Production and egg quality of Lohmann Brown Lite laying hens as influenced by source and dietary inclusion concentration of zinc, copper, and manganese

by

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DECLARATION

I, Petrus Engelbrecht hereby declare that this thesis, "Production and egg quality of Lohmann Brown Lite laying hens as influenced by source and dietary inclusion concentration of zinc, copper and manganese," submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other university.

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Leave this for last:

Novus

Eagles Pride

Tia, Michael & Vicky

Dr. C. JvR

Abstract

The egg formation process is complex and can be influenced by a multitude of factors, one of which includes the availability of vital trace minerals in the hens' diet. Although calcium carbonate makes up the largest part of the eggshell, trace minerals such as zinc, copper, and manganese influence the structure and the strength of the shell. Trace minerals also act as catalysts in various enzymatic processes important for normal homeostasis and egg production. Adding trace minerals to the diets of laying hens, has been a longstanding practice, these minerals were traditionally sourced from various sulphide and oxide salts. However, in the light of the significant increases in the modern layer hens' egg production rate and productive lifespan, the nutritional requirements for trace minerals may not be met by simply increasing the mineral inclusion level in the diet, as this does not necessarily correlate with an increase in the absorption of the mineral. Organic minerals, especially chelated methionine mineral sources, may ameliorate this problem, due to minerals being absorbed inadvertently through amino acid uptake regulators.

This trial was conducted in an effort to determine the effects of long-term inclusion of the trace minerals: zinc, copper, and manganese in the feed on the egg production, egg shell quality, and internal egg quality of Lohmann lite laying hens. This trial compared minerals sourced from chelated hydroxy-analogues of methionine, with conventional sulphide and oxide salt sources of the same minerals. The production parameters that were measured during the trial included egg production, total feed intake, feed conversion ratio, and hen mortality. Egg shell quality was quantified by recording the total amount of eggs rejected per category, egg shell weight, egg weight, and the egg shell breaking strength. Internal egg quality was quantified by measuring the albumin height (Haugh unit), and the mineral concentration in the egg yolk of Zn, Cu and Mn. A total of 1080 Lohmann Brown Lite laying hens were allocated to six treatments. The trial was run for 60 weeks, was initiated at 16 weeks-of-age, and was terminated at 76 weeks-of-age. Each treatment was repeated eighteen times, and a complete randomised block design was used. The treatments were supplemented with Zn, Cu and Mn (mg/kg) as follows, at inclusion level 1 contained Zn 60: Cu 15: Mn 80 as sulphate and Zn 60: Cu 15: Mn 80 from an organic mineral source, inclusion level 2 contained Zn 50: Cu 8: Mn 50 as sulphate and Zn 50: Cu 8: Mn 50 from an organic

mineral source and inclusion level 3 Zn 32: Cu 8: Mn 32 as sulphate, Zn 32: Cu 8: Mn 32, from an organic mineral source.

The feed that contained the organic mineral resulted in a significant decrease in feed intake and feed conversion ratio. Egg production and hen mortality did not differ significantly, irrespective of the mineral source or the inclusion level. The inclusion of organic minerals in the feed failed to produce a significant improvement in egg shell quality, where different inclusion rates also failed to produce any significant changes to the egg shell parameters that were measured during the course of the trial. The inclusion of the organic copper mineral source increased the concentration of copper in the egg yolks significantly, where Cu concentration in the egg yolk was also increased, by increasing the concentration of Cu in the feed, irrespective of the source of Cu.

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LIST OF ABBREVIATIONS

DAFF	The Department of Agriculture Forestry and Fisheries
DGAC US	Dietary Guidelines Advisory Committee
Mn	Manganese
Cu	Copper
Zn	Zinc
SG	standard gravity
HU	Haugh Unit
HMTBa	hydroxy methylthiobutanoic acid
SAPA	South African Poultry Association
mg	milligram
kg	kilogramme
ppm	parts per million
NRC	National Research Council
MHA	Methionine
FI	Feed intake
FCR	Feed conversion ratio

CHAPTER 1:

Introduction

Sustainable protein production for human consumption has become increasingly important as the human population increases worldwide. The lack of essential amino acids in the human diet is even more pronounced in developing nations, where animal protein products may be scarce. In these countries, eggs are often the most affordable source of essential amino acids (Hartmann et al., 2001). Consumption of eggs in the developed world has recently been fuelled by changing views on cholesterol and the increasing popularity of high protein/high fat diets (SAPA report,2016). According to recommendations released by the US Dietary Guidelines Advisory Committee (DGAC) in 2015, cholesterol was no longer considered "a nutrient of concern for over-consumption". An increase has also been noted in so-called fortified eggs; these eggs may contain increased levels of selenium and vitamins A, B_{12}, D, riboflavin, and folate (SAPA report, 2016).

Improved genetic selection, husbandry systems, and feed formulations have enabled commercial egg producers to drastically increase the modern laying hen's productive lifespan. The modern laying hen is capable of reaching 50% production at 20 to 21 weeks-of-age and reaching average peak production of 93-95% at 28 weeks-of-age. Hens are able to produce a total of 360 to 365 eggs per hen at 14 months-of-age, and 405 to 410 eggs by 16 months-of-age. The average egg weight will vary between 62.5 to 63.5 grams over this time period. Vast improvements, through selection and nutrition, have increased egg shell breaking strength to more than 40 Newtons on average, and improved feed conversion to an average of 2.0-2.1 kg feed per kg egg mass (Lohmann Manual, 2013). High levels of egg production are only achievable if nutritional requirements are adequately met. Although trace minerals make up only a small part of the diet, it is considered vital to normal physiological processes. Minerals primarily function as catalysts for enzymatic activity, but also play an important role in optimising growth, immunity, and production (Virden *et al*., 2003). The microminerals copper (Cu), zinc (Zn) and manganese (Mn) are essential elements required by poultry (Lim *et al*., 2003). In the reproducing hen, processes that are dependent on these essential microminerals include: the formation of enzymes; normal tissue and bone development; formation of the egg shell and egg membranes; and normal immune responses (Mabe *et al*., 2003). High levels of Cu, Zn and Mn are vital in

high producing hens that have increased metabolic demands (Sun *et al.*, 2012). Egg shell quality, which is of primary concern to the egg producer as well as to the consumer, is also affected by the dietary availability of essential minerals, by either influencing the catalytic enzymes involved in membrane and shell formation, or by directly influencing the calcite crystals during shell formation (Mabe *et al.*, 2003). Cracked or broken egg shells increase egg reject rates, which increases the cost of egg production significantly (Xiao *et al.*, 2015).

Zinc is crucial to the formation of certain enzymes that function in carbohydrate and protein metabolism (Virden, 2003). Zinc also maintains normal functioning of the immune system, where increased dietary zinc has been demonstrated to enhance immune response during disease challenge (Sun *et al.*, 2012). Zinc is an essential component in carbonic anhydrase, an enzyme that supplies carbonate ions during egg shell formation (Mabe *et al.*, 2003). Inhibition of this enzyme leads to a significant reduction in egg shell weight, due to lower bicarbonate ion secretion (Nys *et al.*, 1999). Copper is essential in the formation of cuproenzymes, a group of metalloenzymes that contain one or more Cu ions. Copper is needed in the formation of the enzyme lysyl oxidase, which is involved in converting lysine to cross-linked demosime and isodesmosine, amino acids that lend elasticity to tissues Chowdhury (1990). Alterations in lysine, like cross-links in laying hens with Cu deficiency, cause egg shell deformation and abnormal mechanical properties (Baumgartner, 1978), due to the abnormal distribution of shell membrane (Mabe *et al.*, 2003). Diets deficient in Mn may cause a decrease in total egg production and egg shell thickness. Manganese deficiency may also affect the ultrastructure of egg shell by changing the mammillary knobs of the egg shells (Xiao *et al.*, 2015). Manganese is a component of the enzyme glycosyl transferase, which is essential in the formation of proteoglycans. Proteoglycans such as keratan and dermatan are essential components of the egg shell matrix, and influence the structure and texture of the egg shell, and thus, the mechanical properties of the egg (Mabe *et al.*, 2003). Supplementation of the diet with Mn may therefore increase egg shell breaking strength (Mabe *et al.*, 2003). Manganese is also an important catalyst in the formation of essential enzymes such as arginase, pyruvate carboxylase and manganese superoxide dismutase, which are essential for protecting the cell walls against oxidation (Lima *et al.,* 2000).

Traditionally, these trace elements have been supplemented in layer diets as inorganic salts. However, inorganic mineral sources are bound to sulphate or oxide salts, reducing the bioavailability of the minerals. Nutritionists may compensate for the low availability of essential minerals from these sources by including levels far exceeding recommendations by the NRC (1994) to maintain maximum performance (Bratz *et al.,* 2013). However, pollution of the environment may be increased through the excretion of faeces with high concentrations of unabsorbed minerals (Abedine *et al.,* 2017). Given the importance of Cu, Mn and Zn in the normal expression of physiological processes, the exact inclusion levels in the feed as well as the source of the minerals used must be considered, in order to avoid either a mineral deficiency, or excess, respectively. Because of the variability and relatively low availability of trace minerals supplied by inorganic sources (Bao *et al*., 2010), there has been an increased interest in the development of various organic sources of trace minerals to improve absorption from the intestine and uptake into the tissues (Baltaci et al., 2017). Chelated minerals can be included in the diet at lower mineral inclusion levels without negative impact on performance, thereby decreasing environmental pollution (Abedine *et al.,* 2017). Recent research has focused on the use of the chelate formed between the mineral element and two molecules of the hydroxy analogue of methionine (hydroxy methylthiobutanoic acid, HMTBa). It has been shown that these complexes are stable and deliver the minerals to the correct site of absorption for improved availability (Sun *et al*., 2012).

