 32.7%. When calcium was fed at 3.1, 3.9 and 4.2% eggshell weight increased significantly by 35.1, 45.0 and 47.0%, respectively. The egg weight increased linearly up to age 40 weeks and there after developed a Plateau (Negoita *et al*., 2018).

Eggshell quality

Gongruttananun, (2011) conducted a study using Rhode Island Red females on the effects of calcium source from eggshell on layers productive performance. In this study, limestone was replaced with ground sterilized eggshell. Layers overall performance and eggshell quality was assessed. Egg specific gravity, thickness and shell percentage were not affected by the treatment diets. In a similar study, Pelícia, *et al.*, (2017) studied an alternative calcium source. The study showed that marine calcium can replace calcitic limestone up to 45% with no effects on performance or egg quality.

Tune and Cufadar, (2014) studied the effect of Ca sources and particle size on layer performance and eggshell quality. They reported that dietary Ca from different sources and the size of the particles had no significant effect on feed intake, FCR, egg production, eggshell weight, specific gravity, thickness and body weight gain but egg weight had significantly affected by the treatments

Similar findings by Valable *et al.*, (2018) using 20-week old Japanese quail and replacing limestone calcium source with oyster shells and calcium premix. They reported that different dietary Ca sources had no significant effect on egg weight, shell weight, shell thickness and shell percentage. Limestone, oyster shell and eggshell dietary calcium had no significant effect on layer weight gain, egg production, egg size, feed intake and feed conversion ratio but had significant effect on egg weight (Tune and Cufadar, 2014)

The particle size effect on eggshell quality is not conclusive. In their study De Witt and associates (2009) looked at the effect of different sizes particles on performance and eggshell quality parameters. Varying limestone particle sizes had no effect on productive performance and eggshell quality. This implies that larger particles of limestone are not always essential to offer sufficient Cato laying hens, provided that the dietary Ca content satisfies the requirements of the laying hen. Similar findings have been reported by Świątkiewicz, *et al*, (2015)

2.3.4 Effect of age on eggshell

In a study by Tumova and associates (2014), it was shown that the age of the bird affects eggshell weight. In this study, the heaviest mean eggshell weight of 7.74g was realized in birds 83 weeks old compared to 6.76g in the birds 22weeks old. In a study using Perkin ducks, hatching eggs were collected at ages 26, 31, 36 and 42 weeks. Determination of their egg weight showed an increase in weight of 11.1g between 21and 31 weeks of age (i.e. from 77.5 to 88.6g) and a decrease between 36 and 42 weeks (from 87.8 to 85.3g) as reported by Samiullah *et al.*, (2016).

2.3.5Effect of ambient temperature on eggshell quality

Tumova *et al*, (2014) using 22 weeks and 83 weeks Lohnmann brown layers and 36 weeks and 64 week old 500 cobb broilers showed high shell Ca of 351 per kg at an environmental temperature of 28^{o}c compared to 343g/kg at 20^{o}c environmental temperature and poorer egg shell strength of 3.32kg/cm^2 at 28^{o}c compared to 3.61kg/cm^2 at 20^{o}c.The study attributed the poor egg shell strength at high temperatures to the more rapid precipitation of calcium carbonate leading to high rate of calcium deposition. The high rate of calcium deposition leads to a large crystal formation resulting to poor eggshell strength

The quality eggshell deteriorates during exposure to high temperatures as an example during summer months and at temperatures above 25^o C. Hens react by increasing their rate of breathing in order to cool itself (Tumova and Gous, 2012). This may lower the blood carbon dioxide a condition referred to as 'respiratory alkalosis' leading to alkaline blood pH which reduces the availability of calcium. This blood acid base imbalance due to high temperatures causes an increase in number of eggs with soft shells (Sharif *et al*., 2019). Research has indicated that exposure of a laying hen to high ambient temperatures leads to significant reduction of feed intake which ultimately affects calcium intake and hence eggshell formation

2.4 Physical and chemical characteristics of limestone

Zhang *et al,* (2017) studied *in vitro* solubility in an experiment where three dietary calcium level feeds i.e. 1.95%, 3.72%, and 5.32% were fed to 88 weeks old Leghorn hens. This study showed that *in vivo* solubility was 88.2, 78.6 and 77.3% for the three levels of dietary calcium, respectively. Retention of limestone in the gizzard was 2.77, 5.98 and 5.81g, respectively. The increase in dietary particle size is directly proportional to amount of limestone retained inside the gizzard. Excessive supply of limestone from a much less in vitro soluble supply decreases limestone in vivo solubility by increasing the excretion of the undissolved limestone (Pelícia *et al*., 2009).

Prolonged retention of Ca particles in the gizzard has been associated with more availability of the mineral throughout shell formation period. The retention time of Ca particles in the layer's gizzard is one of the factors that affect the amount of this mineral deposited on the egg shell hence affecting the eggshell quality. The retention time is dependent on the source of the Ca , size of the particles and the *in vivo* solubility (Zhang *et al,* 2017).The potential of *in vivo* solubility of Ca particles has been estimated using *in vitro* solubility technique defined by Cheng and Coon (1990).A study by Zhang *et al.*, (2017) on *in vivo* and *in vitro* solubility showed that the two are reversibly related. In the same study, large particle size limestone (greater than 0.8 mm) had low *in vitro* solubility of 30-50 %, accumulated in the gizzard and produced a high *in vivo* solubility. Pizzolante *et al*. (2009) reported that limestone with large granules had low *in vitro* solubility and bird fed on its diet produced a smaller number of cracked eggs compared to the fine particle limestone.

In a study by Zhang *et al*., (2017) in which the limestone particle size was defined using the average US Standard Screen numbers (S. No) the results shown in Table 3 were obtained.

Table 3: *In vitro* and *in vivo* solubility of limestones particles of different sizes

Parameter	Average US Standard Screen numbers (S. No and equivalent in mm)			
	5 (4.0 mm)	8 (2.38 mm)	14 (1.4 mm)	27 (630 µm)
In vitro solubility, %	29.8	45.8	49.3	63.1
In vivo solubility, %	86.3	84.0	79.1	78.0
Limestone gizzard retention, g	7.9	6.2	4.5	0.8

Source: Zhang et al., (2017)

The study showed that Ca sources or supplements with low *in vitro* solubilities had increased gizzard retention and increased *in vivo* solubilities. These attributes influence utilization of various sources of Ca and ultimately affect eggshell quality.

2.5 Effect of time of lay on eggshell thickness

Harms *et al*., (2016) tested the impact time of lay on eggshell quality. They showed that specific gravity and shell weight were higher in eggs laid early in the morning than those collected in the afternoon. Ketta and Tůmová (2018) reported that eggshell weight was significantly increased in the thick eggshell category being higher in morning (7.23 g) than in afternoon (5.14 g). Saki, *et al.*, (2019) in their study reported that providing calcium at 9.00 pm resulted in higher shell weight, egg thickness and shell percentage. Oviposition time plays a critical physiological role in determining eggshell traits. The quantity of calcium deposited within the shell depends on time spent inside the shell gland after a pumping (Tumova et al., 2016). Cambo *et al*., (2007) commended that the distribution of oviposition time is restricted to an 8 hour period of light with eggs being laid between 7.30 am to 16.00 pm under standard lighting conditions. This agrees with an experiment by Tumova *et al*., (2014). According to Tumova *et al*., (2015) study high shell calcium was realized in the morning eggs 352g/kg compared to those laid in the midday 344g/kg and those laid in the afternoon 342 g / kg. The reverse was observed in the amount of shell phosphorus and magnesium values which increased with late oviposition with values of 1.20 g / kg P in eggs laid in the morning, 1.21 g / kg P those laid in the midday and 1.43 g / kg P for the afternoon eggs. There was a decrease in eggshell weight and eggshell strength and an increase in eggshell thickness respectively in eggs laid in the morning compared to those laid in the afternoon. The higher shell calcium in the morning eggs was attributed to longer periods of calcium deposition in the medullary bone in the dark period (Kebreab *et al*., 2009).

The role of magnesium is to slow down and prolong the eggshell forming periods in the shell gland and leads to increased shell magnesium and shell thickness in the eggs laid late in the day (Choudhary *et al*, 2015).A study by Daigle *et al*, (2014) introduced environmental stress by moving laying ISA brown adult hens into cages containing unfamiliar hens one hour before the predicted oviposition time for a time ranging from 20 minutes to 360 minutes showed that stress can cause oviposition delays. This oviposition delays were classified as either short term (oviposition delay ended during stress or less than 2hrs after stress ended) and long term (when the oviposition delay ended more than 5 hrs after the stress ended).The eggs that were laid within the short term oviposition delay were evenly dusted and normal while most of the eggs that were laid in the long term delay were slab-sided, some were premature and soft shelled.

There was an observed increase in egg weight (60.3g to 61.8g), shell thickness (0.347mm to 0.352 mm) and a slight decrease in egg shell strength (47.5g/cm^2 to 44.6g/cm^2) and no significant difference in egg shell weight (6.15g Vs 6.19g) with age when ISA brown layers aged 21 weeks were housed in an enriched cage system fed adlib with isonitrogenous and isocalolific feed for up to 51 weeks of age (Vlckova *et al.*, 2018).

2.6 Conclusions

From the review of literature presented above, the following conclusions can be made

i. Calcium is an important mineral required for various physiological processes such as bone structure, osmotic pressure, ionic balance and enzyme systems.
ii. It is required for egg production and formation of the eggshell, which is almost 100% calcium carbonate.
iii. Sources of calcium include oyster shell, limestone, bone meal, dicalcium phosphate. Limestone is widely used in poultry feed because it is readily available and is inexpensive.
iv. The factors that differentiate one source of limestone from another are calcium and magnesium content as well as content of other minerals such as iron and aluminium
v. Particle size of limestone affects the retention time and also the availability of calcium.
vi. Calcium affects egg production and eggshell quality.

vii. Eggs laid in the afternoon have thicker eggshell so long as they are fed limestone with coarse particles.

CHAPTER THREE

3.0 Physical and chemical characteristics of limestone mined in Kenya Coast (UKC) and Athi river(AR).

3.1 Introduction

The objective of this study was to determine the physical and chemical properties of limestone used in poultry feeds in Kenya. Limestone is a sedimentary rock, which mainly contains calcium and magnesium carbonate. It is formed by deposition of skeletons of small creatures and or plants (organic limestones), or by chemical precipitation. Limestone is made up of varying proportions of calcium carbonate ($CaCo_3$), magnesium carbonate ($MgCo_3$), silica, alumina, iron oxide, sulphate, phosphorus and potash soda with $CaCo_3$ and$MgCo_3$ being the two major components. The two main impurities in limestone are silica and alumina with iron as the third. The colour of most limestones varies in shades of grey and tan. The greyness is caused by the presence of carbonaceous impurities and the tan by the presence of iron. Limestone varies depending on the ratio of calcium to magnesium and the amount of Mg levels it contains. Thus, dolomitic limestone (DLS) consists of 5 – 46 % magnesium carbonate and more than 20% magnesium oxide while calcitic limestone (CLS) contains less than 5% magnesium carbonate. CLS is generally considered to contain more than 95% calcium carbonate. However, most commercial high-calcium limestones contain 97- 98% calcium carbonate (Khobondo, *et al*, 2014). Other than the mineral content limestone utilization is influenced by particle size and solubility.

3.2Materials and methods

3.2.1 Source of limestone

The limestone used in this study was purchased from a raw material vendor in Nairobi who had obtained some of the limestone from Athi River near Nairobi and the other from Ukunda in coast region of Kenya. The limestone from Athi River (AR) is white in colour while that from the coast UKC is brown (Plate 1)

PLATE 1: Brown (UKC) and White (AR)) limestone

UKC and AR limestone samples were obtained and used for determination of physical and chemical characteristics.

3.2.2 Determination of particle size

Limestone samples were analysed for particle size using the methods described by Zanotto and Bellaver (1996) and Chakraborty (2016), which involved using a vibrator with ABNT (Brazilian Association of Technical Standards) sieves. The sieves had a mesh ranging in sizes from 2 mm to 0 .063 mm. Below the sieves was the collector. The sieves were stack together from the biggest to the smallest sieve in descending order as shown in Plate 2 below and firmly fixed on the vibrator. One kilogram of the limestone sample was weighed and put into the first sieve (2 mm mesh sieve). The vibrator was rotated for 30 minutes at a speed of 1,450 rpm. The amount of limestone remaining on each sieve was weighed and recorded. Percentage particle size was determined using the formula.

Weight of limestone in the respective sieve (%) = (Particle weight (g) X 100)/1000 g

Decrease in sieve size

Collector at the bottom

PLATE 2: ABN sieves and the collector

3.2.3 Chemical analysis of limestone

Limestone samples were analysed for various minerals. A preliminary investigation was done to determine the minerals present in the samples using an X-Ray Fluorescence (XRF) method This was done at the Geology and Mines department, Ministry of Natural resources GOK, Nairobi, Kenya

After the preliminary investigation using XRF, the samples were analysed for the minerals expected to be present namely: calcium, zinc, iron, potassium, magnesium aluminium, cobalt, copper, chromium, manganese, sodium and phosphorus using (AOAC, 2016) procedures. Half a gram (0.5 g) of sample was weighed into a 100 ml glass beaker and 15 ml of a digestion mixture added. This mixture consisted of three parts 70 % concentrated per-chloric acid and one part 70 % concentrated nitric acid. The glass beakers with the contents were placed on a sand bath and allowed to boil for 3 to 4 hrs until the solution became colourless. The beakers were allowed to cool and 20 ml of distilled water was then added into each beaker.

The contents were then filtered through an ashless Whatman filter paper no.42 into a 100 ml volumetric flask, after which it was topped up to the mark using distilled water. The methods used for determination of the minerals content in the sample were: flame emission spectrophotometer, atomic absorption spectrophotometer and UV-visible spectrophotometer (Table 4). Spectrophotometry was used to determine phosphorus at a wavelength of 400 nm. A commercial phosphorus stock standard (1000 ppm) was purchased from Pyrex East Africa Nairobi, was used to prepare working standards of the range 0 to 10 µg/kg with a 2 µg/kg gradient. The working standards were prepared through a serial dilution of 1000 µg /kg to 500 µg/kg to 250 µg/kg to50 µg/kg (AOAC, 2016). The 50 µg/kg stock standard was used to prepare the working standards. 15 ml of a mixture of ammonium molybdate, ammonium metavanadate and nitric acid was used to develop a yellow colour in both the standards and the samples (AOAC, 2016).

Table 4: Methods used for analysis of minerals in limestone

Method used	Mineral analysed for	Equipment
Atomic absorption spectrophotometer	Ca, Mg, Zn, Cu, Co	Varian, spectra AA
Flame emission spectrophotometer	Na, K	Emission spectrophotometer (PFP7 – JENWAY, UK)
UV-visible Spectrophotometer	P	UV-Visible Spectrophotometer - Hitachi u 2900, Model 2JI-0003 Tokyo

3.2.4 Determination of solubility of limestone samples

The *in vitro* solubility of the two limestones samples was carried out so as to estimate *in vivo* solubility in the gizzard. The solubility was determined by weight loss percentage method as described by Cheng and Coon (2013). Approximately 2.0 g of each of the two limestone samples were weighed in duplicates into 500 ml glass beakers. To each beaker a 200 ml of 0.2N hydrochloric acid was added. The beakers were then heated in a water bath for 10 minutes under slight agitation with the temperature being maintained at 42 ^{o}C. A 200 ml of de-ionized water was added in each of the beakers to stop the reaction (AOAC, 2016). The solutions were filtered through a Whatman 42 ashless filter paper after which, residue dried in an oven at 60 ^{o}C for 20 h and then finally weighed using analytical balance (AOAC, 2016). Percentages *in-vitro* solubility was calculated by weight difference (Zhang, Caldas & Coon, 2017)

3.3: Results and Discussion

Table 5 shows the particle size of the limestone samples categorized according to the sieve size used in the study. From poultry nutrition point of view, the particle size is put into four categories that is course medium, fine and silt (De Witt *et al.,* 2009). Information in Table 5 was used to generate Table 6.

The limestone samples differ in terms of particle size. UKC limestone had a higher proportion of coarse particles than the AR limestone. On the other hand, AR limestone had more medium sized particles than the UKC. Both types have large amounts of fine and silt particles (47 and 52% for UKC and AR respectively).

Table 5: Particle size of limestone samples from Ukunda (UKC) and Athi River (AR)

Sieve size (mm)	UKC limestone (% of sample)	AR limestone (% of sample)
2	8.33	0.43
1	14.31	9.48
0.5	17.36	28.64
0.25	16.71	25.0
0.125	13.52	16.9
0.063	17.78	10.0
<0.063*	11.63	9.6
Total	99.64	99.63

**This is the collector where the finest material falls*

The particle size influences the digestion and absorption of limestone (Świątkiewicz *et. al.*, 2015). Digestion is higher in the fine particles than the coarse ones. The particle size also affects the eggshell quality and egg weight. Tunç and Cufadar (2014) reported that the heavy eggs were produced when hens were fed a mixture of course limestone and fine limestone in equal amounts. In an earlier study Cufadar *et.al., (*2011) suggested that layers should be provided with limestone of medium particle size (2 to 5 mm) in the diet to maintain bone, layers performance and eggshell quality. On the other hand, Bueno *et al.*, (2016) reported that limestone containing large particles release Ca slowly and uniformly during the process of eggshell formation, allowing calcium retention into the medullary bone of the layers. This indicates that it is desirable to have limestone with both fine and course particles.

Table 6: Category of particle size limestone samples

Particle description	Size (mm)	UKC limestone %	AR limestone %
Course	1-2	22.6±0.15	9.9±0.15
Medium	0.5	17.4±0.15	28.6±0.05
Fine	0.125-0.5	29.2±0.1	41.9±0.15
Silt	< 0.063	17.8±0.3	10.0±3.9

3.3.1 Chemical composition

Mineral content

The limestone samples were analysed for ten minerals and were found to contain no Mg, K, Mn and Co. This shows that those minerals which were shown to be present in the preliminary investigation could not be detected using the method applied in this study. The absence of magnesium makes the two sources of limestone to be classified as calcitic limestone (Khobondo, *et al.*, 2014). Table 7 below shows the concentration of minerals in the UKC and AR limestones respectively. The samples contained Ca, Al, Fe, Cr, Cu and Zn. Both UKC and AR limestone had high levels of calcium at 420 and 340 g / kg, respectively. The level of calcium in UKC was higher than the values reported by Scotts *et al*, 1974 and Viem, (2010). A high concentration of calcium is important in that it minimizes the dilution effect of the diet by limestone The UKC limestone had high levels of aluminium compared to AR limestone. The two samples also contained Cr, Cu and Fe.

Table 7: Concentration of analyzed minerals in UKC and AR limestone

Mineral	UKC limestone	AR limestone
Calcium, g/kg	420 ± 0.1	340 ± 0.15
Content in μ/kg		
Aluminium (Al)	1072.5 ± 7.17	302 ± 3.2
Iron (Fe)	42.6 ± 7.9	53.6 ± 2.9
Chromium (Cr)	7.0 ± 1.78	8.6 ± 1.41
Copper (Cu)	3.0 ± 0.01	3.0 ± 0.01
Zinc (Zn)	0.3 ± 0.04	0.0

Solubility

UKC limestone had higher solubility than the AR one (Table8). Solubility of limestone is influenced by particle size and the amount of Acid Insoluble Ash (AIA). The proportion of coarse particles was 22.6 and 9.9% for UKC and AR limestone, respectively. On the other hand, the amount of acid insoluble ash was 3.39 and 23.17% for UKC and AR limestone, respectively (Table 8).

Based on particle size, AR which has 9.9% coarse particles was expected to be more soluble than UKC limestone which contained (22.6% - coarse particles) This however was not the case. This implies that the amount of acid insoluble ash had greater effect on solubility than particle size. Solubility influences absorption of minerals. In terms of calcium availability UKC was therefore, more available to the bird than AR limestone. AR limestone has the potential of longer retention time in the gizzard than the UKC limestone. Retention time is dependent on particle size and *in vivo* solubility of Ca zhang, *et al*., (2017) and Pizzolante *et al*., (2009).

Table 8: *In vitro* solubility and acid insoluble ash (AIA) of UKC and AR limestone

Limestone	*In vitro* Solubility (%)	Acid insoluble ash (%)
UKC	63.04± 0.34	3.39± 0.36
AR	29.43 ± 0.23	23.17± 0.16

3.4 Conclusions

The chemical and physical characteristics of limestone obtained from Athi River (AR) and that from the coast (UKC) were assessed. The following conclusions were made:

i. The calcium content in the limestone analysed was 42% for UKC and 34% for AR.

ii. UKC limestone had higher proportion of coarse particles (1-2 mm) than AR limestone.

iii. UKC had a higher solubility than AR.

iv. The content of AIA had a greater effect on solubility of limestone than particle size UKC and AR limestones contained trace amounts of Fe, Cr, Cu and Zn, but UKC limestone had a high content of Aluminium (1.07 mg /kg)

3.5 Recommendation

Based on physical and chemical attributes UKC limestone is recommended for use in layer feed. Both the UKC and AR limestone can be used in poultry feeds. UKC limestone is however superior to the AR one and where available it should be used.

CHAPTER FOUR

4.0 Effect of source of limestone and level of calcium in the diet on layers performance and eggshell quality in layers

4.1 Introduction

This experiment was carried out to assess the efficacy of two sources of limestone on layer performance and eggshell quality in layers. The producer is interested in obtaining eggs that will withstand breakage during handling. Eggshell quality affects water retention in the eggs, which influences internal quality of table eggs and hatchability of fertile eggs (Buzala, *et al.,* 2016 and Ketta and Tumova, 2015). The eggshell also keeps away pathogenic bacteria from gaining entry to the inside of the egg (Mazzuco and Bertechini 2014). Indicators of eggshell quality include thickness, specific gravity, eggshell weight and shell percentage (Ricke et al.,2015). These parameters are inter-related. The properties of the eggshell are described by the eggshell structure which is a bio-complex resulting from controlled interaction between both minerals and organic matrix constituents (Hunton, 2005). The eggshell is almost one hundred percent calcium carbonate. Therefore, inadequate intake of calcium will affect its formation in the shell gland.

The quality of the eggshell is determined by factors such as genotype, age of the hen, size of the egg, environmental temperature and housing system, time of lay and nutrition of the layers (Pavlovski *et al,* 2012). Minerals such as calcium, magnesium and phosphorus as well as vitamin D influence eggshell quality (Scott *et al*., 1982). To ensure high level of production and good eggshell quality, the laying hen must receive an adequate supply of Ca in the diet. The Kenyan bureau of standard specifies dietary Ca level for laying hens as 3.3 % (KEBS, 2014). Limestone, dicalcium phosphate, bone meal and fish meal are examples of Ca sources used in poultry feeds. Limestone is widely used because it is inexpensive and readily available. However, limestone is mined from different places which can influence its physical and chemical characteristics. Such characteristics are likely to influence its utilization by the laying hens. In this study the efficacy of limestone mined in the Kenyan coast (UKC) and Athi River, near Nairobi was assessed.

4.2 Materials and methods

4.2.1. Chemical analysis of raw materials

Limestone was the main source of calcium in the diets used in this study. It was obtained from two sources namely, Ukunda in the coast region of Kenya (UKC) and Athi River, near Nairobi (AR). The other main raw materials used in the diets were maize grain, soybean meal and wheat pollard. These were analysed for proximate composition consistent with the AOAC procedures of 2018. They were also analysed for phosphorus (P) and calcium (Ca) content whereby UV-visible spectrophotometer and atomic absorption spectrophotometer were used for determination of P and Ca, respectively.

4.2.2 Experimental diets

Six experimental diets and one depletion diet were formulated. The depletion diet was formulated to contain less than 1% calcium and no limestone was used. UKC limestone was used for the first three diets while the AR was used in the others. Each limestone source was included in the diet so as to supply 1, 2 or 4% Ca (Table 9). The experimental diets were formulated to satisfy the nutrient requirements for the layers (NRC, 1994). The chemical composition of the formulated diets was determined to confirm the calculated nutrient composition prior to the start of feeding. Each diet was randomly assigned to a group of six birds each replicated four times, making a total of twenty-four birds per treatment.

Table9:Composition of layer diets used in the experiment

Ingredient (%)	Depletion diet	UKC limestone 1%	UKC limestone 2%	UKC limestone 4%	AR limestone 1%	AR limestone 2%	AR limestone 4%
Maize grain	57.7	58.92	61.59	65.3	59.2	62.0	65.9
Soya bean meal	13.4	13.92	15.9	19.3	14.04	16.2	20.15
Wheat pollard	26.04	23.07	15.7	3.83	22.42	14.39	0.3
Corn oil	0	0	0	0	0	0	0.4
UKC limestone	0	1.24	3.9	8.6	0	0	0
AR limestone	0	0	0	0	1.5	4.5	10.3
DCP	1.7	1.7	1.8	1.9	1.7	1.8	1.9
Premix	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Toxin binder	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Coccidiostat	0.1	0.1	0.1	0.1	0.1	0.1	0.1
HCl lysine	0.09	0.08	0.05	0.02	0.07	0.05	0
DL methionine	0.07	0.07	0.06	0.05	0.07	0.06	0.05
Salt	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total	100	100	100	100	100	100	100

Source of the premix was Unga feeds Ltd Nairobi Kenya. Vitamin mineral premix composition : Vitamin D_3 -900,000IU/Kg, Vitamin A-4,500,000 IU/Kg; Vitamin K-1000mg/Kg; Vitamin E-12000mg/Kg; Vitamin B_2-1750mg/Kg; vitamin B_1-700mg/Kg Vitamin B_6-1500mg/Kg ;Vitamin B_{12}-0.024mg;Pntonthenic acid-4000mg/Kg; Nicotinic acid -32mg;Cholinechloride-350mg/Kg; Vitamin C-40,000mg/Kg;Fe-12800mg/Kg; Folic acid-400mg/Kg;Mn-4800mg/Kg;Cu-1600mg/Kg;Zn-14,400mg/Kg;I-448mg/Kg;Co-72mg/Kg;Se-40mg/Kg;Antioxidant-1600mg/Kg

4.2.3 Experimental birds

ISA Brown laying hens used in the experiment were obtained from a laying flock at the Poultry Unit, University of Nairobi. A total of 144 hens in their 25th week of age were selected, from a flock of 500 birds. Each of the birds was weighed using a spring balance and allocated randomly to the experimental cages in a completely randomized block design.

Housing of the birds was in metallic wire mesh battery cages with a floor measuring 32 cm by 38 cm and a height of 40 cm. The cages were fitted with feeding and drinking troughs. Each cage held two birds. The cages were in a staircase configuration consisting of two tiers. The lower tier of cages near the wall of the house formed the first block while the upper one formed the second block. To the inside of the house the lower and the upper tier, formed the third and fourth block, respectively (Plate 3)

The cages were placed in a well ventilated house with a natural lighting for a relatively constant period of about 12hrs per day. This was provided through open sides fixed with chicken wire mesh. Each experimental unit had three cages housing a set of six hens.

The hens were initially fed on a depletion diet; deficient in calcium, for ten days until laying of thin-shelled eggs was observed. After this, they were fed experimental diets and water *ad libitum* for 60 days. The water troughs were thoroughly cleaned daily to remove contamination with feed particles and fresh clean water added. The feed troughs were filled daily to three quarter level to prevent feed spillage. The excreta were removed three times per week to prevent build up. Sawdust was sprinkled every day on the concrete floor to help identify easily any fresh cracked egg that fell on the floor.

PLATE 3: Cages used in the experiment showing the lower and upper tiers

4.2.4 Experimental design

The design of the experiment was a 2 by 3 factorial, whereby two sources of limestone were used to supply dietary calcium at three levels (i.e. 1, 2 and 4%). Each of the dietary treatments was randomly allocated to each of the 4 blocks

4.2.5 Data collection

Data on egg production, feed intake and eggshell quality were collected. Data on egg production was obtained daily, while those on eggshell quality and feed intake were collected on weekly basis.

(i) Feed intake

A labelled plastic bucket of known weight was assigned to each replicate. At the beginning the experiment and subsequently each week, six kg of feed were put into each bucket. At feeding, the feed was obtained from the respective bucket and put into the trough. At the end of the week, any feed remaining in the trough was collected and returned into the respective bucket. The bucket and feed were then weighed. The difference in weight of the bucket and feed at the beginning and end of week represented the feed consumed during the week.

(ii) Weight of the birds

The birds were individually weighed at the onset and at the end of the experiment. The average weight for each replicate was calculated.

(iii) Egg production

Eggs were collected three times a day early morning (9.00 am), late morning (12.00 noon) and afternoon (3.00 pm). They were of different sizes (Plate 4). The number of eggs laid per replicate was recorded and categorized as good or broken. The hen-day egg production per replicate per week was calculated as: -

% Hen day egg production = (Number of eggs produced/Number of live hens) X 100

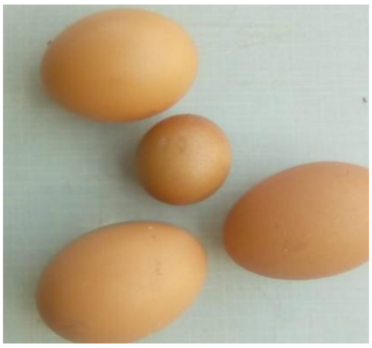

PLATE 4: Eggs of different sizes obtained during experiment

Eggshell quality

For each replicate all eggs laid were collected and checked for cracks and breakage. The number of eggs broken or with cracks in a replicate were noted and eliminated. All the eggs in a replicate whole egg were individually labelled and weighed. After this, various determinations as shown below were done.