The aim of this study was to determine whether the inclusion of organic minerals could improve egg production and egg quality in comparison to the conventional inorganic mineral sources of Zn, Cu and Mn. Furthermore, the study was aimed at determining the optimal supplementation level of organic Zn, Cu and Mn in order to correctly meet the nutritional needs of the modern laying hen. The objective of the study was to measure production parameters such as: total egg production; feed conversion ratio; feed intake; and hen mortality, when feeding diets that contained different levels and different sources of Zn, Cu and Mn. Egg quality was quantified by measuring: egg shell weight; egg shell breaking strength; egg reject rate; albumin height (Haugh unit); and mineral concentration of the egg yolk.

The following hypotheses were tested:

H_0: Egg production and egg quality will not be affected by the dietary source of the trace minerals zinc, copper, and manganese.

H1: Dietary supplementation of an organic source of the trace minerals zinc, copper, and manganese will improve egg production and egg quality when compared to the use of inorganic sources.

H_0: Egg production and egg quality will be negatively affected when supplementing organic mineral sources at lower levels of trace minerals than inorganic sources of the same minerals.

H1: Due to the higher availability of trace minerals obtainable from organic sources, lower supplementation levels will sustain egg production and egg quality to the same extent than higher levels of an inorganic source.

CHAPTER 2:

Literature review

2.1 Introduction

Minerals, and particularly trace minerals, are important nutrients in the diets of laying hens as they participate in many physiological processes required for normal growth, egg production, and eggshell formation (Tang et al., 2006). Minerals such as calcium and phosphorus alone contribute over 95% of the total mineral content of the eggshell (Coto *et al*., 2008), however the quality of eggshell depends not only on the mineral quantity but also on the composition of the mineral matrix (Viguet-Carrin *et al*., 2006). Therefore, trace elements such Zn, Mn and Cu, are essential in ensuring good eggshell quality and normal egg production (Mabe *et al*., 2003). Egg production and eggshell quality can be manipulated and improved upon by increasing the concentration of these trace elements in the feed (Zamani *et al*., 2005). A better understanding of organic sources of micro minerals and their inclusion levels is needed (Stefenallo *et al*., 2014), as the availability of these minerals for absorption and tissue deposition depend on their chemical form and interactions with other minerals, as well as anti-nutritional factors, such as phytic acid (Watts, 1990). Organic minerals are best defined as metal ions chemically bonded to organic molecules such as amino acids, thereby forming chemical structures that are stable and more absorbable in the gastrointestinal tract. The bioavailability of an organic trace mineral source is higher in comparison to inorganic sources and organic mineral source promotes higher trace mineral retention (Sun *et al*., 2012) and lower excretion (Leeson et al., 2005). In addition to the normal ion absorption mechanisms in the intestines, organic minerals are also absorbed through pathways similar to the organic molecules, such as amino acids, thereby avoiding competition among different minerals for the same carrier, making them more readily absorbable (Mabe *et al*., 2003). Thus, the use of organic trace minerals may have a beneficial effect on egg production and egg shell quality (Klecker *et al*., 2002). The focus of this literature review is to discuss factors that influence egg production, eggshell quality, and internal egg quality, with special interest on the effect of organic and inorganic sources of Zn, Cu, and Mn.

2.2 The poultry industry in South Africa: An overview

Commercial layer hens make up a quarter of the poultry population in South Africa (SAPA Report, 2018) and the poultry industry is the largest producer of animal protein in South Africa. This sector alone is responsible for producing 65.3% of the total animal protein produced in the country per annum (excluding milk production). Poultry are used primarily for meat production, with broilers and broiler breeders accounting for 76% of the total poultry population in the country (SAPA Report, 2018). The remaining 24% of the population consist of commercial egg producing hens which includes layer breeder and laying hens.

The poultry industry is the largest agricultural industry in South Africa and contributes 18% to the total agricultural production in the country. Poultry meat producing birds contribute 14.1% while commercial egg production accounts for 3.9 percent. In 2018, the total production of poultry meat and eggs was estimated at 2.3 million, and 0.446 million tons, respectively (SAPA Report, 2018). The Department of Agriculture Forestry and Fisheries (DAFF) estimated that the gross farm income for the combined poultry sector averaged R47.96 billion for the year 2018. The poultry industry is also an important player in rural employment. An estimated 111822 workers are employed in this industry. The outbreak of highly pathogenic avian influenza H5N8 in 2017 led to a dramatic decrease in egg production in the second half of that year. In 2018, as farms were repopulated, egg production increased steadily. The average number of cases produced per week was 386 997 during 2018. This was an increase of 8 058 cases (+2.1%) per week from the previous year. Total egg production in 2018 amounted to 20.19 million cases in total (SAPA Report, 2018). In 2018, there was a 9.8% growth in day-old pullets' placements. This reflected the industry's efforts to recover from the mass culling due to HPAI in 2017. The consumption of poultry products exceeds the consumption of all the other protein sources combined. The per capita consumption of poultry meat and eggs in 2018 was 39.53 kg and 7.33 kg, respectively, with a combined per capita consumption of 46.87 kg (this includes backyard consumption). South Africa remains the largest table egg exporter to the SADEC countries. The main destinations for South African egg exports are Mozambique (82.9%), Swaziland (14%) and Botswana (1.4%) (SAPA report, 2018). Exports to Angola, Botswana and Namibia have steadily been dropping since 2015 as local production increases in these countries (SAPA Report, 2016).

2.3 The process of egg formation

Both the left and right ovaries and oviducts are present in the embryonic development of all birds. By day four of incubation, the distribution of primordial germ cells to the ovaries of the chicken become asymmetrical, and by day ten, regression of the right ovary and oviduct is initiated under the influence of Mullerian inhibiting substance. Thus, the Galliformes reproductive tract consist only of the left ovary and its oviduct (Johnson, 2010). The infundibulum, magnus, isthmus, shell gland, and vagina represent the five distinguishable regions of the hen's oviduct. After the ovum is ovulated from the ovary, it is engulfed by the infundibulum, where it will remain for approximately 18 minutes. It is in the infundibulum where the perivitelline membrane and chalazae are formed (Ahmadi *et al*., 2011). The perivitelline membrane and chalazae ensure the structural integrity of the yolk. Should the ovum fail to be picked up by the infundibulum, it will be reabsorbed within 24 hours, in a process known as internal ovulation. The first layer of albumen is produced in the infundibulum, which is also the site of fertilisation. Following ovulation into the infundibulum, the yolk moves into the magnus. The magnus consists of three morphologically different cell types: tubular gland cells, ciliated cells, and goblet cells. These different cells are formed under the influence of oestrogen, as the bird reaches sexual maturity. The magnus is the largest structure of the oviduct, and it is in the magnus where the most albumin is formed. Tubular gland cells produce oval-albumin, lysozyme and conalbumin, whilst goblet cells produce avidin, that are secreted into the albumin. The different layers of albumin proteins will provide mechanical and bacterial protection to the yolk as well as creating a template for formation of the shell. The process of albumin formation in the magnus takes approximately 2-3 hours. From the magnus, the egg enters the isthmus, where the inner and outer layer shell membranes are formed. This process lasts for approximately 1-2 hours.

The final part of egg formation takes place in the tubular shell gland, where water and electrolytes enter the albumen. Prior to calcification, the egg takes up salts and approximately 15 g of fluid into the albumin, in a process called plumping. This fluid contains carbonic anhydrase, acid phosphatase and esterase, as well as bicarbonate and various ions (Salevsky *et al*., 1980). The primary function of these additives is to buffer the egg content from pH changes. It is estimated that the egg will be retained in this part of the reproductive tract for approximately 15 hours (Ahmadi *et al*., 2011). Prior to oviposition,

shell pigments (biliverdin and protoporphyrin) are deposited via ciliated cells onto the shell. The vagina and the shell gland are separated by the utero vaginal sphincter, which terminates at the cloaca. The aforementioned structures have no role in the formation of the egg, bar the coordination of expulsion of the egg. The only other function of the vagina is sperm storage in the utero vaginal tubules. Following oviposition of the egg, sperm is released and moves to infundibulum, where fertilisation takes place (Zavaleta *et al*., 1987).

2.4 Factors that affect egg production

2.4.1 Breed

For decades, modern laying hens have been selected for egg production and quality (Lui *et al*., 2011). Egg production remains the most important trait in commercial laying hens as it determines the total production efficiency over the lifespan of the flock. Many other factors such as internal and external egg quality traits are important, however total number of eggs produced determines the return on invest and efficiency of the production unit (Wolc *et al*., 2010). Breeding companies have placed major emphasis on the rate of lay as a selection trait. The rate of egg production, peak egg production, and the persistency of production are the most important factors that contribute to the total eggs laid per hen. Maintaining a high rate of lay and the age when production will start to decrease is largely attributed to genetic differences between strains (Silversides *et al*., 2006).

2.4.2 Photo-stimulation

Laying hens are photoperiodic and will respond to increased daylength by activation of the reproductive hormone cascade (Shi *et al*., 2019). Photo-stimulation results in activation of the hypothalamo–pituitary–gonadal cascade. Gonadotropin releasing hormone (GnRH) is released by GnRH neurons. GnRH in turn will stimulate gonadotropin secretion from the anterior pituitary (Sharp, 1993), which activates the secretion of gonadal steroids hormones (Renema *et al*., 2007). Sexual maturation is hastened by providing photo-stimulation once the birds have reached the minimum required age and body weight (Katanbaf *et al*., 1989). The onset of lay does not advance when hens receive photostimulation before they reach a critical age (van der Klein et al., 2018). Layer hen breeds reach sexual maturity at different ages. Light stimulation before the optimal age will have a direct influence on their laying performance (Farghly et al., 2019). Point of lay pullets that are underweight at photostimulation will subsequently cause lower egg production (van der Klein et al., 2018*).