(a) Specific gravity

All whole the eggs in a replicate were used to determine the specific gravity. This was done by immersing the eggs in saline solutions with specific gravities between 1.060 and 1.100g/cm3, with gradients 0.005 (Butcher and Miles, 2017). The saline solutions were made by dissolving specified amounts of common salt (NaCl) in three litres of water as shown in Table 10

Table10: Weight of NaCl dissolved in three litres of water for given specific gravity

NaCl dissolved (g)	Specific gravity (g/cm^3)
278	1.060
296	1.065
320	1.070
344	1.075
367	1.08
391	1.085
413	1.090
436	1.095
466	1.100

The eggs were then immersed in each saline solution starting from the lowest specific gravity to the highest. Before immersing into the next saline solution, they were rinsed off traces of the previous solution by immersing them in distilled water. The salt solutions were placed in plastic buckets which were well labelled according to the saline solution's specific gravity. The specific gravity of the solution in which each egg first floated was recorded. The concentration of the saline solution at which most of the eggs floated was taken to be the specific gravity for that replicate. Treatment means was calculated as the average of the four replicates

(b) Eggshell weight

The eggs were carefully broken, the albumin and yolk were separated from the eggshell (Plate 11). The Eggshells were washed under running tap water to remove any traces of albumin remaining on its surface and then dried in an oven at of 60ºC for 12 hours, after which they were left to cool to room temperature and finally weighed using a 0.0001g precision analytical balance.

PLATE 5: Cleaned and dried eggshells for determination of shell weight and thickness

(c) Eggshell percentage

The eggshell percentage was calculated by dividing its weight by egg weight and multiplying the result by 100 as shown in the formula below: -

Eggshell % = [Weight of the eggshell (g)/Egg weight (g)] X 100

(d) Egg shell thickness

Eggshell percentage and thickness were assessed using the same shells. The thickness of the eggshell with the membrane was measured at three positions at the egg equator using a 0.001mm precision micro meter screw gauge. The average value of the three measurements was then determined. These observations were pooled together per week. The treatment means were calculated as the average of the four replicates.

4.3 Data analysis

Data analysis was done using GenStat Statistical package and significant treatment means separated using Tukey's test.

4.4 Results and discussion

4.4.1 Chemical composition of raw materials and experimental diets

The dry matter content of the three main raw materials used ranged between 87.31 and 89.18% (Table 12), which was within the specifications for laying hens (KeBS, 2014).

Table 11: Chemical composition of the main raw materials used in experimental diets

Composition	Soy bean meal	Maize grain	Wheat pollard
Dry matter, %	87.66	87.31	89.18
Composition on dry matter basis			
Ash, %	4.97	1.24	3.48
Crude fibre, %	6.61	3.91	8.51
Crude Protein, %	48.22	10.1	16.93
Ether Extract, %	2.49	4.55	6.59
NFE, %	37.71	50.2	64.49
ME, Kcal/kg*	2814.82	2775.52	2919.11

The dry matter content of the seven diets ranged between 88.8 and 90.2, which were within the Kenya Bureau of Standards (KeBS) (2014) and NRC (1994) specifications. All diets had adequate levels of crude protein (KeBS, 2014) and also met specifications for crude fibre and Ether extract (6% or less) as shown in Table 13.

Table 12: Chemical composition (% air dry basis) and cost of the depletion and experimental diets

Composition	Depletion diet	Experimental diets					
		UKC limestone			AR limestone		
Dietary Ca level, %	<1	1	2	4	1	2	4
Dry matter, %	88.8	88.9	89.9	90	89.9	89.2	90.2
Crude protein, %	15.8	15.3	15.3	16.1	15.8	15.8	15.9
Crude fibre, %	5.8	6.2	6.1	6.3	5.7	6.2	4.9
Ether extract, %	5.4	3.3	4.5	5	3.2	4.5	4.6
NFE, %	68.5	69.7	66.8	58.2	69.5	65.7	62.2
Calcium, %	-	1.1	2.1	4.1	1.2	2	3.9
Total phosphorus, %	-	0.7	0.6	0.7	0.5	0.6	0.5
ME, Kcal/kg*	2881.2	2720.3	2811	2852.6	2723.8	2817.8	2844.4
Cost, Kshs /Kg		33.9	34.5	37.1	33.9	34.5	37.1

*Calculated metabolizable energy

In the experimental diets the NFE declined as the dietary calcium level increased with subsequent increase in limestone. This means that limestone has a dilution effect on the nutrients content in the diet. Diets 2 and 5 were formulated to contain a minimum of 1 % Ca while diets 3 and 6 were formulated to contain 2 % Ca. Diets 4 and 7 were to contain 4 %. The analysed Ca level of the diet was within the stipulated range. Total P was within the expected levels ranging between 0.5 to 0.7 %. To meet Ca requirements of 3.5% for a laying hen, then 7.2 kg and 9.1 kg of UKC and AR limestone will be required per 100kg of feed, respectively.

Table 13: Effect of dietary calcium source and level on egg production, feed intake, egg breakage, egg weight and feed conversion ratio in laying birds

	UKC limestone			AR limestone						Main effect		Interaction
Ca in the diet, %	1	2	4	1	2	4	Mean	LSD	SEM	Source	Level	Source *Level
Calcium intake (g/day)	1.3	2.7	4.6	1.4	2.4	4.6						
Feed consumed (g/hen/d)	115[a]	129.6[d]	112[a]	117[c]	118.7[a]	118.3[a]	118.5	3.1	2.325	0.417	<.001	<.001
Hen-day egg production (%)	69[a]	87[c]	77[b]	64[a]	77[b]	82[b]	76	4.6	2.438	0.023	<.001	<.001
Egg breakage, %	13.1[c]	2.3[a]	2.1[a]	21.9[d]	8.7[b]	4.5[a]	8.8	3.56	1.242	0.882	0.905	0.036
F CR (kg feed/dozen eggs)	2.1[b]	1.8[a]	1.8[a]	2.3[c]	1.9[a]	1.8[a]	2.0	0.11	0.042	0.034	<.001	0.085
Weight of whole egg(g/egg)	55.89[a]	57.93[b]	59.73[c]	57.27[d]	56.91[ad]	60.44[c]	58.03	1.65	1.446	<.001	0.636	0.019
Feed cost/dozen eggs (KES)	71.19[b]	61.74[a]	66.78[a]	77.97[c]	65.55[a]	66.78[a]	68.49	3.505	2.27	<.001	0.063	0.40

a, b, c, d Means followed by same superscript per row, do not differ significantly (*P*<0.05). LSD-Least significant difference

Table14: Effects of calcium source on performance of layer chicken

Parameter	UKC limestone	AR limestone	LSD	%CV	P-value
Feed consumed (g/hen/d)	119.10[a]	118.00[a]	2.60	7.70	0.417
Hen-day egg production (%)	77.65[a]	74.21[a]	5.14	13.70	0.230
Weight of whole egg(g/egg)	57.85[a]	58.21[a]	0.71	4.30	0.320
FCR (kg feed/dozen eggs)	1.90 [b]	2.00 [a]	0.05	15.00	0.034
Egg breakage, %	5.82[b]	11.72[a]	2.00	80.10	0.036
Feed cost/dozen eggs (KSH)	66.57[a]	70.10[a]	3.92	67.20	0.591

Means in a row with similar superscripts do not differ significantly (P > 0.05)

Table 15: Effects of dietary calcium level on performance of layer chicken

Parameter	4%	2%	1%	LSD	%CV	P-value
Feed consumed (g/hen/d)	115.15 [a]	124.15[b]	116[a]	0.0045	7.7	<.001
Hen-day egg production (%)	79.13[a]	81.89[a]	66.78[b]	5.141	13.7	<.001
Weight of whole egg(g/egg)	60.09[a]	57.42[b]	56.58[b]	0.295	3.5	<.001
FCR (kg feed/dozen eggs)	1.06[c]	1.10[b]	1.29[a]	0.085	15	0.085
Egg breakage, %	3.33[b]	5.50[b]	17.49[a]	3.46	80.1	0.036
Feed cost/dozen eggs	66.78[b]	63.64[c]	74.58[a]			

(i) Feed intake

The source of limestone did not have a significant effect on feed intake P>0.05 (Table 14). There was a significant interaction between the level and source of calcium for this parameter (Table 13, fig 1). For the birds fed on UKC limestone, the highest feed intake was recorded where layers were received diets containing 2% calcium (Fig 1). The feed intake lower for birds receiving 1% and 4% calcium respectively (Table 15). For birds fed on AR limestone, the highest feed intake was recorded among the groups receiving diets containing 2% and 4% calcium (Table 13). Increasing the level of Ca to 4% in the diet did not have effect on feed intake. The performance was significantly better than for those fed on diets containing 1% calcium.

The average feed consumption was 118.5 g/bird/day (Table 13), which was within the stipulated level for the layer chicken (Scott Neishem and Young, 1982). In the UKC limestone-based diets, feed intake was highest *(P<0.05)* when Ca level was set at 2% compared to 1 and 4% levels (Table 13). This was in agreement with Pelicia *et al.*, (2009) who reported that, increased limestone levels reduced feed intake. Increasing levels of calcium in AR limestone had no effect *(P>0.05)* on feed consumption. Similar results have been reported by Świątkiewicz, *et al.,* (2015) and An *et al*., (2016) showed that dietary Ca level did not affect the total feed intake and laying performance in layers.

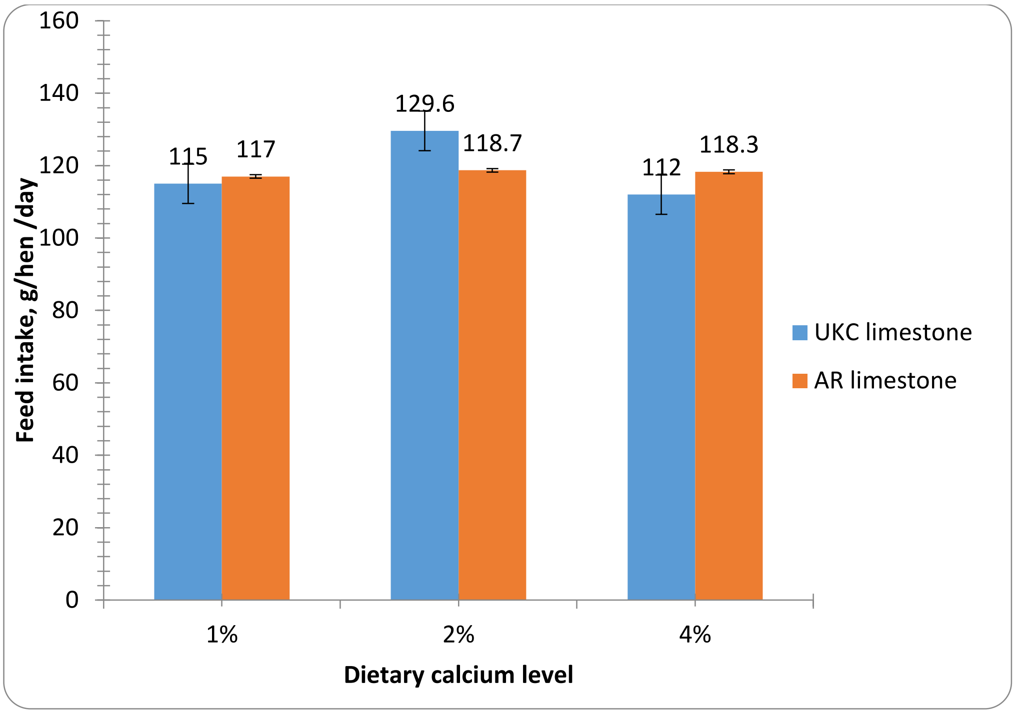

Figure 1:Effect of source of limestone and dietary calcium level on feed intake

It has been reported that including limestone at more than 7 % in the diet affects feed intake negatively due to the high level of dust (Pilicia *et al*., 2011). Feed intake is also affected by the particle size of limestone. The fineness and coarseness of limestone grains affect feed intake (Świątkiewicz *et al*, 2015). UKC limestone utilized in this study had higher level of course particles than AR limestone. High level of limestone seemed to reduce feed intake probably due to the high dust content (Fig 1). This could partially explain the significantly higher intake of feed at 2% compared to 4% for layers fed diets based on UKC limestone.

(ii) Egg production

The source of limestone had no effect (P>0.05) on hen-day egg production (Table 14). The overall effect of Ca in the diet showed that egg production significantly increased between 1 and 2% (Table 13). There was significant interaction between limestone source and Ca level on hen-day egg production (Table 13). Hen-day egg production was significantly higher *(P<0.05)* in the birds fed UKC limestone at 2% than at 1% Ca level (Table 13). However, increasing Ca level to 4%

resulted in a significant decline *(P<0.05)* in egg production and a reduction in feed intake (Table 15, Fig 2). Decreased feed intake will affect egg production because of availability of required nutrients. For the AR limestone increasing dietary Ca from 1 to 4 % resulted in significant increase *(P<0.05)* in egg production (Table 13, Fig 2). The difference in performance could be due to the physical characteristics of the two types of limestone.

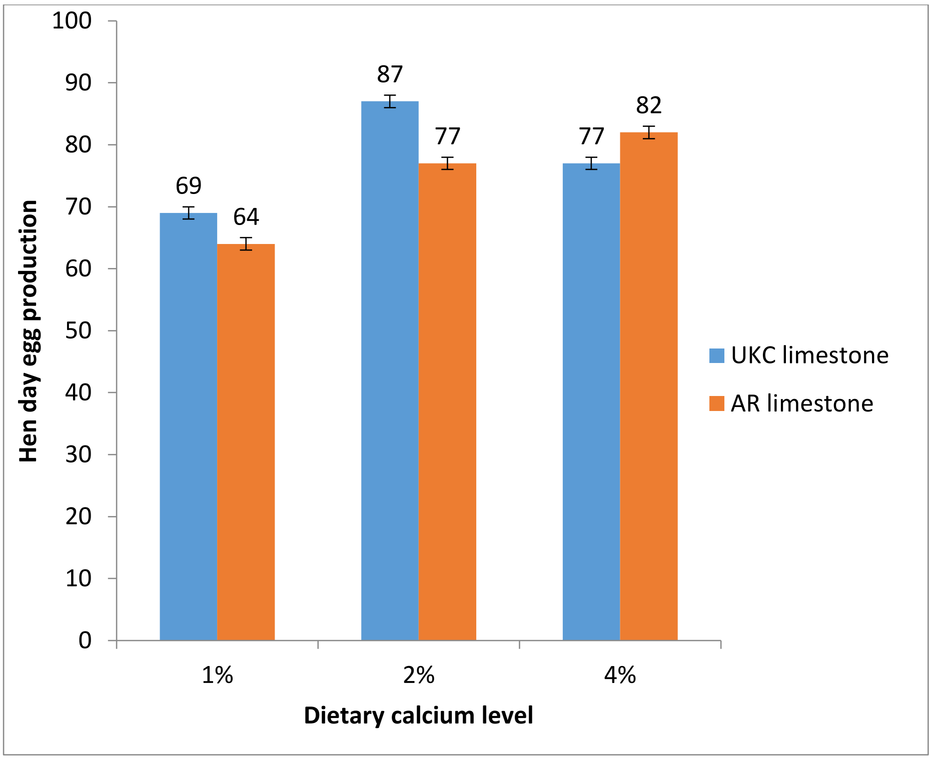

Figure 2: Effects of dietary calcium level on hen-day egg production

Commercial laying hens require approximately 3 g of calcium per day for maximum egg production (Nzioka *et al.*, 2017). When dietary Ca was set at 1, 2 and 4% the calculated Ca intake per bird on daily basis in the UKC limestone based diets was 1.3, 2.7 and 4.6 g, respectively. Similar values for AR limestone based diets were 1.4, 2.4 and 4.1 g (Table 14 and fig 3). The birds receiving 4% dietary calcium had more than the required amount for egg production.

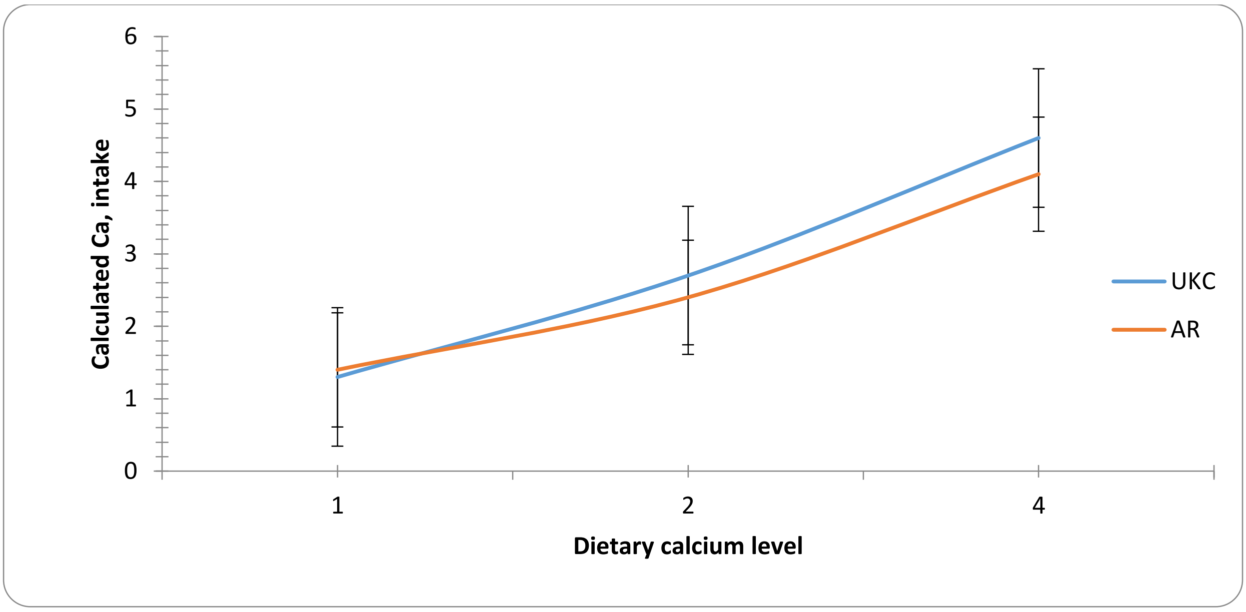

Figure 3: Calculated calcium intake at set dietary calcium level.

When dietary calcium level was set at 1%, its intake was 43 and 47 % of the requirement for UKC and AR limestone, respectively (fig 3). But when increased at 2 % the intake was 90 and 80 % for UKC and AR limestone respectively (Fig 3). For both types of limestone, when dietary calcium was set at 4% the intake was in excess of the requirement by 47 and 37 % for UKC and AR, respectively (Fig 3). Low levels of calcium lead to reduced egg production. Mild deficiency generally decreases egg production but does not stop it completely. However, sometimes the laying rate is high, even though the birds are fed on a low calcium diet (Onono, 2018), which is due to calcium mobilization from the bones.

(iii) Egg Breakage

The mean egg breakage was 8.8 % (Table 13). There was a significant interaction between the source and level of Ca for this parameter. The birds fed AR limestone had a significantly higher *($P<0.05$)* egg breakage than those on UKC (Fig 4). Egg breakage was significantly *($P<0.05$)* higher in layers fed diets containing 1% calcium than those on diet containing 2 and 4%, respectively (Table 15).

Among the birds fed on diets containing the UKC limestone egg breakage was higher *($P<0.05$)* when dietary calcium level was set at 1% than in 2 and 4% levels (Table 13). There was no difference ($P > 0.05$) in egg breakage between birds fed on diets based on UKC limestone containing 2 and 4% calcium respectively. Among the birds fed on AR limestone, egg breakage was highest for those fed on 1% calcium which was significantly higher than those fed on other diets. It reduced significantly ($P < 0.05$) as the level of calcium increased in the diets to 2 % and 4 % respectively. Pavlovski *et al.*, (2012) reported that about 6-8% of the total egg production is not always marketable because of low quality shells, and it's expected that between 14.3 and 21.3% of the total eggs laid internationally, are either broken or cracked earlier before they reach the consumer (Buzała, et al., 2015). Consequently, laying hens should be given adequate amount of calcium for strong eggshell formation (Onono, 2018). Saki *et al.*, 2019 supplemented a calcium-adequate diet with 3 g/hen/day and obtained a significant reduction in egg breakage. This study shows that increasing dietary Ca level resulted in reduced egg breakage. When dietary Ca is fed at 2% or more egg breakage is reduced to almost Zero. In terms of reducing egg breakage UKC was observed to be better than AR limestone. This is due to (a) relatively high Ca content in the UKC than in AR limestone (b) relatively higher proportion of coarse particles in UKC than AR limestone. Coarse limestone particle size results in better availability of Ca during egg shell formation.

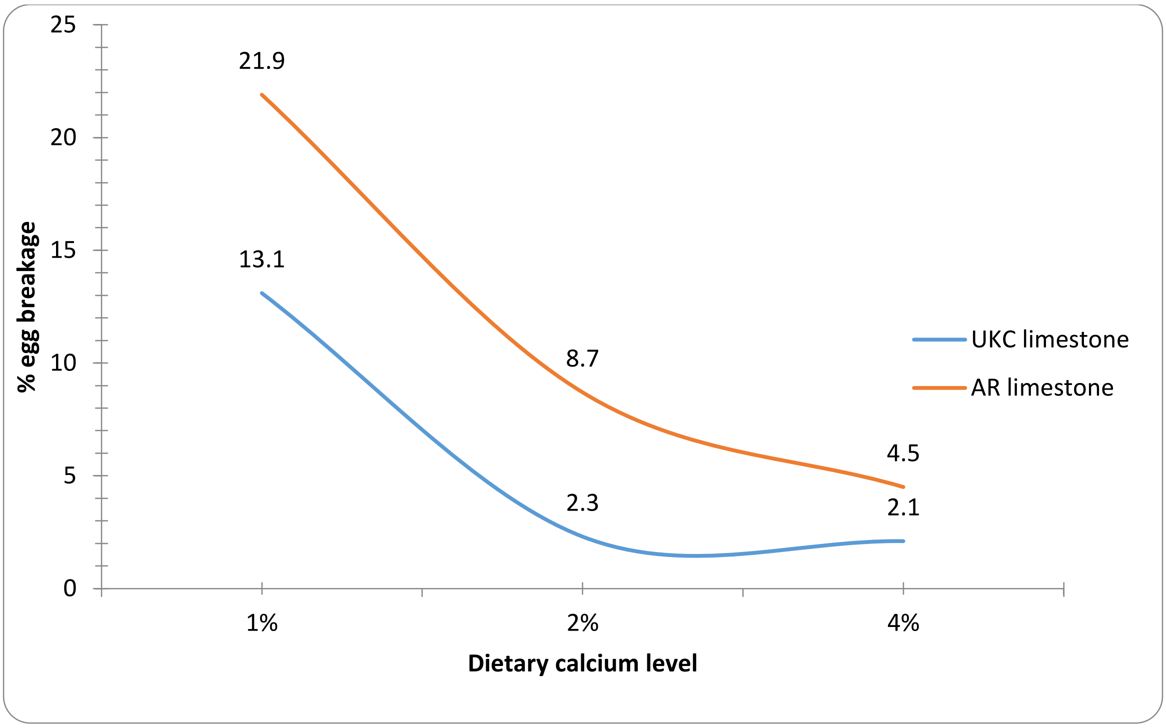

Figure 4: Percentage egg breakage for a set dietary calcium level

(iv) **Feed conversion ratio (FCR)**

The mean feed conversion ratio was 2.0 (Table 13). The interaction between source and level of calcium was not significant for this parameter. The limestone source and level in the diets had a significant effect on FCR *(P<0.05)*.When dietary calcium was 4 % the FCR was 1.8 which was significantly *(P<0.05*lower than 2.2 and 1.9 for birds fed diets containing 1% and 2%, respectively (Table 15). For the birds receiving UKC limestone, FCR decreases slightly with increase in dietary calcium level (P<0.05) (fig 5). The average FCR was 2.2, 1.9 and 1.8 for 1, 2 and 4 % dietary Ca level, respectively. The effect of level of calcium on FCR in AR based diets was similar to that obtained in UKC. Similar results have been obtained by Tune and Cufadar (2014) who reported that the source and level of Ca had no significant effects on FCR Świątkiewicz *et al*, (2015) reported that the fineness and coarseness of the grains affect the utilisation and FCR. However, in this study the effect of fineness of limestone was not assessed.

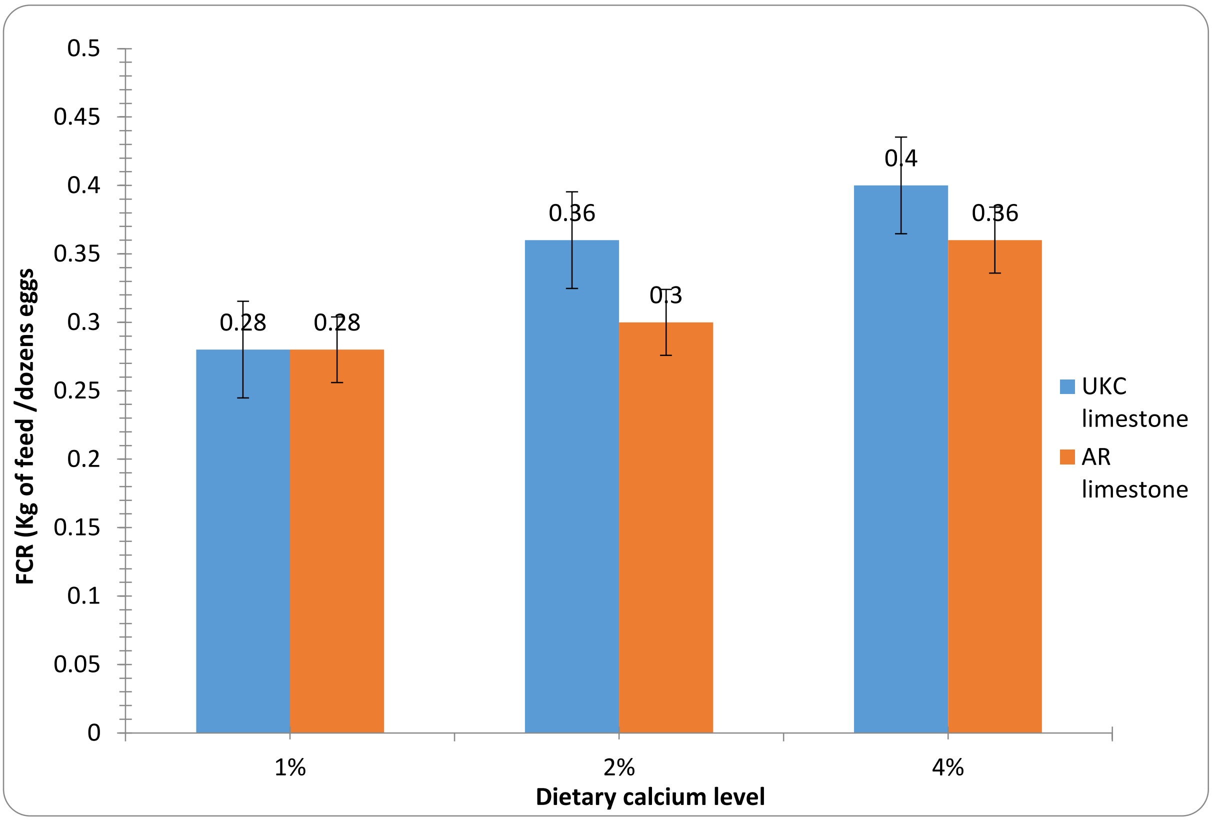

Figure 5: Effect of dietary calcium level on feed conversion ratio

(v) Feed cost

The average feed cost per dozen eggs was ksh.68.49 (Table 13). The UKC based diets were significantly cheaper ($P < 0.05$) than those formulated using AR limestone (Fig.6). The level of Ca inclusion had a significant effect *($P<0.05$)* on the cost of eggs (Table 13). For diets based on both UKC and AR limestone, the most economical level of Ca inclusion was 2% (Table 14). The duration of feeding had no effect on feed cost.