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The uniformity of the flock at the start of photo-stimulation will narrow the range of onset of lay and increase the persistency of production (Robinson *et al*., 1996). Delayed onset of photo-stimulation will ensure that a greater percentage of the hens are sexually mature enough to respond to photo-stimulation, and will result in a more uniform production (Hocking, 1996). Delayed photo-stimulation may narrow the length of the productive egg-laying period (Shi *et al*., 2019).

2.4.3 Disease

There are many poultry diseases that can adversely affect egg production. Disease can either directly cause an egg production drop by infecting the reproductive system, or cause a decrease in egg production by affecting the health of the bird, such an airsacculitis, that is caused by respiratory pathogens, and then in turn infects the ovary and oviduct, causing lowered egg production (Nunoya *et al*., 1997).

Mycoplasma gallisepticum (MG) is a pathogen that causes chronic respiratory disease (CRD) in chickens, and can often occur as a co-infection with other viral respiratory pathogens (Ley, 2008). MG has been implicated in egg production drops, and may also cause a decrease in hatchability. In contrast, *Mycoplasma synoviae* (MS), primarily affects joints and tendon sheaths in chickens, but can also cause respiratory disease (Kleven, 2008). Both these pathogens have a negative effect on egg production and quality. Egg drop syndrome (EDS) is caused by duck adenovirus A. The disease will cause a severe drop in egg production and an increase of thin shelled eggs. Despite the severe decrease in production birds remain healthy and show no other clinical signs. The total drop in egg production varies but can be as high as 40 percent. The drop in production is rapid and can last up to 10 weeks (McFerran and Smyth, 2000). Swollen head syndrome (SHS), a viral disease caused by an avian pneumovirus, can also cause respiratory distress and a drop in egg production (Gough and Jones, 2008). Hens that have been infected with this virus can experience a decrease in egg production of up to 30 percent. The disease lasts from two to three weeks. Adult birds infected with avian encephalomyelitis virus will not exhibit any neurological signs. However, infected laying hens show a temporary drop in egg production of up to 75 percent. Production returns to its previous level within two weeks (Meroz *et al*., 1990). Clinical signs of avian influenza vary, depending on species infected, bird age, and the virulence of the viral subtype. An outbreak of highly pathogenic influenza can cause a mortality rate of up

to 90%, without any clinical signs of illness observed. However, low pathogenic avian influenza virus may also cause a drop in egg production, but not severe mortality. Newcastle virus clinical signs vary, depending on the virus strain. Mesogenic strains of New Castle Disease can cause respiratory and nervous symptoms, and has a lower mortality rate that of other strains found in adult birds. This strain of New Castle Disease can cause a partial to complete drop in egg production. Infectious laryngotracheitis is a respiratory disease of chickens, and results in production losses due to mortality, morbidity, and decreased egg production (Guy and Garcia, 2008). The chicken is the primary natural host and, although the disease may affect chickens of all ages, it is mostly observed in hens. The onset of lay can affect the rate of shedding of carrier birds (Hughes *et al*., 1989). The decreased in egg production appears to be secondary to the effects on other body systems.

Bacteria such as paratyphoid salmonellae are often asymptomatic in avian species, but can cause zoonotic disease in humans (Gast, 2008). The resistance of birds to avian enteric salmonellosis varies between birds of different age and genetic strains (Beal *et al*., 2005). *Salmonella gallinarum* and *Salmonella pullorum* are two species of salmonella that cause septicaemic disease, resulting in decreased egg production and hatchability, as well as morbidity and mortality (Shivaprasad, 2000). *Escherichia coli* causes colibacillosis in all avian species. Young birds are more susceptible to *E.coli* (Barnes *et al*., 2008). E. coli affects production in laying hens by causing colibacillosis with salpingitis (Trampel *et al*., 2007). Multiple serotypes of *E. coli* are found in poultry, but it is only the avian pathogenic E. coli (APEC) that possess specific virulence factors capable of causing salpingitis and peritonitis (Landman and Cornelissen, 2006). Salpingitis may originate from a systemic infection, or by ascending infection from the cloaca. The stress associated with the onset of lay may act as a precipitating factor in colibacillosis outbreaks (Zanella *et al*., 2000).

2.4.4 Nutrition

The effect of dietary metabolisable energy (ME) and crude protein (CP) levels on production varies due to differences between genetic strains and the dietary requirements at different ages of a birds life (Hiep *et al.,* 2017). It was demonstrated by El Manylawi *et al*. (2012) that egg production of Bovmans white hens was not influenced by ME levels between 2600 kcal and 2800kcal. In this breed, an ME level of 2600 kcal and a CP level of 16% produced the highest number of eggs in the early production period (21-40 weeks-of-age). An increase

in the ME level fed to older laying hens, however, produced an increase in egg production (Hiep *et al*., 2017). This was also demonstrated by Depersio *et al*. (2015), who found that both egg weigh and egg production could be increased by increasing the ME levels of Hy-line Brown hens during the last phase of their production period. The ME level should be adjusted according to breed, age, and environmental temperatures (Hiep *et al*., 2017). Increased energy density can assist to maintain egg production in sub-tropical areas, with high ambient temperatures and lowered feed intake (Rao *et al*., 2013). In order to optimise the production potential of hens, balanced dietary protein and amino acid levels should be fed (Li *et al*., 2013). Protein requirements differ according to genotype and laying phase. This was demonstrated in a study by Rao *et al*. (2014), who showed for instance that White Leghorn layers at their laying periods 21-32, 33-52, and 53-72 weeks-of-age the optimal CP levels were 18%, 18% and 15% respectively, whereas, the CP value of 16% was optimal for Lohmann Brown and Bovans White laying hens to produce highest number of eggs at 26 weeks-of-age (El-Manylawi *et al*., 2012). Low protein diets in later phase of lay produced the more hatching eggs than those on a high protein diet (Teuling *et al*., 2015). More importantly, laying hens must be supplied with sufficient and balanced ratio of essential amino acids (Hiep *et al*., 2017). Simultaneous reduction of crude protein and amino acids can reduce the egg weight and egg production in older laying hens (Mohhaddam *et al*., 2012). A digestible lysine level of 0.70% is recommended for Hy-Line Brown layers at 25-41 weeks-of-age (Souza *et al*., 2014). Further to this, in a study by Rocha *et al*. (2013) it was found that a diet with digestible threonine, viz.: a digestible lysine ratio of 78% for Hy-Line hens resulted in the highest egg production, whereas, 92% digestible methionine and cystine/lysine ratio is ideal for Isa Brown laying hens from 34 to 42 weeks-of-age, to ensure the highest production (Nicodemus et al., 2011). Thus, similar to diet energy level, the requirements for protein and essential amino acids also differ between breeds and laying age.

The inclusion of lipid sources in layer diets can assist in increasing the nutrient density of a diet (Oliveira et al., 2010). The literature is contradictory on the effects of lipid supplementation on the production performance of laying hens (Hiep *et al*., 2017*)*. Kucukersam *et al*. (2010) investigated the influence of different dietary oil sources in layer diets on egg production, and found that the inclusion of 3% soy oil inclusion increased layer performance. High energy diets or a high inclusion of dietary lipids may predispose birds to

fatty liver haemorrhagic syndrome (FLHS). FLHS is a metabolic disease and affects mainly caged hens in peak production. The disease can also be complicated by mycotoxins and hot weather. It is characterised by fat infiltration into the liver, haemorrhages, and mortality (Saif, 2008). Clinical signs include an increased mortality rate of hens in full production and sudden a drop in egg production (Julian, 2005).

Anti-nutritional factors, such as toxic agents and contaminants, can result in decreased production in laying hens. Feed that is contaminated with mycotoxins has the potential to reduce production and egg quality (Pandey and Chauhan, 2007). Feed contaminated with mycotoxins results in an increase in plasma uric acid concentrations, due to effects of the toxins on the liver, and may also cause immunosuppression (Chowdhury et al., 2005). Some of these effects are mediated due to a reduction in feed intake of the contaminated feed (Suksupath et al., 1989).

2.5 Egg Shell Quality

Egg quality and longevity of egg content are ensured by egg shell integrity and strength. Egg shell strength ultimately affects the soundness of the shell, with weaker shelled eggs more prone to cracks and breakages, and subsequently microbial contamination (Mabe *et al*., 2003). Most commercial eggs are intended for retail table eggs; cracked eggs cannot be sold and are at best used for egg powder or other byproducts. A high proportion of cracked eggs or eggs with poor shell quality will have a negative effect on profitability for the egg producer.