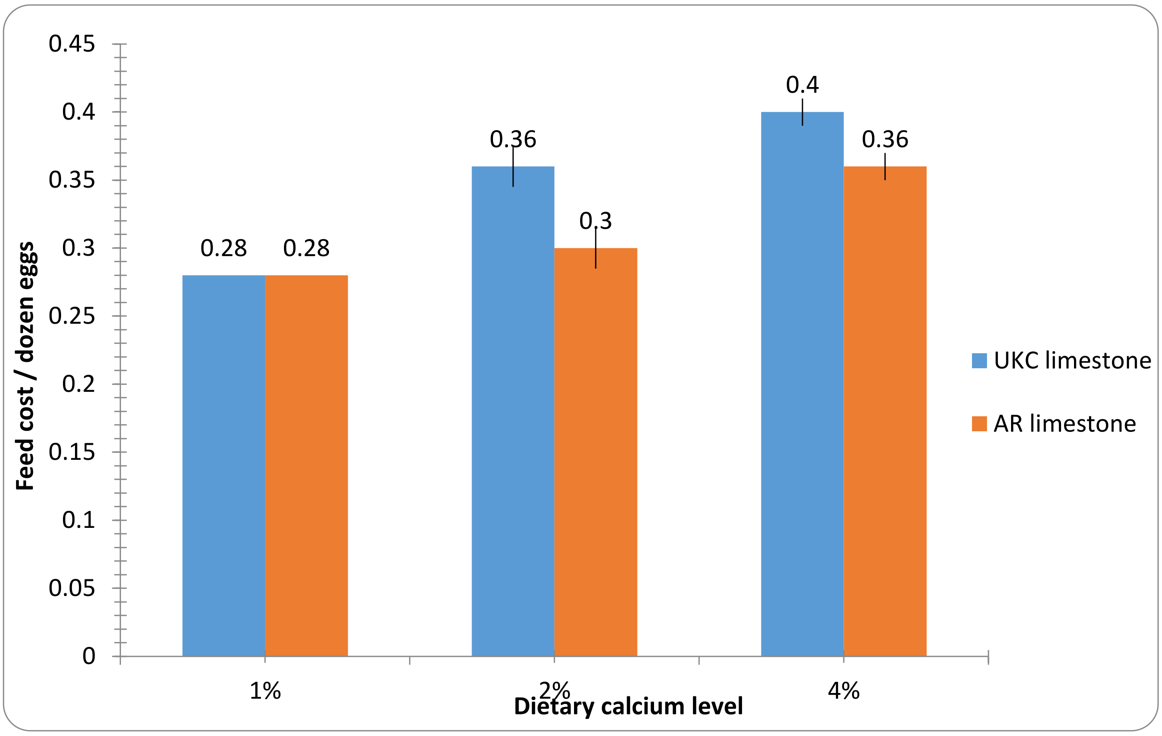

Figure 6: Feed cost per dozen eggs

(vi) Egg weight

The average weight egg was 58.03g (Table 13). There was a significant ($P < 0.05$) interaction between source and level of Ca for this parameter. For birds fed diets based on UKC or AR limestone, the highest egg weight was observed among those receiving diets containing 4% Ca (Table 15). The mean egg weight was 59.73 and 60.44 g for UKC and AR limestone-based diets, respectively. Birds that produced eggs with the least weight were those fed Ca at 1% level (UKC) and 2% level (AR) respectively (Table 13). There was a significant interaction between level and source

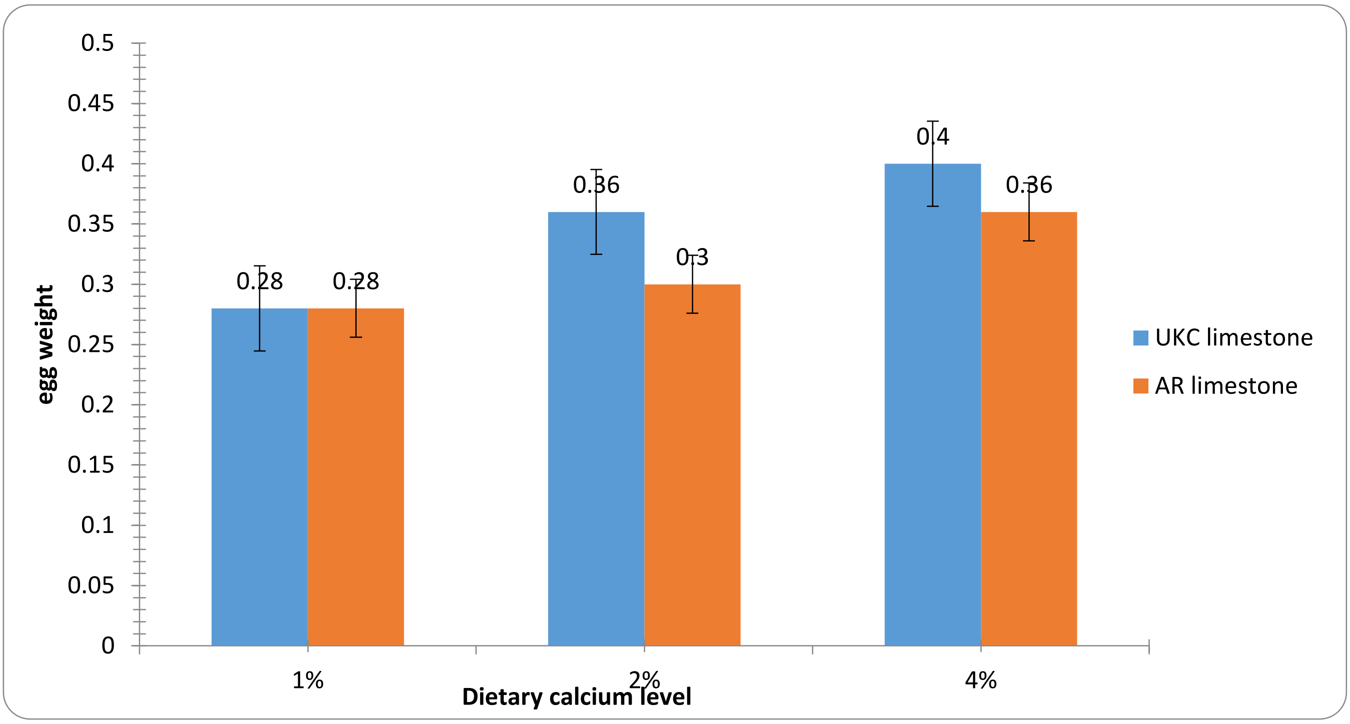

Figure 7: Effect of dietary calcium level on egg weight

Egg weight depends on the breed, size, age and nutrition of the bird (Tůmová, 2016). The consistently higher egg weight for layers feed UKC limestone could be attributed to high level calcium in this feedstuff. These findings however, contradict what has been reported by Tune and Cufadar (2014 and 2015) and Świątkiewicz *et al*, (2015) who indicated that source of Ca had no effect on egg weight. However, such a difference can only arise if there are specific differences in the chemical and physical attributes in the two sources. From this study UKC limestone is higher in solubility, calcium content and lower acid insoluble ash than AR limestone which could explain the differences observed.

4. 4. 3 Eggshell quality

The eggshell quality is defined by several factors including shell weight, shell percentage, egg's specific gravity and thickness (Pavlovski *et al*. 2012).These factors are shown in Table 16.

Table16: Effect of dietary calcium source on specific gravity, shell weight, g, shell thickness and eggshell percentage in layers

	UKC limestone (% dietary Ca)			AR limestone (% dietary Ca)						Main effect		Interaction
Parameter	1	2	4	1	2	4	Mean	LSD	SEM	Source	Level	Level*source
*SG of egg, g/cm³	1.06[a]	1.06[a]	1.10[a]	1.06[a]	1.08[a]	1.09[a]	1.06	0.13	0.1103	0.798	0.056	0.585
Shell weight, g	3.84[a]	5.2[d]	5.91[e]	3.81[a]	4.17[c]	5.18[d]	4.68	0.27	0.239	<.001	<.001	<.001
Shell thickness, mm	0.28[a]	0.36[b]	0.40[a]	0.28[a]	0.30[a]	0.36[b]	0.33	0.03	0.047	<.001	<.001	<.001
Eggshell percentage, %	6.87[b]	8.97[d]	9.90[e]	6.66[a]	7.33[c]	8.57[d]	8.05	0.44	0.385	<.001	<.001	<.001

**SG=Specific gravity:*
a, b, c, d, e Means followed by same superscript per row, do not differ significantly(P>0.05);LSD: Least significant difference

Table 17: Effect of source of limestone eggshell quality

Parameter	UKC limestone	AR limestone	LSD	%CV	P-value
Specific of gravity of egg, g/cm3	1.07[a]	1.06[a]	0.08	14.6	0.58
Shell weight, g	4.98[a]	4.39[b]	0.19	8.3	<.001
Shell thickness, mm	0.35[a]	0.31[b]	0.02	13.0	<.001
Eggshell percentage, %	8.58[a]	7.52[b]	0.29	7.3	<.001

Table 18: Effect of level of calcium in the limestone on eggshell quality

Parameter	4%	2%	1%	LSD	%CV	P-value
Specific of gravity of egg, g/cm3	1.09[a]	1.07[ab]	1.03[b]	0.02	14.7	0.056
Shell weight, g	5.54[a]	4.68[b]	3.83[c]	0.16	8.3	<.001
Shell thickness, mm	0.38[a]	0.33[b]	0.28[c]	0.01	10.2	<.001
Eggshell percentage, %	9.23[a]	8.15[b]	6.76[c]	0.08	6.8	<.001

(i) **Specific gravity**

The mean specific gravity of the eggs was 1.06 g /cm^3(Table 16). The source of limestone had no effect (P> 0.05) on the specific gravity of the eggs (Table 17). The level of dietary Ca had a significant (P<0.05) effect (Table 18). Birds fed on 4 % dietary calcium level had a higher specific gravity than 1 and 2%. Similar findings have been reported by Pelicia *et al*., (2009), Gongruttananun, (2011), Tune and Cufadar, (2014) and Harms *et al*., (2016).

(ii) **Shell weight**

The average shell weight was 4.68g (Table 16). Eggshells from layers fed UKC limestone were significantly heavier than those from birds fed on AR limestone with means of 4.98 and 4.39 g, respectively (Table 17). At 1, 2 and 4% dietary Ca level the eggshell weight was 3.83, 4.69 and 5.55 g, respectively (Table 18). On average increasing dietary calcium level resulted in significantly heavier shell weight *(P<0.05).* At the onset of feeding the shell weight was 4.54 g, which increased to 4.89 g at 2 weeks of the experiment period. Between the 2^{nd} and 8^{th} week the shell weight ranged between 5.00 g and 5.18 g for UKC and between 4.24 g and 4.44 g for AR limestone.

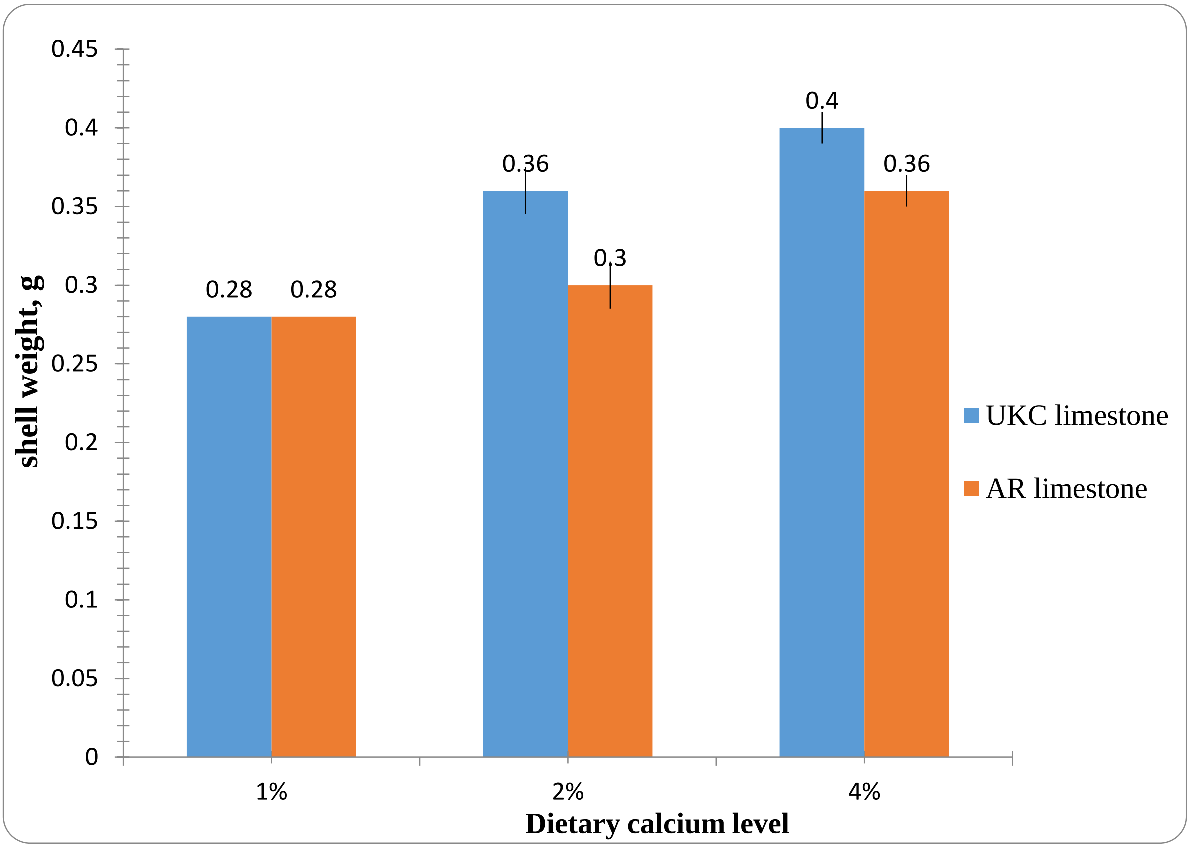

Figure 8: Effect of dietary calcium level on shell weight

The results obtained in this study show that there is a clear difference in the physical and chemical attributes in the UKC and AR limestone. UKC limestone has a higher calcium content, higher solubility and higher proportion of coarse particles than the AR limestone. This implies that more calcium is available for eggshell formation in UKC than AR limestone. A study by Pelicia *et al* (2009) showed an increase in eggshell weight with increase in dietary calcium level from 3.0 to 4.5 %. Robert *et al.*, (2013) reported that eggshell weight may increase from early laying (25-40 weeks) to mid lay (40-55 weeks) as the egg size is increasing.

(iii) Shell thickness

The average eggshell thickness was 0.33mm (Table 16). The thickness of the eggshells derived from hens fed UKC limestone was 0.35mm, which was thicker ($P < 0.05$) than those fed the AR limestone at 0.31mm (Table 17). Eggshell thickness increased with increase in dietary Ca (P< 0.05). This trend was also seen when the source of limestone was considered (Fig 9).

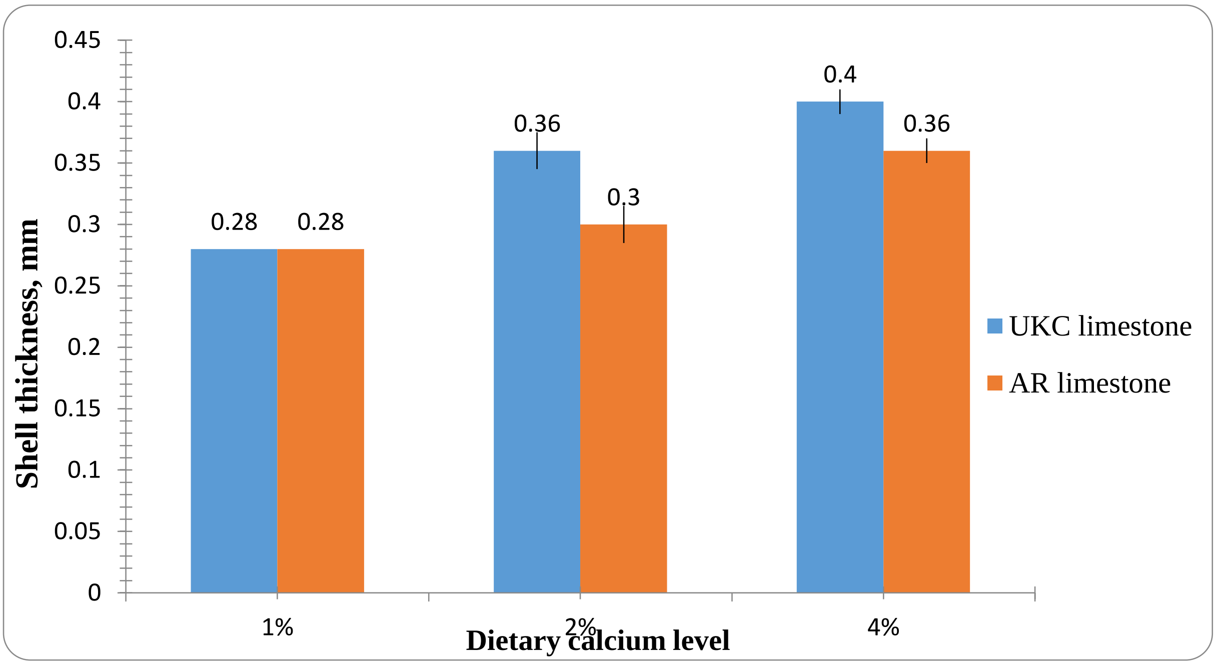

Figure 9: Effect of dietary calcium level on shell thickness

When the duration of feeding the two sources of limestone was considered it was seen that UKC limestone resulted in consistently thicker shells than the AR limestone. The duration of feeding had a significant (*P<0.05*) effect on the eggshell thickness.

Prior to the start of the experiment the birds had been fed a diet with less than 1% Ca. At one week of the experiment the shell thickness was 0.27 mm, which increased to 0.34 mm at 2 weeks. Between the 2nd and 8th week of the experiment the shell thickness ranged between 0.34 mm and 0.37 mm for UKC limestone and 0.27 mm and 0.32 mm for AR limestone (Fig 10). Calcium intake affects shell thickness (Buzała *et al*., 2015), which in turn affects shell strength. Consequently, eggshell thickness was noted to rise from the second week of the feeding. Eggshell thickness was slightly low in the first two weeks which could be due to effect Ca depletion prior to the onset of the study.

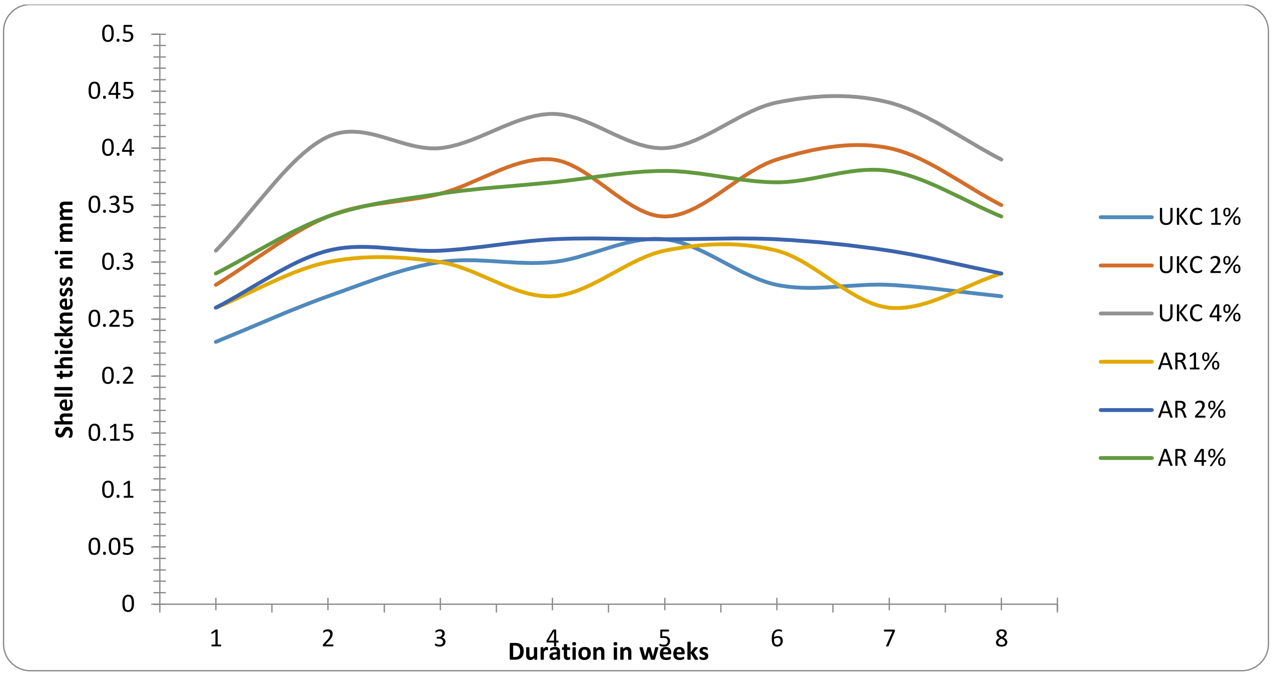

Figure 10: Effect of time, source of limestone and dietary calcium level on shell thickness

The eggshell is almost 100% calcium carbonate and accounts for nearly 2.2g of calcium in the egg (Rodriguez, 2013). Factors that affect eggshell thickness includes calcium and vitamin D intake, ambient temperature, size of the egg and genotype of the bird. In this study Ca intake and its source have been shown to affect eggshell thickness. These findings are in agreement with those of (Robert *et al*., 2013, Sekeroglu *et al*., 2014 and Arenas *et al.*, (2018).

(iv) Shell percentage

The average eggshell percentage was 8.05 (Table 16). The shell percentage was 8.58 for the eggs from birds fed the UKC limestone, which was higher than 7.52 for those fed the AR one ($P< 0.05$) (Table 17). For UKC and AR limestone shell percentage increased with level of Ca in the diet (Table 18). Duration of feeding had a significant ($P<0.05$) effect on the shell percentage (Fig 11)

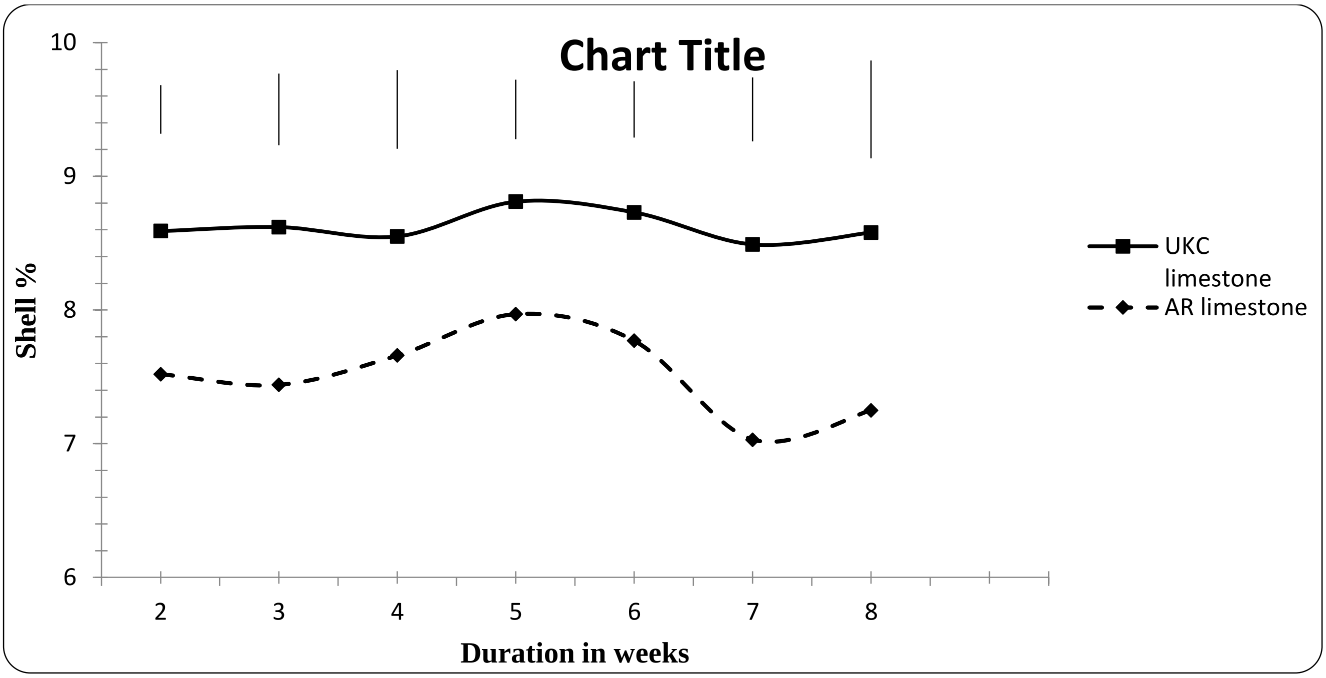

Figure 11: Effect of dietary calcium level on shell percentage with time

At one week of the experiment the shell percentage was 8.25, which increased to 8.59 at 2 weeks. However, between the 2nd and 8th week the shell percentage ranged between 8.62 and 8.59 but at week 5 the shell percentage increased drastically to 8.81% for UKC limestone. For the same period the shell percentage was between 7.52, and then increased to 7.97 at the 5th week and then declined to 7.25 in the 6th week of feeding for AR limestone.

The correlation between the shell thickness and shell percentage was 0.78, while that between egg size and shell percentage was -0.05. Therefore, the factors that influence eggshell thickness also influences shell percentage. Calcium is the primary factor affecting eggshell thickness and hence shells percentage (Pelicia *et al*., 2009) and (Rodriguez, 2013).

4.5 Conclusions

There were two objectives in this study as shown below. The conclusions from this study are shown under the specific objectives.

4.5.1 To determine the effect of source of limestone and dietary calcium level on egg production in laying hens

- Limestone from the coastal region of Kenya (UKC) or from the Athi plains (AR) had no effect on feed intake, hen-day egg production and egg weight but had an effect on FCR and egg breakage.
- When dietary Ca was set between 1 and 4 %, egg production increased linearly in birds receiving AR limestone based diets. However, in UKC limestone based diets highest hen-day egg production was obtained at 2 % dietary Ca level.
- As the dietary Ca increased the FCR improved and was better at 4% in UKC and AR limestone.
- As dietary Ca level was increased the egg size also increased
- Egg breakage decreased with increase in dietary Ca level, with UKC limestone having a lower egg breakage than the AR one.

4.5.2 To determine the effect of source of limestone and dietary Ca level on eggshell quality in laying hens

- The source of limestone (UKC or AR) in the diet had no significant effect on specific gravity but affected eggshell quality in terms shell weight, shell thickness and shell percentage.
- The level of dietary Ca had an effect on specific gravity shell weight, shell thickness and shell percentage.
- The correlation coefficient between egg weight and shell weight, shell percentage and shell thickness were 0.60, 0.38 and 0. 52 while that between shell thickness and shell percentage was 0.78

CHAPTER FIVE

5.0 Effect of time of lay and limestone source on eggshell quality

5.1 Introduction

This study was carried out to investigate the effect of time of lay on eggshell quality. The quality of the eggshell is affected by many factors, which includes age of the bird and its genotype, nutrition, ambient temperature and time of oviposition (Tumova and Gous, 2012). The nutritional factors include dietary calcium and vitamin D levels. The particle size of limestone used as a source of calcium has an effect on eggshell quality.

The time of lay has an effect on egg weight, thickness and shell percentage (Tůmová *et al*., 2017). Oviposition, ovulation and eggshell formation time are all interrelated. This means that there are times when eggshell formation occurs at night when the bird is not feeding and has to rely on calcium from medullary bone for eggshell formation (Tůmová and Ledvinka, 2009).Various researchers have reported that eggs laid in the early morning are usually heavier than to those laid at mid and late in the day (Ledvinka *et al*., 2012, Ketta and Tůmová 2016 and Tůmová *et al*, 2017). The distribution of oviposition times in domestic hens is within the 8hrs period of the day.

5.2 Materials and method

Eggs from a flock of 144 birds were obtained for this study. The birds were fed diets, formulated using limestone from two sources namely: Ukunda at the Kenyan coast (UKC limestone) and Athi River (AR limestone) near Nairobi.

5.2.1 Experimental diets

Two isocaloric and isonitrogenous diets were formulated and were based on two sources of limestone, Athi River and Ukunda limestones respectively. The composition of the experimental diets is shown in Table 20 below. Each diet was replicated 12 times and was fed to a group of six hens for a period of 8 weeks.

Table 19: Chemical composition (% air dry basis) and costs of experimental diets

Dietary Parameters	Sources of limestone	
	UKC limestone	AR limestone
Dry matter, %	89.8	89.3
Crude protein, %	15.8	15.6
Crude fibre, %	5.6	6.2
Ether extract, %	4.1	4.3
NFE, %	65.8	64.9
Calcium, %	2.4	2.43
Total phosphorus, %	0.53	0.67
ME, Kcal/kg*	2795	2794.6
Cost, Kshs/Kg	35.2	35.2

5.2.2 Data collection

Eggs were collected for this study in the 1st, 4th and 7th week of the experimental period. On weekly basis all the eggs laid in the third day were collected. This was done three times in a day, which were early morning (EM) between 0730 hrs and 0900 hrs; late morning (LM) between 901 and 1200 hrs and the afternoon (A) between 1201 and 1600 hrs. For all the eggs collected, specific gravity, egg weight, eggshell weight, shell thickness and eggshell percentage were evaluated.

All the eggs collected in each replicate were labelled to show the replicate number, treatment and time of lay and were immediately weighed individually using a precision analytical balance. To determine eggshell weight, eggs were broken and the contents removed. The eggshells were then put in an oven set at 60 ^{o}C. for a period of 12 hours as described in section 4.2.5 (b). They were then dried in a dessicator and weighed. The eggshell percentage (%) was calculated as a proportion of eggshell weight and egg weight. The thickness of the shell together with the membrane was measured at three points at the egg equator using a 0.001mm precision micro meter screw gauge.

5.3 Results and Discussion

5.3.1 Egg weight

Table 20 shows the effect of time of lay on egg weight. Eggs laid in early morning (EM), weighed 59.24 g and were heavier ($P < 0.05$) than those laid in the afternoon (57.64 g). The source of limestone had no significant effect on egg weight ($P>0.05$) (Table 21). Similar findings have been reported by Świątkiewicz, *et al*, (2015). The key factors that influence egg size are age and genotype of the bird as well as dietary protein and essential amino acids level.