Egg shells are composed of an organic and inorganic matrix. The organic matrix is made up shell membranes, the mamillary cores, the shell matrix, and the cuticle, whilst inorganic matrices are comprised of the mamillary knob layer, palisade layer, and outer surface crystal layer (Rahn, 1979). It is the organic matrix that determines the rate of egg shell formation (Nys *et al*., 1999). The shell matrix is a series of layers of mucopolysaccharides that are the initial site of calcification. Although shell membranes constitute only a small fraction of the egg shell, its integrity is critical to the egg strength and formation. Shell membranes are organised into an inner and outer membrane, and are produced in the isthmus region of the oviduct. The membranes are composed of collagen (10%) and a fibrous component of protein and glycoprotein (Johnson, 2010). The membranes permit the passage of gases and water. There is no correlation between the thickness of the shell and the thickness of the

shell membrane, but membrane thickness does decrease with age of the hen (Johnson, 2010). Mamillary cores, which are proposed as the initial sites of calcification, represent the largest proportion of the organic material in the eggshell and are projections from the outer membrane surface. Manganese is required for the development of the mammillae, due to its role in synthesis of mucopolysaccharides (Leach *et al*., 1983). The cuticle is a wax-like outer layer of the shell. It consists of protein, mucopolysaccharides and lipids. Its function is to protect the egg from water, bacterial infection, and evaporation (Ahmadi *et al*., 2011) Calcium carbonate and magnesium carbonate make up the inorganic part of the egg. The palisade layer of the shell is formed by calcified crystals that have formed in the shell matrix. Carbonic anhydrase is an important enzyme in the matrix that has a vital role in carbonate formation. Zn constitutes are vital for the formation of carbonic anhydrase (Silyn-Roberts *et al*., 1986). The surface crystal layer consists of crystals in various arrangements. More research is needed on the surface crystal layer as it is likely to have a more important role in egg shell strength than previously thought (Nys *et al*., 1999).

2.5.1 Measurements of shell quality

Any damage to the egg shell is directly related to shell strength. The strength of the shell is determined by shell thickness (calcium carbonate content) and shell matrix organisation (Ahmadi et al., 2011). Damage to the egg shell mainly occurs at point of lay, or movement of the egg from the point of lay to the egg grading area. The process of washing, packaging, and transport may also damage the egg shell.

Various techniques measuring egg shell quality have been developed, where some methods involve the destruction of the egg. Destructive methods for measuring egg shell quality include compression force, measuring egg shell thickness, and egg shell weight. Non-destructive methods also exist, such as the specific gravity (SG) tests. Standard gravity is defined as the ratio of the density of a given substance, to the density of water (Sloan *et al*., 2000). Determining an egg's SG is accomplished by floating the egg in salt solutions. An average SG greater than or equal to 1.080 indicates acceptable shell quality (these are eggs that do not float in the saline solution). Egg SG declines as the hen ages. This is partly due to the egg size increasing more rapidly than the shell weight. Thus, differences in SG among eggs of similar weight are mainly due to variations in the amount of shell. Specific gravity and egg shell thickness are positively correlated to one another. Fresh eggs will have higher SG than eggs in storage for a long period of time, because there will be an increasing loss

of moisture from the eggs over time, which is then replaced by air, therefore increasing the total air in the egg (Sloan et al 2000).

Shell breaking strength is measured by the static compression force needed before the egg shell breaks or cracks. According to the Lohmann Manual (2013), egg laying breeds have a Newton strength of above 40 Newton. The total amount of shell and thickness of the shell have been found to be directly related to egg shell strength. Shell weight may be measured by breaking open an egg and carefully rinsing and then drying the pieces of shell before measuring the egg shell weight. However, this is destructive, and losses of small pieces of egg shell may occur. Shell thickness may be measured by a suitable gauge and is usually measured on three pieces of shell around the equator of the egg. In field circumstances, the SG measurements can provide accurate results more rapidly than the destructive tests. The SG and egg shell thickness are highly positively correlated, and SG measurements are usually sufficient (Ahmadi *et al*., 2011). Recently, studies on egg shell quality have focused on the arrangement of the crystal structures in the shell by using electron microscopy. It was found that variability in egg strength can be explained by differences in this shell ultrastructure, even when shell weight and shell thickness have not changed (Ahmadi *et al*., 2011).

2.5.2 Factors that affect egg shell quality

2.5.2.1 Nutrition

An egg shell can contain up to three grams of Ca (Ahmadi *et al*., 2011). Breed recommendations are quite clear on the Ca inclusion level in feed for the different production stages during the laying period. Most hens are reared on a Ca inclusion level of 1% in the rearing feed level (Sohail *et al*.,2002) at the onset of lay Ca increases to 3.8% at the onset of lay (Lohmann Manual, 2013). The hen's greatest calcium demand is during egg shell formation, whilst the egg is in the shell gland. This period is usually at night and feeding practices have evolved to try and increase the hen's calcium intake at night. One such practice includes an interrupted lighting programme that wakes the hen up during the dark period, inducing the hen to take in feed and increase her blood calcium levels. Other programmes include the feeding of grit into the feed, or whole grit fed to the birds in the late afternoon. Due to the increased grit particle size, the release of calcium from the grit is slower, thus providing the much-needed Ca when the egg is being formed (Balnave *et al*,.

1997), however there is no indication that suggest that increasing the Ca level above the recommended level has any beneficial affect (Altan *et al*,. 2000). During calcium release from bone during periods of low blood calcium deficiency, parathyroid hormone will release calcium from the bone in order to supplement the bodies need for calcium. Adequate phosporous levels are needed in the feed for normal bone formation as it is a component of hydroxyapetetite, which in turn is a major component of bone.

The hen's ability to produce an increased amount of egg shell may be related to the activity of 25-hydroxy-cholecalciferol-l-hydroxylase $(1,25\text{-}(OH)_2D_3$ (Nys, 1987). The inability of the birds to mobilise adequate Ca from the bones may lead to a condition known as cage layer osteoporosis. Osteoporosis is a metabolic disorder affecting bone structure and reduction in bone mass (Roberts, 2004). In the bones of birds that are affected by this condition, bones are more fragile and susceptible to fractures. Osteoporosis can be induced by various factors such as inadequate dietary Ca, vitamin D_3 and phosphorus (P) (Julian, 2005). During times when Ca is insufficient in the diet, hens withdraw the mineral from cortical bone, which leads to an inadvertent depletion of bone phosphorous.

2.5.2.2 Genetic and environmental factors

Due to rigorous genetic selection, many different breeds of laying hens now exist, which has led to significant variation in egg shell quality, colour, size and production rate (De Ketelaere, 2002). Whilst selection has mainly focused on increasing the amount of eggs produced and longevity of the birds, other characteristics have also been affected (Curtis *et al*., 1995). Selection for a single characteristic such as production or egg weight can affect other characteristics, such as egg shell quality (Roberts. 2004). Bird age is directly proportional to bigger eggs, which results in higher egg mass and lower proportion of total egg relative to the amount of shell (Ahmadi *et al*., 2011). As the birds age, egg weight is not accompanied by proportional increase in shell weight, thus the ratio of egg to shell decreases over age. More exercise may result in better bone quality, but may not decrease the fracture incidence. Genetic lines that are resistant to osteoporosis have been selected (Whitehead and Fleming, 2000).

Environmental temperatures, such as extremely hot weather, may reduce the feed intake of layer hens (Solomon *et al*., 1987). The reduced intake of feed limits the availability of blood

calcium for egg shell formation. In order to deal with exposure to heat stress, the hen can only reduce her temperature by increasing her respiration rate. This results in lowering the partial pressure of arterial blood carbon dioxide (paCO2) and arterial blood bicarbonate ion (HCO3-), but will increased arterial blood pH and plasma lactate (Johnson, 2010). This results in respiratory alkalosis, which is caused by the loss of carbon dioxide from the blood, and an increase in blood pH. This change in acid-base balance during heat stress reduces the level of ionised calcium level in the blood, which may in turn limit the availability of calcium for egg shell formation. Increased panting may also reduce the activity of carbonic anhydrase, an enzyme that results in the formation of bicarbonate, which contributes the carbonate to the egg shell (Solomon *et al*., 2010). Carbonic anhydrase is required to form bicarbonate that passes through the shell gland to form calcium carbonate. Under high temperature, blood flow within the body is changed, and more blood flows to peripheral tissues to transfer more heat from the body core to the surface. The effect of aging on shell quality can be reversed, partially by the process of forced moulting (Roberts, 2004). The success of a forced moult is largely determined by the severity and nature of the moult, as well as the age of the birds.

2.5.2.3 Diseases that affect egg shell quality

There are many common poultry diseases that compromise the health of the bird and may result in egg shell changes, or even defective eggs, such as misshapen eggs being formed (Saif, 2008). Viral diseases such as egg drop syndrome, infectious bronchitis, New Castle Disease, and infectious laryngotracheitis cause severe discoloration of the egg, resulting in white eggs (in brown egg laying hens) and soft shells. These changes in egg shell are a result of virus replication in the epithelial cells of the oviduct that disrupts the normal calcification process (Saif, 2008). Diseases such as *Mycoplasma gallisepticum* and avian encephalomyelitis produce deformities of the egg shell and soft or rough shell eggs (Saif, 2008.). *Mycoplasma gallisepticum* and MS both have the potential to cause salpingitis in laying birds (Domermuth et al., 1967). *Mycoplasma synoviae* isolated from the oviduct of birds producing abnormal eggs, can induce egg shell apex abnormalities (EAA) (Feberwee et al., 2009). These abnormalities are characterised by changes in the colour and texture of the shell in the region of the air sac. When this region of the egg shell is observed under the scanning electron microscope, the mammillary layer and the lower part of the palisade layer

are missing. The EAA ceased after an injection of long acting oxytetracycline but reoccurred 12 days later. The incidence of EEA was higher in the presence of infectious bronchitis virus. The presence of EAA was accompanied by shell thinning, increased translucency, and reduced shell breaking strength. A later experiment with SPF white layers (Feberwee et al., 2009) investigated the ability of an MS vaccine to protect against egg shell abnormalities in the presence of an infectious bronchitis challenge. Egg shell apex abnormality were produced only in birds that were challenged with M. synoviae. Again, this eggshell abnormality was associated with reduced shell strength. Vaccination reduced the incidence and delayed the appearance of EAA in eggs from birds challenged with MS, but did not completely prevent the occurrence of EAA.

2.6 Internal egg quality

The yolk is vital for embryonic growth in fertilised eggs containing large amounts lipids and proteins. Lipids contribute 33% of the total mass of the yolk, whilst protein contributes 17 percent. The balance of the yolk content is made up of 48% water, carbohydrates 0.2% and organic elements 1 percent. Lipid content of the yolk is comprised of triacelglycerols, phospholipids, and cholesterols (Johnson, 2010). Albumen provides the embryo with essential amino acids, and it also acts as barrier preventing bacterial infection of the yolk and embryo. Bacterial growth is inhibited by the enzyme ovotransferrin, which acts as an iron chelator, thereby preventing bacterial replication (Burley et al., 1989).

Yolk quality is determined by measuring the colour texture, firmness, and smell of the yolk. The egg should also not have any internal blemishes, such as blood spots, pigment spots, and meat spots (Ahmadi, 2011). The egg yolk integrity is determined by the strength of the perivitaline membrane. If the perivitaline membrane is weak, the yolk is more likely to break (Dale et al., 1998). Yolk colour is primary determined xanthophyl present in the diet consumed (Butler, 1972). Xanthophyll is a pigment that cannot be formed by the bird and derived from grains or synthetic xanthophyll precursors added to the feed (Mabe et al,. 2003).

The quality of the albumin is measured by measuring the height of the albumin, which in turn is converted to Haugh units (Tangere et al., 2001). Haugh units are a measure of the viscosity and thickness of the albumin. A minimum measurement for HU units for reaching

the consumer is 60. Most eggs should leave the farm with Haugh unit between 75 and 85 HU (Doyon et al., 1986).

2.6.2 Factors that affect internal egg quality

2.6.2.1 Storage

The egg shell is a porous structure, which allows for respiration during the incubation process. The albumin will progressively become watery and transparent, as the egg ages and carbon dioxide is lost through the egg shell. Increased ambient temperature increases the rate of respiration through the egg shell pores, thus increasing the rate of deterioration of the albumin.

Storage of eggs at a higher temperature increases the loss of carbon dioxide, where the result is that the albumin quality deteriorates faster. Decreasing shed temperatures in warmer months, combined with regular collection of eggs, will reduce detrition of the albumin before collection (Ahmadi et al., 2011). According to work done by Okeudo et al. (2003), eggs stored at ambient temperatures and humidity lower than 70% will lose 10-15 HU every few days from point-of-lay. Storage of eggs at temperatures of 7-13 $^{\circ}$C and humidity of 50-60% will reduce the rate of degeneration of thick albumen proteins, and consequently, egg albumin will be maintained for longer (Natalie, 2009).

2.6.2.2 Age and breed

Haugh unit and bird age are inversely proportional to one another. In work done by Ahmadi et al. (2011), the Haugh unit decreased with 1.5 – 2 units for each month in lay. The ideal Haugh unit should be an average of 102 HU at 20 weeks-of-age, whereafter the Haugh unit will fall to an average of 74 HU by 78 weeks-of-age, a constant decreasing rate of 0.0458 units per day of lay (Doyon et al., 1986).

2.6.2.3 Nutrition

Albumen quality is not greatly influenced by bird nutrition (Williams, 1992), although albumen quality decreases and increases with protein and amino acid content (Zaman, 2005). Albumen quality increases with increased dietary lysine content (Williams, 1992). Albumen quality increases with the inclusion of ascorbic acid (Balnave, 2000), especially at high temperatures and increasing with vitamin E supplementation (Dale 1998).

2.6.2.4 Disease

Various viral poultry diseases, such as New Castle disease and Egg drop syndrome, may disrupt egg shell formation or decrease egg production, but infectious bronchitis virus, a member of the corona virus family, is the only virus that may cause variation in albumin quality (Nys *et al*., 2001*)*. During the formation of the egg, albumin is incorporated into the albumen of the egg, where this process takes place in the magnum of the oviduct (Abd-Elrazig *et al*., 1998). Bronchitis virus disrupt the synthesis of albumen proteins by causing histological changes in the epithelium of the oviduct (Ekweozor, 2002). Infectious bronchitis virus has tropism for both respiratory and genital epithelial cells, and although the virus may only cause respiratory signs in broilers, it can cause both drops in egg production and deterioration in egg quality in layers. Sevoian and Levine (1957) have observed internal and external egg quality alteration in laying hens experimentally infected with Massachussetts IB strain. The severity of the production drop may vary with the period of lay and with the causative virus strain. When White Leghorn layers were infected with an Arkansas strain, the hens laid fewer eggs, and the shell quality and internal quality were inferior (Muneer *et al*., 1986). Infected hens laid eggs with a watery albumen consistency and the yolks were smaller. Studies by Chousalkar and Roberts (2009) did not show any decline in egg production. There was however a deterioration of egg internal quality (albumen) and a loss of egg shell colour (Chousalkar and Roberts, 2009). Further in this study, the egg shape changed, with IB causing eggs to become more elongated. Studies of the histopathology of the oviduct of White Leghorn (Chousalkar *et al*., 2007), Hy-Line Gray (Chousalkar *et al*., 2007) and Isa Brown hens (Chousalkar *et al*., 2009) confirmed that IB induces pathology in various regions of the oviduct of laying hens. Coronavirus infection in the shell gland cells causes declines in egg shell quality: thin, soft, misshapen, or pale unpigmented shells. The thin and watery albumen occurs when the coronavirus affects the cells of the magnum, resulting in lower Haugh unit values. A day-old coronavirus infection can also cause false layers, where hens are then unable to lay due to the oviduct damage. This situation has been described in France in breeders and layers associated with a new variant strain, Qx (Robineau and Moalic, 2009).

2.7 Mineral nutrition in layer hens

A mineral is an inorganic element that is essential in the body in various physiological processes. Mineral absorption is realised from the gastrointestinal tract and absorption availability differs between mineral composition (Ammerman *et al*., 1995). Macro minerals, essential for bone, muscle, production and nervous tissue function are required in greater amounts and are therefore included in greater quantities in the diet. Macro minerals include calcium, phosphorous, sodium, potassium, chloride, magnesium, and sulphur. Trace minerals, or micro minerals, are required in small quantities which are included at rates of mg/kg of the diet, some of most important trace minerals included in poultry diets are Zn, Cu, Mn, Fe, I, Se and Mo. Dietary supplementation of these minerals in poultry diets are essential despite raw material contribution to the diets (McNamara, 2006).

2.7.1 Inorganic mineral sources

Inorganic trace minerals are the most common source of trace minerals supplemented in poultry diets (Leeson *et al*., 2005). The overall benefits of including these minerals in poultry diets are increased growth rate, health, and reproduction. The rate of dietary trace mineral supplementation is approximately 0.25% of the total diet (Boa *et al*., 2007). Inorganic trace minerals are added to feed in the form of inorganic salts, such as sulfates, oxides and carbonates (Nollet *et al*., 2007). Sulfates and chloride salts are more soluble than the carbonate and oxide salts (Miles *et al*., 2008). Excessive use of inorganic trace minerals in poultry diets may reduce their relative biological availabilities due to mineral interactions which may cause environmental pollution due to increased mineral excretion (Aksu *et al*., 2010).

2.7.2 Organic mineral sources

Recent research on organic trace minerals has focused on the biological value and absorption availability in several species of livestock and poultry. According to AAFCO (2000), organic trace minerals are described as specific amino acid complexes formed by the combination of a soluble metal salt with an amino acid. These chelates are thus molecules that are the result of a covalent binding between a metal ion and a ligand, such as a protein or carbohydrate (Vieira, 2008). There are many forms of organic minerals such as metal

complexes, metal amino acid complexes, metal amino acid chelates, metal proteonates, metal polysaccharide complexes, metal propionates and yeast derived complexes (AAFCO, 1998).

The use of organic minerals results in improved intestinal absorption, due the smaller organic molecules and the reduction of the formation of insoluble complexes with other ionic trace elements (Peric *et al*., 2007). Further to this, micromineral homeostasis is controlled at cellular and subcellular level by a number of proteins. Zinc homeostasis in controlled by zinc transporter proteins and metallothionines (Baltaci, 2017). Zn transporter proteins, based on their location in the cell, can be divided into two major groups, namely Zrt and Irt-like protein or ZIPs and Zinc transporter protein ZnT (Hojoy, 2016; Baltaci,2017). Both ZIP and Znt transporters have opposite roles in controlling cellular zinc levels and transport Zn in opposite directions across cell membranes. Intracellular levels of Zn are increased by the ZIP transporter by promoting uptake of Zn from the lumen of the the gastrointestinal tract or alternatively from the release of Zn from intracellular stores. (Kimura, 2016). ZnT transporters, in contrast, mediate the efflux of Zn from the cells into the extracellular fluid, such as blood plasma and mediate the uptake of Zn into intracellular vesicles (Wang, 2009). Should the requirement for Zn increase, or a diet low in Zn is consumed, the number of ZIP transporters at the plasma membrane may be upregulated in order to significantly increase absorption of zinc. In the case of diets high in zinc, these may be down-regulated to protect the bird from Zn toxicity (Baltaci, 2017). Availability of metal transporters and metallothionines are thought to be the limiting factor in the uptake of Cu and Zn at a cellular level. These binding partners are necessary for absorption (Sauer *et al*., 2017).