Table 20: Effect of time of lay on eggshell quality

	Time of lay *				Statistics		
Parameter	EM	LM	A	Mean	Time	Source	Interaction Time*source
Egg weight	59.24^{b}	58.05^{ab}	57.64^{a}	58.31	0.034	0.112	0.447
Shell weight	4.93^{b}	4.59^{ab}	4.44^{a}	4.65	0.014	<0.001	0.431
Shell thickness	0.36^{b}	0.33^{a}	0.32^{a}	0.34	<0.001	0.002	0.051
Shell %	8.28^{b}	7.87^{ab}	7.68^{a}	7.94	0.051	<0.001	0.495

EM =early morning, 7.00 to 9.00 Hrs; LM = Late Morning, 9.01 to 12.00hrs; A = Afternoon, 12.01 to 4.00hrs; a, b, c means with different superscripts are significantly different. Means followed by same superscript per row, do not differ significantly (P<0.05

Table 21: Effect of limestone source and time of lay on eggshell quality

Parameter	UKC Limestone				AR Limestone			
	EM	LM	A	Mean	EM	LM	A	Mean
Egg weight	59.29^{ab}	57.46^{a}	56.95^{a}	57.90	59.20^{ab}	58.65^{a}	58.34^{a}	58.73
Shell weight	5.29^{b}	4.73^{ab}	4.68^{ab}	4.90	4.56^{a}	4.44^{a}	4.20^{a}	4.40
Shell thickness	0.38^{b}	0.34^{ab}	0.31^{a}	0.35	0.33^{a}	0.32^{a}	0.32^{a}	0.32
Shell percentage	8.88^{b}	8.18^{ab}	8.20^{ab}	8.42	7.68^{ab}	7.55^{a}	7.15^{a}	7.46

EM =early morning, 7.00 to 9.00 hrs; LM = Late Morning, 9.01 to 12.00hrs; A = Afternoon, 12.01 to 4.00hrs; a, b, c means with different superscripts are significantly different. Means followed by same superscript per row, do not differ significantly ($P<0.05$)

The time of lay had significant *(P<0.05)* effect on egg weight (Table 22). Eggs laid in early morning (EM) were heavier (59.24g) than those laid in the afternoon (57.64g). Limestone source had no effect on egg weight (P>0.05). In this study there was no significant interaction between time of lay and the source of Ca (P> 0.05) (Table 22). Świątkiewicz, *et al*, (2015) and Valable *et al*, (2018) reported that different dietary Ca sources had no effect on egg weight. Some of the factors affecting egg weight include: age of the bird (Samiullah *et al.*, 2016), dietary calcium level (Kermanshahi and Hadar, 2006), quality and level of dietary protein (Scott *et al*., 1982)

5.3.2 Eggshell weight

Feeding UKC limestone resulted in heavier *(P<0.05)* eggshells of 4.90g compared to AR limestone (4.40g) (Table 22). Time of lay had a significant (P<0.014) effect on the eggshell weight. Eggs laid early in the morning (EM) had heavier *(P<0.05)* eggshells than those laid in the afternoon (Fig 12). This is in agreement with the studies by Tumova *et al*., (2014) and Ketta and Tůmová (2018), showed that eggshell weight for eggs laid in the morning was higher than those laid in the afternoon (A). The amount of calcium deposited in the eggshell is the primary factor influencing its weight (Leeson *et al*, 2015). There was no significant interaction between time and limestone source on shell weight

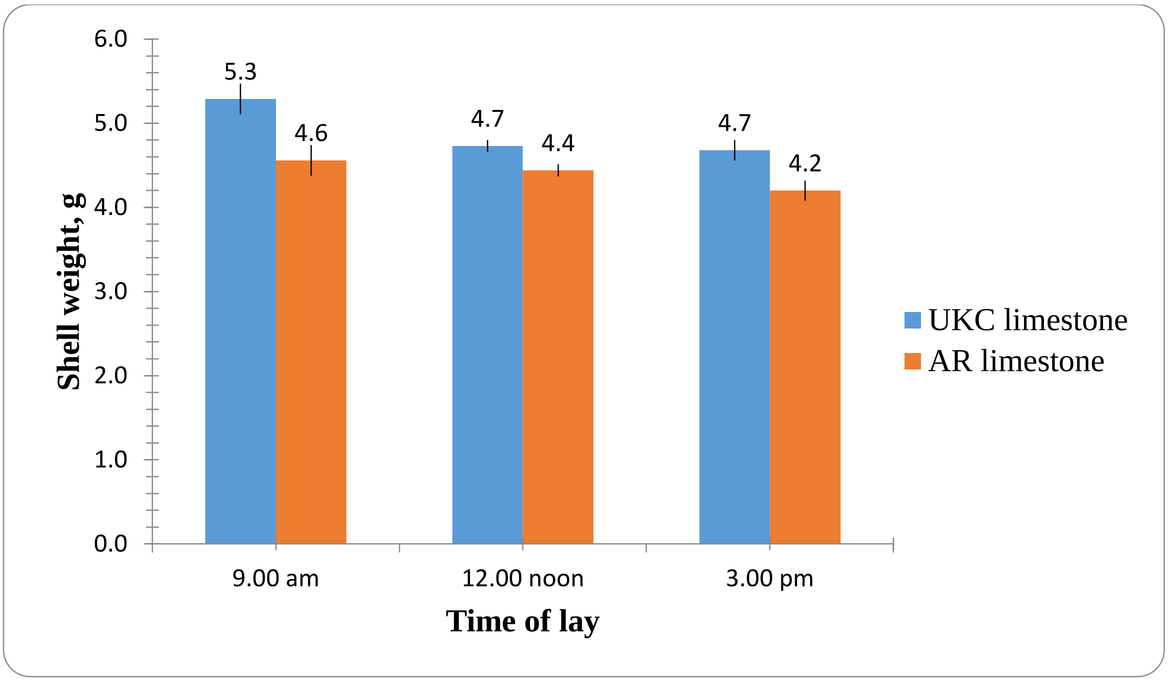

Figure 12: Effect of source of limestone on eggshell weight

5.3.3 Shell percentage

The average eggshell percentage was 7.94. Eggs from birds fed diets containing UKC limestone had significantly higher *($P<0.05$)* egg shell percentage at 8.42 % than those fed diets containing AR limestone at 7.46 % as shown in (Table 22.) The eggs laid early in the EM, had higher *($P<0.05$)* eggshell percentage at 8.88 % than those laid in the afternoon (8.20 %) for UKC based diets. The corresponding values were 7.68 % and 7.15 % respectively for AR limestone. On average time of lay did not affect eggshell percentage ($P>0.051$), while limestone source had a significant ($P<0.001$) effect on eggshell percentage.

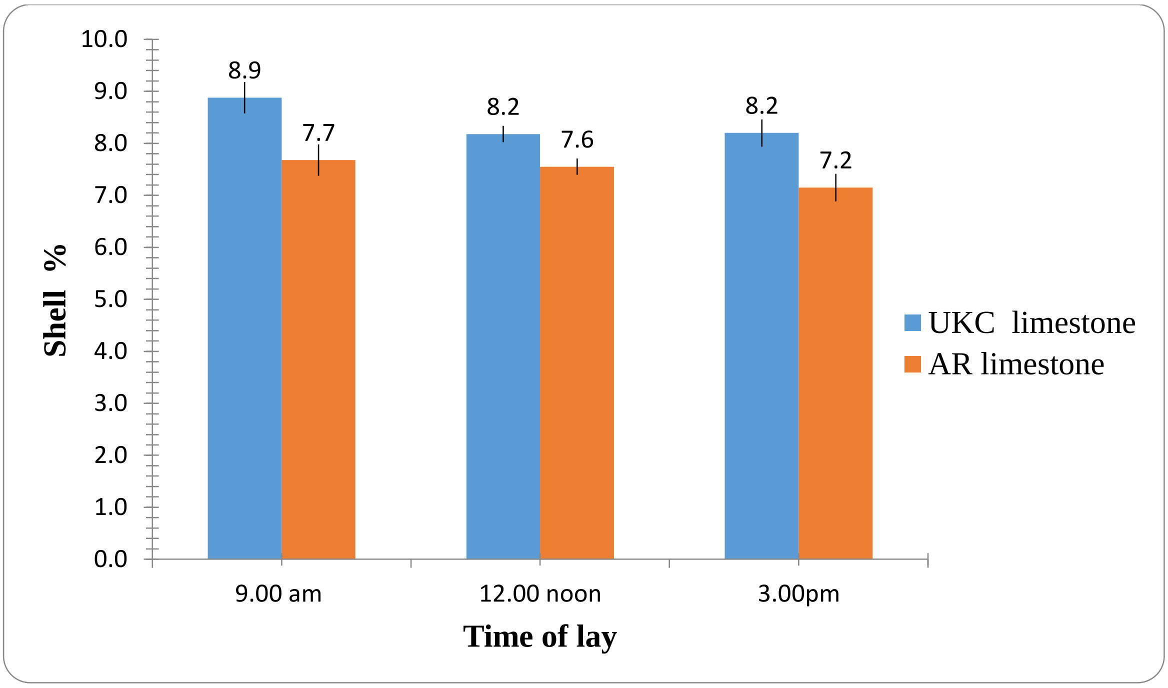

Figure 13: Effect of source of limestone on shell percentage.

Calcium is a major component of the egg shell (Rodriguez, 2013) and therefore an increased dietary supply contributes to its accumulation in the shell. A study by Pelicia *et al*., (2009) indicated that, an increase in egg shell weight and shell percentage per surface area with increase of dietary Ca level. There was no significant interaction between the time of lay and the limestone source.

5.3.4 Shell thickness

The average shell thickness was 0.34 mm (Table 22). Eggs from birds fed limestone from UKC had a shell thickness (0.35 mm) which was higher *($P<0.05$)* than those fed limestone from AR (0.32mm). The eggs laid in the EM had higher *($P<0.05$)* shell thickness, (0.35 mm) than those laid in the afternoon (A), (0.32 mm) (Fig 14).

Ketta and Tůmová (2018) reported that eggshell thickness contributed to eggshell strength and was higher for eggs laid in the morning (0.64), ($P<0.001$) than in those laid in the afternoon (0.48, $P<0.001$). According to Harms, Douglas and Sloan (2016), the eggshell strength increased with increase in eggshells thickness.

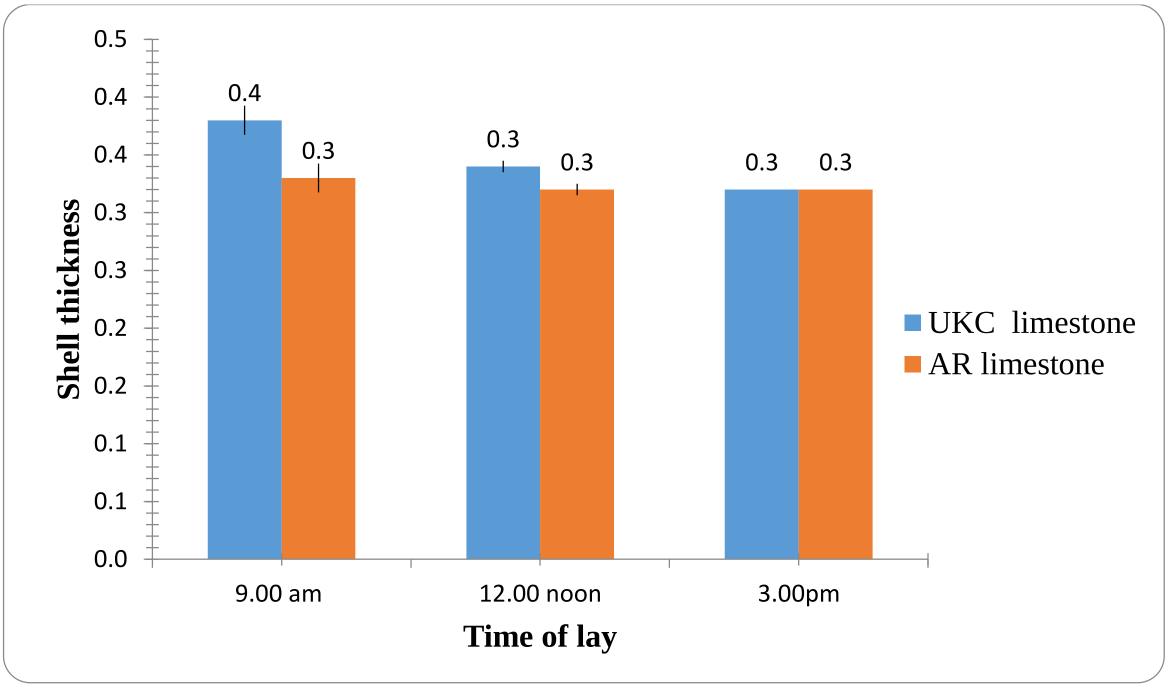

Figure 14: Effect of source of limestone on shell thickness.

5.4 Conclusion

The objective was to evaluate the effects of time of lay and source of Ca on egg weight, eggshell weight, eggshell thickness and eggshell percentage. From the results, conclusions were made as follows:

i. Time of lay had significant effects on all the parameters studied.

ii. Eggs laid early in the morning (EM) had higher weights than those laid in late afternoon (A). In addition, they had stronger shells expressed as shell percentage and thickness than those laid in the afternoon.

iii. There were no significant differences in egg weight, shell thickness and shell percentage between eggs laid early in the morning (EM) and those laid mid- morning (LM)

iv. The source of limestone did not have a significant effect on egg weight, but layers fed on UKC laid eggs with stronger shells than those fed on AR limestone.

v. There was no significant interaction between the time of lay and source of calcium for the parameters studied.

This study shows that UKC limestone is a better source of Ca than AR limestone. It is therefore recommended that UKC be used instead of AR limestone where possible.

CHAPTER SIX

6.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

6.1 General discussion

Limestone is an important feedstuff used in production of poultry feed as a source of calcium. The nutritional value of this feedstuff depends on its physical and chemical characteristics. In Kenya limestone is mined from different parts of the country. For this study, the nutritional value of two sources of limestone i.e. Ukunda in Kenyan coast (UKC) and Athi river (AR) near Nairobi was assessed. Thus, the physical and chemical composition of UKC and AR limestone was determined as well as the effect on the layer performance and eggshell quality.

This determination was based on four hypotheses which are: (i) source of limestone has no effect on eggshell quality (ii) source of limestone has no effect on layer performance and egg shell quality (iii) dietary level of calcium derived from limestone has no effect on layer performance and eggshell quality (iv) time of lay and dietary level of calcium has no effect on eggshell quality.

The Ca content in the UKC and AR limestone was 42% and 34%, respectively. Both sources are rich in calcium compared to 38 % reported by Scott et al, 1982 and NRC, 1994. Both source of limestone had trace elements of Fe, Zn, Cu and Cr with an average of 47, 0.25, 3.0 and 7.8 µ /kg, respectively. The first three elements i.e. Fe, Zn and Cu are essential nutrients and were in very low concentrations compared to that in the commercial premix used in this study. In the premix, concentrations of Fe, Cu and Zn were 12800, 1600 and 14400 mg /kg, respectively. Different authors have looked at the particle size in limestone (De Witt *et al*., 2009, Pizzolante et *al.,* 2009, Cufadar *et al*., 2011, Tune and Cufadar, 2014, Bueno et *al*., 2016, Zhang *et al*., 2017). The importance of the particle size is that it influences the solubility and availability of calcium. UKC limestone had higher proportion of coarse particles (1-2 mm), higher solubility than AR limestone. AR limestone had a higher acid insoluble ash than UKC limestone. It is clear that the level of insoluble ash influences invitro solubility of limestone (Pelicia et al., 2009, Tumova et al., 2016, Zhang et al., 2017)

Birds fed UKC limestone, had heavier eggs and eggshells, higher eggshell percentage and thicker eggshells than those receiving AR limestone. This means that the UKC limestone had physical and chemical attributes which were more favorable for egg production than the AR one (Ketta and Tůmova, 2016). As dietary calcium level was increased the egg size also increased (Tune and Cudafar, 2015, Negoita et al., 2017). Dietary level of calcium affected egg production and eggshell quality parameters but did not affect the specific gravity of the eggs. Various physiological processes in a laying hen do not necessarily respond in the same manner for a given nutrient.