Recent research has indicated that feeding laying hens or broiler breeder hens organic mineral sources can improve general egg quality and specifically shell quality. Further organic trace minerals can improve embryo development, hatchability, chick quality, egg production and growth (Uni *et al*., 2012). Moreover, several studies indicate that organic sources are absorbed more efficiently in comparison to inorganic sources, which results in better production (Nollet *et al*., 2007). In studies performed by Yenice *et al*. (2015), post-peak Barred Rock layers were fed inorganic and organic sources of trace minerals Zn, Cu, Mn, an Cr. Results showed a significant increase in the serum concentration of all four the trace elements, an increase in the egg concentration of Zn, Cu, Mn, and Cr, and an increase

in the Zn and Cr content of egg shells, from the hens that were fed the organic mineral source. Gheisari et al. (2011), using Hy-line W-36 layers at 38 weeks-of-age, tried to evaluate the effect of organic and inorganic sources of Zn, Cu and Mn on egg production and egg shell qualities. A maize-soybean diet supplemented with organic form of these minerals were fed, with concentrations at 50% and 75% lower than the NRC (1994) recommendation. It was found that these lower levels of Zn, Cu, and Mn were sufficient to maintain laying performance and resulted in improved egg shell and albumin qualities. Studies on broiler breeder hens showed that feeding diets containing organic mineral sources increased egg shell weight and thickness; these results improved further when a combination of organic and inorganic minerals were fed (Favero *et al*., 2013).

2.7.3 Zinc

Zinc is an essential trace element for humans and animals alike; it is necessary for normal growth, development, and health. Zinc is distributed throughout the body and plays a role in reproduction, development of blood cells, immune system function, and bone development (Hudson *et al*., 2004). Egg production decreases with a Zn deficiency, as Zn is a critical cofactor in calcium metabolism. This is due to Zn being a critical component in the enzyme carbonic anhydrase, which is an essential enzyme for egg shell formation and calcification of bone from carbonate ions. Carbonic anhydrase is found in the shell gland of the oviduct of laying hens (Leeson *et al*., 2001). Carbonic anhydrase supplies carbonate ions during egg shell formation. Inhibition of this enzyme results in lowered bicarbonate ion secretion and reduces the egg shell weight. This is especially relevant to older laying hens as production starts to decline (Mabe *et al*., 2003). Additionally, Zn deficiency can result in poor growth and abnormal bone development in chicks (Leeson *et al*., 2001). Metalloenzymes such carboxypeptidases and DNA polymerases also contain Zn, these enzymes are essential for normal skin, wound healing and hormone production (Favero *et al*., 2013). A Zn deficiency can have a severe effect on the process of erythropoiesis, due to Zn playing a major role in the activation and synthesis of alfa-amilovunilic acid dehydratase an enzyme essential to the formation of red blood cells (Aksu *et al*., 2010). A Zn deficiency can also cause reduced hatchability (Favero *et al*., 2013) due to poor sperm quality.

Zinc is absorbed in the small intestine of monogastric animals, and a small amount of absorption occurs in the proventriculus of chicks (McDowell, 2003). The chemical form and the inclusion rate of Zn is important in preventing mineral antagonism during the digestion

process. This is especially true in the case of high calcium intake, which reduces Zn absorption (Mabe *et al*., 2003). Similarly, excess Zn in the diet can reduce Cu absorption (Dobrzanski et al., 2003). Hudson et al. (2004) fed diets supplemented with inorganic $ZnSO_4$ or organic sources of Zn. Their results indicated that there was an increased absorption of Zn, increased Zn retention, and enhanced performance of hens that were supplemented with both organic and inorganic sources of Zn. Common sources of organic Zn include metal-amino acid metal-proteinates.

2.7.4 Copper

Copper is essential for growth, egg shell membrane formation, egg shell structure, shell texture, and egg shape (Wang *et al*., 2014). The micro mineral is responsible for the formation of cross-links of the microstructure within the eggshell via the enzyme lysyl oxidase-Cu proenzyme, which is involved in converting lysine to cross-linked desmosine and isodesmosine. The lack of Cu in a diet results in abnormal eggshell formation. According to Mabe *et al*. (2003), the eggshells of Cu deficient hens are characterised by abnormal distribution of the shell membrane fibers due to alterations in lysine derived cross-links, which results in egg shape deformation and abnormal mechanical properties. Copper is also necessary for bone formation, especially cartilage structure and integrity (Leeson *et al*., 2001). Copper also improves gut health, as it has antimicrobial properties that work to depress the growth of pathogenic bacteria, and it can thereby act as a growth promoter (Ewing *et al*., 1998). Copper is also essential for the synthesis of hemoglobin, erythrocuprein and other plasma proteins necessary for lipid metabolism and hepatic lipogenic enzymes (Favero *et al*., 2013). Copper is also present in certain pigments, such as turacin, a pigment that is found in feathers.

Anemia is the first clinical sign of a Cu deficiency in poultry (McDowell. 2003). This condition may only occur if Cu is not supplemented in the diet. The onset of anemia is preceded by severe neutropenia (Linder, 2013). Copper deficiency in layer hens can result in abnormal eggshell formation, which includes irregular distribution of the shell membrane, causing increased incidence of shell-less eggs and irregular egg and shape (Mabe *et al*., 2003). An excess concentration of Zn in the diet can also result in a copper deficiency, due to competitive exclusion of the uptake regulators between the two minerals (Watts, 1990). The absorption of Cu occurs in the duodenum and is attached to a protein carrier hemocyanin

that facilitates diffusion of molecules across the cell membrane (Leeson et al., 2001). Retention of Cu is approximately 6% in broiler chickens.

2.7.5 Manganese

Manganese plays a significant role in growth, reproduction amino acid metabolism and biosynthetic processes. It is also vital to the normal regulation of the immune system and blood cell formation (Boa *et al*., 2007). Manganese is essential to mucopolysaccharide synthesis, which in turn is vital in eggshell formation. Manganese is a co-factor in the enzyme glycosyl transferases, which is involved in the formation of mucopolysaccharides. These mucopolysaccharides are components of proteoglycans, which are involved in the eggshell matrix formation (Zamani *et al*., 2005). Keratin and dermatan proteoglycans are present in the eggshell matrix and may be involved in the control of its ability to withstand compressive forces, consequently they may influence mechanical properties of the egg's shell (Nys *et al*., 1999). Hens that are fed Mn deficient diets produced eggs with thinner shells with translucent areas and abnormalities of the eggshell ultra-structures, particularly in the mammillary layer (Mabe *et al*., 2003). Manganese is essential for the synthesis of polysaccharides, where a deficiency of this mineral results in a decrease in the eggshell content of hexosamine and hexuronic acid (Leeson *et al*., 2001). Furthermore, a diet marginally deficient in Mn may cause a decrease in hatchability, and an absolute deficiency of the mineral may cause shortened long bones, parrot beak and wiry down in chicks (Favero *et al*., 2013). Zamani *et al*. (2005) evaluated the inclusion of both Zn and Mn in combination on performance and egg quality in commercial laying hens. Hy-line W-36 layers received a maize-soybean diet that contained 50 mg/kg Zn and 30 mg/kg Mn. The results indicated that Mn and Zn alone, and in combination, reduced the number of broken eggs, but did not affect the total eggs produced, feed consumption or feed conversion ratio. In laying hens, Yildiz *et al*. (2011) evaluated the effects of organic and inorganic Mn sources at different doses for a 12-week period. The results showed that organic Mn sources improve growth, egg weight, and bone solidity.

Absorption of manganese in the intestinal tract is low (Leeson *et al*., 2001) and is absorbed by binding divalent metal ion transporters (Aksu *et al*., 2010). Retention of manganese in the body is as low as 0.2% in broilers (Peric *et al*., 2007). In poultry feed organic sources of Mn consist of amino-acid complexes, chelates, and proteinates. The bioavailability of

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chelated and complexed Mn is related to chemical characteristics that are improved by chelation effectiveness and the percentage of organic ligand (Yildiz *et al*., 2011). Current studies are contradictory regarding weather organic Mn is more absorbable than inorganic Mn sources (Li *et al*., 2004). Mn can cause toxicity, by interfering with magnesium absorption, and causes excessive sodium and potassium concentrations in the tissues (Watts, 1990), consequently there is a high inclusion rate of Mn, which can interfere with the synthesis of aminovalinant that averts Fe metabolism (Aksu *et al*., 2010).

2.8 Conclusion

Zinc, copper, and manganese are three of the trace minerals required in poultry diets for normal physiological processes, such as production and egg shell formation. Research indicates that in the laying hen, the dietary supplementation of organic trace mineral sources of Zn, Cu, and Mn result in improved performance and egg quality (Tang et al., 2006). There are, however, many other factors that can play a role in layer hens' performance and egg quality. The objective of this research was to evaluate the effect of different inclusion rates and sources of Zn, Cu and Mn on the performance and egg quality of laying hens.

Chapter 3

Materials and Methods

3.1 Facilities and experimental design

The protocol for this trial was approved by the University of Pretoria's Animal Ethics Committee (Project number: EC016-14). The trial was conducted on the Hillcrest Experimental Farm, at the University of Pretoria. The naturally ventilated layer house consisted of four rows of a two-tiered system of cages designed to house layer hens individually. The lower tier was 52 cm from the concrete floor, with the upper tier 27 cm above the lower tier. Dimensions of the cages were 46 cm x 22 cm x 43 cm, floored with sloped wired mesh (mesh size 3 cm x 5 cm). A replicate (experimental unit) comprised of 10 adjacent hens, with one hen per cage, separated from the next experimental unit by two open cages. The study had a randomised complete block design. Six different dietary treatments in a 2 x 3 factorial arrangement were used in this trial with each of the treatments replicated eighteen times. Treatments included an organic or an inorganic mineral source, with three different concentrations of Zn, Mn, and Cu. Ratios of inclusion were fixed for Zn, Mn, and Cu and did not change between the different dietary phases. Each of the six treatments were divided into two blocks over the length of the house and each block contained nine replications of a treatment.

3.2 Animal husbandry

Lohman Brown Lite point-of-lay hens were purchased from a commercial company, situated near Pretoria. A total of 1080 point-of-lay pullets at 16 weeks-of-age were received and placed at a stocking rate of one hen per cage. Water was supplied by one nipple drinker per cage. Feed was fed *ad libitum* in a steel trough in the front of the cage. A two-phase feeding programme was applied with the first phase fed from placement at 16 weeks-of-age up to 48 weeks-of-age, after which the second phase feed was fed up to the end of the experimental period at 76 weeks-of-age. Manure was removed once a week by means of a scraper that ran underneath the tiers. Birds were fully vaccinated during the rearing period against all important poultry diseases as shown in Appendix 1. The only vaccination done during the laying period was a five weekly Newcastle disease virus vaccination administered by eye drop. The following lighting programme was used during the trial: at 16 weeks-of-age, the hens received nine hours of constant light. During the first four weeks (17-20 weeks-of-age), lighting was increased by one hour of light per

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week. From 21 weeks-of-age, 14 hours of light was provided until termination of the trial at 76 weeks-of-age (Appendix 2).

3.3 Experimental diets

The trial included six dietary treatments. A 2 x 3 factorial arrangement of treatments were used. Zinc, Mn and Cu from two different sources, i.e inorganic and organic, were used at three different inclusion levels. The organic mineral sources used were Mintrex Zn, Mintrex Cu, and Mintrex Mn (Novus International Inc, Missouri, USA). The inorganic mineral sources used were zinc sulphate, copper sulphate, and manganese oxide salts. The contribution of additional methionine in the organic mineral source was subtracted from the total methionine in the diet formulations so as to ensure similar methionine concentrations for all treatments. Typical South African maize-soya based diets were formulated based on nutrient specifications recommended for Lohman hens. Feed ingredient composition and calculated nutrient concentration for the diets are shown in Tables 3.1 – 3.3. Zinc, Cu, and Mn were added to Treatments 1 and 2 at 60, 15 and 80 mg/kg, respectively. Treatments 3 and 4 contained 32,8 and 32 mg/kg of supplemented Zn, Cu, and Mn, respectively. Supplementation levels of Zn, Cu, and Mn in Treatments 5 and 6 was 50, 8 and 50 mg/kg respectively (Table 3.4). Zinc, Cu, and Mn were derived from inorganic salts in Treatments 1, 3 and 5. For Treatments 2, 4 and 6, an organic mineral source was used (Table 3.4). Minerals were added at a specific inclusion rate, irrespective of the mineral contribution of the raw materials. This is also the practice in commercial feed formulations where the mineral premix is added to the feed formulation, irrespective of the raw material contribution.

Table 3.1 Raw material composition (g/kg) of phase 1 of the experimental diets (fed from 16 to 48 weeks-of-age)

Raw material	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Maize (ground)	600	600	600	600	600	600
Soy oilcake	165	165	165	165	165	165
Sunflower oilcake	80	80	80	80	80	80
Soya oil	5	5	5	5	5	5
Wheat bran	45	45	45	45	45	45
Limestone	90.5	90.5	90.5	90.5	90.5	90.5
Monocalcium phosphate	6.5	6.5	6.5	6.5	6.5	6.5
Salt	3.5	3.5	3.5	3.5	3.5	3.5
Methionine (MHA)	0.9	0.159	0.9	0.558	0.9	0.385
Choline chloride	0.7	0.7	0.7	0.7	0.7	0.7
Axtra® Phy phytase	0.6	0.6	0.6	0.6	0.6	0.6
Layer premix	2.5	2.5	2.5	2.5	2.5	2.5

Table 3.2 Raw material composition (g/kg) of Phase 2 of the experimental diets (fed from 48 to 76 weeks of age)

Raw material	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Maize ground	600	600	600	600	600	600
Soy oilcake	155	155	155	155	155	155
Sunflower oilcake	78	78	78	78	78	78
Soya oil	5	5	5	5	5	5
Wheat bran	50	50	50	50	50	50
Limestone	98	98	98	98	98	98
Monocalcium phosphate	5.5	5.5	5.5	5.5	5.5	5.5
Salt	4	4	4	4	4	4
Methionine (MHA)	0.7	0.041	0.7	0.358	0.7	0.185
Choline chloride	0.7	0.7	0.7	0.7	0.7	0.7
Axtra® Phyphytase	0.6	0.6	0.6	0.6	0.6	0.6
Layer premix	2.5	2.5	2.5	2.5	2.5	2.5

Table 3.3 Calculated nutrient values (%) of the basal diets for Phase 1 and Phase 2

Nutrient	Phase 1	Phase 2
Crude protein	18.70	17.95
ME Layer	11.43	11.37
DM	89.10	89.16
Fibre	4.79	4.75
Fat	3.28	3.28
Ash	11.66	12.38
Calcium	4.10	4.40
Phosphorous(total)	0.6	0.58
Available Phosphorous	0.42	0.4
Sodium	0.18	0.17
Chlorine	0.18	0.17
Lysine (total)	0.88	0.84
Digestible lysine	0.72	0.69
Methionine (total)	0.44	0.42
Digestible methionine	0.36	0.35
Methionine/cystine (total)	0.8	0.77
Digestible Methionine/cystine	0.66	0.63
Arginine (total)	0.91	0.88
Digestible arginine	0.75	0.72
Valine (total)	0.74	0.71
Digestible valine	0.63	0.60
Tryptophan (total)	0.18	0.18
Digestible Tryptophan	0.15	0.14
Threonine (total)	0.61	0.59
Digestible threonine	0.50	0.48
Isoleucine (total)	0.70	0.67
Digestible isoleucine	0.57	0.55
Linoleic acid	2.0	1.6

Table 3.4 Added mineral inclusion concentrations (mg/kg) per treatment

Treatment source	Zn	Cu	Mn
1 Inorganic	60	15	80
2 Organic	60	15	80
3 Inorganic	32	8	32
4 Organic	32	8	32
5 Inorganic	50	8	50
6 Organic	50	8	50

3.4 Measurement of production parameters

Feed intakes were measured on a weekly basis during the trial period. Feed conversion and total hen-housed egg production percentage were calculated at the end of the experimental period. To measure feed intake, feed per replication was added to a sealable container at the start of the week, and the weight was recorded. Birds in a replicate only received feed from their allocated container. Total feed intake was calculated by deducting the orts at the end of the week from the total feed in the container at the start of the week. The total eggs per hen housed was calculated by dividing the total number of eggs laid over the experimental period, by the number of the hens placed at the onset of the trial for each replication. Feed conversion ratio was calculated for each replicate by dividing the total feed consumed by the number of total dozen eggs produced over the duration of the trial. Eggs were collected on paper trays. Mortalities were recorded as it happened. A post-mortem examination was performed to ascertain cause of death.

3.5 Measurement of internal and external egg quality

Egg quality was measured at 45, 70, and 75 weeks-of-age. Egg shell quality was measured by quantifying egg weight, egg shell weight, and breaking strength. Internal egg quality measurements included the Haugh units of albumin and the mineral concentration of the egg yolk. Four eggs per replicate were randomly collected and all measurements were taken one day after collection.

Two eggs per replicate were weighed and the weight recorded. These eggs were then broken onto a flat clean plate, and the albumin height relative to egg weight (Haugh unit) was measured using an Ames Waltham, Mass USA Haugh unit meter. The egg yolks were then removed and freeze-dried for analysis of Zn, Mn, and Cu concentrations. The dried and

ground egg yolks were first digested in a mars microwave accelerated reaction system. After this process, Cu, and Mn were measured by Atom Absorption using the Varian SpektrAA instrument (used according to the manufacturer's instructions). The Zn concentration was measured using the GBS 905 atom absorption instrument.

Egg shell integrity was measured by using an Instron apparatus to test the breaking strength of egg shell. The remaining two eggs of the four eggs that were randomly selected at 45 and 70 weeks-of-age were tested one day after the eggs were collected using the Instron apparatus at the Civil Engineering laboratory University of Pretoria. Egg breaking strength was not tested at 75 weeks-of-age. Each egg was tested individually, and an average value for breaking strength was obtained for each replicate. The egg was placed in a steel cup that negated any movement of the egg, and placed with its sharp side pointing downwards. The machine was set to move at a speed of 2 mm/minute to apply pressure on the egg surface. The probe measured the amount of pressure in Newtons, that could be applied to the egg shell before it would crack. The point at which the egg cracked was seen as the maximum amount of pressure required to crack the egg's shell.

Daily production of reject eggs was also recorded as indication of egg shell quality. Egg rejects were subdivided into three sub-categories: cracked eggs, soft-shell, and misshapen eggs. Cracked eggs included all eggs withcracked shells that were not suitable for packaging or human consumption. Eggs with a not properly calcified shell and were pliable to the touch were classified as soft-shell eggs. The total average reject percentage was calculated per replication, by dividing the total number of reject eggs per category by the total number of eggs produced over the experimental period for that particular replicate.

3.7 Statistical Analysis

Data was analysed statistically as a randomised block design with the GLM model (Statistical Analysis Systems, 2017) for the average effects over time. Repeated Measures Analysis of Variance with the GLM model was used for repeated week or period measures. Means and standard error were calculated and significance of difference ($P<0.05$) between means was determined by Fischer's test (Samuels 1989).

The linear model used is described by the following equation:

$Y_{ijk} = \mu + L_i + T_j + LT_{ij} + B_k + e_{ijk}$

Where Y_{ijk} = variable studied during the period

μ = overall mean of the population

L_i = effect of the ith level

T_j = effect of the jth treatment

LT_{ij} = effect of the ij[th] interaction between level and treatment

B_k = effect of the kth block in house

e_{ijk} = error associated with each

CHAPTER 4

RESULTS

4.1 Production parameters

The results of the production parameters measured are summarised in Tables 4.1-4.4. Although neither mineral source nor mineral inclusion level had any effect on egg production, a significant improvement in feed intake and feed conversion ratio was seen in hens that received the organic mineral source. Mortality rate was not influenced by either the level or the source of minerals.