The ssource of limestone had no effect on feed intake, hen-day egg production but had an effect on feed conversion ratio, egg breakage. The highest hen-day egg production was obtained when UKC limestone was fed at 2% dietary calcium. When limestone from Athi River (AR) was used, egg production increased as dietary calcium level increased. As the dietary calcium increased the FCR improved and was better at 4% in both UKC and AR. UKC limestone resulted in lower egg breakage than the AR one. The breakage decreased with increase in dietary calcium. Similar findings were reported by Świątkiewicz, *et al.,* (2015) and An *et al*., (2016) who showed that dietary Ca level did not affect the total feed intake and laying performance in layers. On the other hand, Pelicia *et al*., 2009, indicated that the level of limestone reduced the feed intake and hence calcium intake. Pavlovski *et al*., 2012 reported that 6-8 % of the total eggs laid is not always marketable because of low eggshell quality. It is accepted that laying hens should be given adequate amount of calcium for strong eggshell formation (Scott *et al*., 1982 and Onono, 2018).

When comparing the effect of time of lay on eggshell quality, it was noted that; eggs laid in the early morning were heavier than those laid in late morning and afternoon and as dietary calcium level was increased egg weight, shell weight, shell thickness and shell percentage also increased. According to Tumova *et al*., (2014), eggshell weight significantly increased when hens had been given the midnight feeding and eggs were collected at 09:00 hr. However, when eggs were collected at 1500hr, eggshell weight significantly decreased. Ketta and Tůmová (2018) indicated that Eggshell weight significantly increased in the morning hours (7.23 g) than in afternoon (5.14 g).

Based on the above evaluations it can be concluded that UKC limestone is superior than AR limestone for use in diets of laying hens.

6.2 General recommendations

From the study, it is recommended that UKC limestone can be used as source of calcium at 2 and 4% levels while AR limestone can only be used at 4% dietary calcium level.

6.3 Way forward

A study on the UKC limestone to be done to establish its effect on layers growth and tibia mineral content. There is also need to look unto the effect of particle size on digestibility of limestone.

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APPENDICES

APPENDIX 1**:** ANOVA table showing the effect of calcium level on feed consumed on layers feed with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Level	2	5.4116	2.7058	19.12	<.001
Lime_colour	1	0.0974	0.0974	0.69	0.408
Week	7	0.8227	0.1175	0.83	0.563
Level.lime_colour	2	4.4538	2.2269	15.74	<.001
Level.week	14	3.4506	0.2465	1.74	0.053
Lime_colour.week	7	0.3061	0.0437	0.31	0.949
Level.lime_colour.week	14	2.4125	0.1723	1.22	0.268
Residual	144	20.3738	0.1415		
Total	191	37.3285			

APPENDIX 2: ANOVA table showing the effect of calcium level on egg production on layers feed with limestone from different sources.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Level	2	8283.02	4141.51	47.74	<.001
Level.Lime_Colour	3	2554.83	851.61	9.82	<.001
Level.Lime_Colour.Week	42	7716.31	183.72	2.12	<.001
Residual	144	12492.91	86.76		
Total	191	31047.07			

APPENDIX 3: ANOVA table showing the effect of calcium level on feed conversion ratio on layers feed with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Breed.Level	2	5.10758	2.55379	30.72	<.001
Breed.Level.Lime_Colour	3	0.74223	0.24741	2.98	0.034
Breed. Level. Lime_Colour.Week	42	4.99344	0.11889	1.43	0.063
Residual	144	11.96912	0.08312		
Total	191	22.81238			

APPENDIX 4: ANOVA table showing the effect of calcium level on egg weight on layers feed with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Level	2	429.034	214.517	51.30	<.001
Lime_colour	1	6.091	6.091	1.46	0.229
Week	7	334.808	47.830	11.44	<.001
Level.lime_colour	2	49.489	24.745	5.92	0.003
Level.week	14	106.886	7.635	1.83	0.040
Lime_colour.week	7	56.284	8.041	1.92	0.070
Level.lime_colour.week	14	38.787	2.770	0.66	0.807
Residual	144	602.196	4.182		
Total	191	1623.575			

APPENDIX 5: ANOVA table showing the effect of calcium level on specific gravity on layers feed with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Level	2	0.14309	0.07155	2.94	0.056
Lime_colour	1	0.00160	0.00160	0.07	0.798
Week	7	0.10360	0.01480	0.61	0.748
Level.lime_colour	2	0.02621	0.01310	0.54	0.585
Level.week	14	0.29647	0.02118	0.87	0.592
Lime_colour.week	7	0.19193	0.02742	1.13	0.349
Level.lime_colour.week	14	0.36180	0.02584	1.06	0.397
Residual	144	3.50336	0.02433		
Total	191	4.62806			

APPENDIX 6: ANOVA table showing the effect of calcium level on shell weight on layers feed with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Level	2	94.2378	47.1189	412.88	<.001
Lime_colour	1	17.1100	17.1100	149.93	<.001
Week	7	4.3244	0.6178	5.41	<.001
Level.lime_colour	2	8.4741	4.2371	37.13	<.001
Level.week	14	4.6134	0.3295	2.89	<.001
Lime_colour.week	7	1.1397	0.1628	1.43	0.199
Level.lime_colour.week	14	1.9488	0.1392	1.22	0.267
Residual	144	16.4337	0.1141		
Total	191	148.2819			

APPENDIX 7: ANOVA table showing the effect of calcium level on shell thickness on layers feed with limestone from different sources.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Level	2	0.314543	0.157271	138.34	<.001
Lime_colour	1	0.054239	0.054239	47.71	<.001
Week	7	0.110667	0.015810	13.91	<.001
Level.lime_colour	2	0.024964	0.012482	10.98	<.001
Level.week	14	0.033500	0.002393	2.10	0.015
Lime_colour.week	7	0.019940	0.002849	2.51	0.018
Level.lime_colour.week	14	0.015529	0.001109	0.98	0.481
Residual	144	0.163705	0.001137		
Total	191	0.737088			

APPENDIX 8: ANOVA table showing the effect of calcium level on shell percentage on layers feed with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Level	2	196.0750	98.0375	331.42	<.001
Lime_colour	1	54.0252	54.0252	182.64	<.001
Week	7	6.9480	0.9926	3.36	0.002
Level.lime_colour	2	18.2533	9.1267	30.85	<.001
Level.week	14	6.5912	0.4708	1.59	0.088
Lime_colour.week	7	2.6802	0.3829	1.29	0.257
Level.lime_colour.week	14	4.5293	0.3235	1.09	0.368
Residual	144	42.5963	0.2958		
Total	191	331.6985			

APPENDIX 9: ANOVA table showing the effect of calcium level in layers feed and time lay on egg weight with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	3	92.3	30.8	0.09	0.967
Lime	1	83.7	83.7	0.23	0.629
Level	2	8278.3	4139.2	11.62	<.001
Time	2	3304.5	1652.3	4.64	0.012
Week	2	3974.6	1987.3	5.58	0.005
Replicate/lime	3	3315.9	1105.3	3.10	0.030
Replicate/level	6	1295.4	215.9	0.61	0.725
Lime/level	2	2313.0	1156.5	3.25	0.043
Replicate/time	6	9213.2	1535.5	4.31	<.001
Lime/time	2	135.0	67.5	0.19	0.828
Level/time	4	11960.6	2990.2	8.39	<.001
Replicate/week	6	2468.8	411.5	1.15	0.338
Lime/.week	2	1951.5	975.7	2.74	0.070
Level/week	4	757.2	189.3	0.53	0.713
Time/week	4	4188.3	1047.1	2.94	0.025
Replicate/.lime/.level	6	2063.2	343.9	0.96	0.453
Replicate/lime/time	6	3248.9	541.5	1.52	0.180
Replicate/level/time	12	3366.4	280.5	0.79	0.662
Lime/level/time	4	8883.3	2220.8	6.23	<.001
Replicate/lime/week	6	4977.8	829.6	2.33	0.039
Replicate/level/week	12	7481.3	623.4	1.75	0.069
Lime/level/week	4	5368.8	1342.2	3.77	0.007
Replicate/.time/week	12	6007.9	500.7	1.40	0.178
Lime/time/week	4	1212.0	303.0	0.85	0.497
Level/time/week	8	4441.9	555.2	1.56	0.148
Residual	92	32784.4	356.4		
Total	215	133168.1			

APPENDIX 10: ANOVA table showing the effect of calcium level in layers feed and time lay on eggshell weight with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	3	13.808	4.603	1.61	0.192
Lime	1	17.813	17.813	6.25	0.014
Level	2	155.827	77.913	27.32	<.001
Time	2	53.226	26.613	9.33	<.001
Week	2	35.928	17.964	6.30	0.003
Replicate/lime	3	19.367	6.456	2.26	0.086
Replicate/level	6	16.269	2.712	0.95	0.463
Lime/.level	2	15.749	7.874	2.76	0.068
Replicate/time	6	41.410	6.902	2.42	0.032
Lime/time	2	2.177	1.089	0.38	0.684
Level/time	4	38.321	9.580	3.36	0.013
Replicate/week	6	18.825	3.138	1.10	0.368
Lime/week	2	10.192	5.096	1.79	0.173
Level/.week	4	21.438	5.360	1.88	0.121
Time/week	4	30.560	7.640	2.68	0.037
Replicate/lime/.level	6	44.751	7.458	2.61	0.022
Replicate/lime/time	6	20.131	3.355	1.18	0.326
Replicate/level/time	12	17.400	1.450	0.51	0.904
Lime/level/time	4	36.252	9.063	3.18	0.017
Replicate/lime/week	6	37.129	6.188	2.17	0.053
Replicate/level/week	12	22.284	1.857	0.65	0.793
Lime/level/week	4	24.242	6.060	2.12	0.084
Replicate/time/week	12	23.720	1.977	0.69	0.754
Lime/time/week	4	3.010	0.752	0.26	0.900
Level/time/week	8	34.315	4.289	1.50	0.167
Residual	92	262.411	2.852		
Total	215	1016.554			

APPENDIX 11: ANOVA table showing the effect of calcium level in layers feed and time lay on shell % with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	3	12.514	4.171	0.54	0.659
Lime	1	63.772	63.772	8.19	0.005
Level	2	295.889	147.944	18.99	<.001
Time	2	75.003	37.502	4.81	0.010
Week	2	98.205	49.103	6.30	0.003
Replicate/lime	3	18.643	6.214	0.80	0.498
Replicate/.level	6	37.935	6.323	0.81	0.564
Lime/level	2	11.431	5.716	0.73	0.483
Replicate/time	6	41.184	6.864	0.88	0.512
Lime/.time	2	3.337	1.669	0.21	0.808
Level/time	4	134.812	33.703	4.33	0.003
Replicate/week	6	114.945	19.157	2.46	0.030
Lime/week	2	30.346	15.173	1.95	0.148
Level/week	4	96.796	24.199	3.11	0.019
Time/week	4	85.134	21.284	2.73	0.034
Replicate/lime/.level	6	86.091	14.348	1.84	0.100
Replicate/lime/time	6	53.752	8.959	1.15	0.340
Replicate/level/time	12	160.582	13.382	1.72	0.075
Lime/level/time	4	90.964	22.741	2.92	0.025
Replicate/lime/week	6	75.299	12.550	1.61	0.153
Replicate/level/week	12	122.821	10.235	1.31	0.224
Lime/level/week	4	49.266	12.317	1.58	0.186
Replicate/time/week	12	103.048	8.587	1.10	0.368
Lime/ime/week	4	7.553	1.888	0.24	0.914
Level/time/week	8	97.631	12.204	1.57	0.146
Residual	92	716.728	7.791		
Total	215	2683.682			

APPENDIX 12: ANOVA table showing the effect of calcium level in layers feed and time lay on shell thickness with limestone from different sources

Source of variation	d.f.	S.s.	M.s.	V.r.	F pr.
Replicate	3	0.00647	0.00216	0.13	0.943
Lime	1	0.08700	0.08700	5.20	0.025
Level	2	0.40882	0.20441	12.21	<.001
Time	2	0.31147	0.15574	9.30	<.001
Week	2	0.04827	0.02414	1.44	0.242
Replicate/lime	3	0.06318	0.02106	1.26	0.294
Replicate/level	6	0.03987	0.00664	0.40	0.879
Lime/level	2	0.06459	0.03229	1.93	0.151
Replicate/time	6	0.12109	0.02018	1.21	0.311
Lime/time	2	0.00478	0.00239	0.14	0.867
Level/time	4	0.16687	0.04172	2.49	0.048
Replicate/week	6	0.16309	0.02718	1.62	0.149
Lime/week	2	0.03016	0.01508	0.90	0.410
Level/week	4	0.12400	0.03100	1.85	0.126
Time/week	4	0.30240	0.07560	4.52	0.002
Replicate/lime/level	6	0.27801	0.04633	2.77	0.016
Replicate/lime/time	6	0.22531	0.03755	2.24	0.046
Replicate/level/time	12	0.40782	0.03399	2.03	0.030
Lime/level/time	4	0.15227	0.03807	2.27	0.067
Replicate/lime/week	6	0.13527	0.02255	1.35	0.245
Replicate/level/week	12	0.18761	0.01563	0.93	0.517
Lime/level/week	4	0.06848	0.01712	1.02	0.400
Replicate/time/week	12	0.23021	0.01918	1.15	0.334
Lime/time/week	4	0.04462	0.01115	0.67	0.617
Level/time/week	8	0.30732	0.03842	2.29	0.027
Residual	92	1.54032	0.01674		
Total	215	5.51931			

APPENDIX 13: ANOVA table showing the effect of calcium level on egg breakage on layers feed with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Breed.Level	2	487.719	243.859	56.52	<.001
Breed.level.lime_colour	3	140.578	46.859	10.86	<.001
Breed.level.lime_colour.week	42	116.406	2.772	0.64	0.952
Residual	144	621.250	4.314		
Total	191	1365.953			

APPENDIX 14: ANOVA table showing the effect of calcium level on cost of layers feed with limestone from different sources

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Level	2	2930.5	1465.2	14.56	<.001
Level.lime_colour	3	855.3	285.1	2.83	0.040
Level.lime_colour.week	42	6040.5	143.8	1.43	0.063
Residual	144	14489.2	100.6		
Total	191	24315.5			