4.1.1 Hen-housed egg production

Mineral inclusion Level 2 (Zn 50: Cu 8: Mn 50) produced the most eggs per hen housed, irrespective of the mineral source. Inclusion Level 1 (Zn 32: Cu 8: Mn 32) produced the lowest total number of eggs, irrespective of the mineral source. The highest mineral inclusion level, inclusion Level 3 (Zn 60: Cu 15: Mn 80), did not produce more eggs than inclusion Level 1 (Zn 32: Cu 8: Mn 32) or inclusion Level 2 (Zn 50: Cu 8: Mn 50), despite containing a higher concentration of Zn, Cu, and Mn. A significant difference was observed between the three organic mineral treatments groups. The inclusion of organic minerals at Level 2 (Zn 50: Cu 8: Mn 50) in the feed resulted in the highest number of eggs, although this was not statistically different in comparison to the total eggs produced by hens that received feed that contained inclusion Level 3 (Zn 60: Cu 15: Mn 80). Hens that were fed organic mineral inclusion Level 1 (Zn 32: Cu 8: Mn 32) produced the lowest number of eggs across all the treatments. No differences in total eggs produced was recorded between the different inclusion levels derived from inorganic mineral sources.

Table 0.1 **The effect of mineral source and mineral inclusion rate on hen-housed production measured as total eggs per hen-housed at 76 weeks-of-age (± standard error of the mean)**

Mineral inclusion level	Source		
	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	357.38[b] (± 3.63)	364.1 (± 3.63)	360.74[b] (± 2.5)
Level 2 (Zn 50: Cu 8: Mn 50)	368.11[a] (± 3.63)	368.03 (± 3.63)	368.07[a] (± 2.5)
Level 3 (Zn 60: Cu 15: Mn 80)	366.10[ab] (± 3.63)	360.06 (± 3.63)	363.08[ab] (± 2.5)
Mean	363.86 (± 2.1)	364.06 (± 2.1)	

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

4.1.2 Feed intake

The total feed intake of hens (Table 4.2) that were fed the organic mineral source was significantly lower in comparison to the feed intake of hens that were fed the inorganic mineral source. At inclusion Level 3 (Zn 60: Cu 15: Mn 80), feed intake of hens that received feed from an organic mineral source was significantly lower in comparison to hens that received feed that contained the inorganic mineral source. Increasing the inclusion rates of the organic minerals resulted in a lower feed intake. Irrespective of source, inclusion of minerals at the highest level reduced feed intake significantly when compared to Level 2.

Table 0.2   **The feed intake (kg) per hen measured in kg per hen from point-of-lay to 76 weeks of age (± standard error of the mean)**

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	59.79[b] (± 1.19)	62.96 (± 1.19)	61.37[ab] (± 0.84)
Level 2 (Zn 50: Cu 8: Mn 50)	63.32[a] (± 1.19)	63.10 (± 1.19)	63.21[a] (± 0.84)
Level 3 (Zn 60: Cu 15: Mn 80)	55.80[cA] (± 1.19)	64.17[B] (± 1.19)	59.98[b] (± 0.84)
Mean	59.63[B] (± 0.68)	63.40[A] (± 0.68)	

[a-c] Means within the same column with no common superscript differ significantly ($P<0.05$)

[A,B] Means within the same row with no common superscript differ significantly ($P<0.05$)

4.1.3 Feed conversion ratio

A significant difference in feed conversion ratio was observed between the two mineral sources. The hens that received the organic mineral source had a better feed conversion of 1.97 kg feed per dozen eggs produced, in comparison to the hens fed the inorganic mineral source, which in turn had a feed conversion ratio of 2.09 kg feed per dozen eggs produced. The different mineral inclusion levels had no significant effect on feed conversion ratio.

Table 0.3   **The effect of mineral source and mineral inclusion level on feed conversion ratio[1] from point-of-lay to 76 weeks-of-age (± standard error of the mean)**

Mineral inclusion level	Source		
	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	2.00 (± 0.04)	2.08 (± 0.04)	2.04 (± 0.3)
Level 2 (Zn 50: Cu 8: Mn 50)	2.06 (± 0.04)	2.06 (± 0.04)	2.06 (± 0.3)
Level 3 (Zn 60: Cu 15: Mn 80)	1.83 (± 0.04)	2.14 (± 0.04)	1.98 (± 0.3)
Mean	1.97[B] (± 0.02)	2.09[A] (± 0.02)	

[a-c] Means within the same column with no common superscript differ significantly (P<0.05)

[1]   Feed conversion ratio was calculated as total feed consumed divided by the dozen of eggs produced

4.1.4 Egg weight

Neither the mineral source nor the mineral inclusion level produced any statistically significant differences for egg weight at 45 weeks-of-age for egg weight (Table 4.5).

Table 0.4   **The effect mineral source and inclusion level on egg weight (g) at 45 weeks-of-age (± standard error of the mean)**

Mineral inclusion level	Source		
	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	58.69 (± 0.94)	58.14 (± 0.94)	58.41 (± 0.66)
Level 2 (Zn 50: Cu 8: Mn 50)	59.27 (± 0.94)	60.18 (± 0.94)	59.72 (± 0.66)
Level 3 (Zn 60: Cu 15: Mn 80)	58.97 (± 0.94)	60.63 (± 0.94)	59.8 (± 0.66)
Mean	58.97 (± 0.54)	59.65 (± 0.54)	

Egg weight did not differ significantly between mineral inclusion level or the mineral source at 70 weeks-of-age (Table 4.6).

Table 0.5   **The effect mineral sources and inclusion level on egg weight (g) at 70 weeks-of-age (± standard error of the mean)**

Mineral inclusion level	Source		
	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	60.90 (± 1.27)	61.69 (± 1.27)	61.3 (± 0.9)
Level 2 (Zn 50: Cu 8: Mn 50)	61.30 (± 1.27)	64.26 (± 1.27)	62.79 (± 0.9)
Level 3 (Zn 60: Cu 15: Mn 80)	61.54 (± 1.27)	62.57 (± 1.27)	62.06 (± 0.9)
Mean	61.25 (± 0.73)	62.84 (± 0.73)	

At 75 weeks-of-age, eggs produced by hens that were fed higher mineral inclusion levels, were significantly heavier than eggs produced by hens that received the lowest level of minerals (Zn 32: Cu 8: Mn 32), irrespective of the mineral source (Table 4.7).

Table 0.6   The effect mineral source and inclusion level on egg weight (g) at 75 weeks-of-age (± standard error of the mean)

	Source		
Mineral inclusion level	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	64.02 (± 0.86)	62.74 (± 0.86)	63.38[b] (± 0.86)
Level 2 (Zn 50: Cu 8: Mn 50)	63.95 (± 0.86)	63.12 (± 0.86)	63.53[ab] (± 0.86)
Level 3 (Zn 60: Cu 15: Mn 80)	67.36 (± 0.86)	64.32 (± 0.86)	65.84[a] (± 0.86)
Mean	62.84 (± 0.7)	63.39 (± 0.7)	

[a-b] Means within the same column with no common superscript differ significantly ($P<0.05$)

4.1.5 Total mortality

As shown in Table 4.4 neither mineral source nor the mineral inclusion rate had any statistically significant effect on total hen mortality.

Table 0.7 The total mortality rate expressed as a percentage of total hens placed (± standard error of the mean)

	Source		
Mineral inclusion level	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	5.00 (± 1.29)	3.89 (± 1.29)	4.44 (± 0.91)
Level 2 (Zn 50: Cu 8: Mn 50)	2.22 (± 1.29)	1.67 (±1.29)	1.94 (± 0.91)
Level 3 (Zn 60: Cu 15: Mn 80)	3.33 (± 1.29)	3.33 (±1.29)	3.33 (± 0.91)
Mean	3.51 (± 0.75)	2.96 (± 0.75)	

4.2 Egg shell quality

Egg quality was measured at three points during the hens' production cycle i.e., 45, 70, and 75 weeks-of-age. The measurements taken to quantify egg shell strength egg shell weight (Tables 4.8 - 4.10), reject percentage (Tables 4.11 - 4.13). Egg shell breaking strength was only measured at 45 and 70 weeks-of-age (Tables 4.14 - 4.15).

4.2.1 Egg shell weight

At 45 weeks-of-age, the inorganic mineral source at inclusion level 2 (Zn 50: Cu 8: Mn 50) resulted in a higher egg shell weight in comparison to the organic source at the same level. Within the organic treatment group, Level 1 (Zn 32: Cu 8: Mn 32) produced significantly higher egg shell weight compared to treatment Levels 2 (Zn 50: Cu 8: Mn 50). In the inorganic treatment group, inclusion Level 1 (Zn 32: Cu 8: Mn 32) produced a significantly lower egg shell weight than did Level 2 (Zn 50: Cu 8: Mn 50).

Table 0.8   The effects of different mineral sources and inclusion level on egg shell weight (g) at 45 weeks-of-age (± standard error of the mean)

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	7.71[a] (± 0.14)	7.46[b] (± 0.14)	7.59 (± 0.1)
Level 2 (Zn 50: Cu 8: Mn 50)	7.47[bB] (± 0.14)	7.93[aA] (± 0.14)	7.71 (± 0.1)
Level 3 (Zn 60: Cu 15: Mn 80)	7.58[ab] (± 0.14)	7.74[ab] (± 0.14)	7.66 (± 0.1)
Mean	7.59 (± 0.08)	7.72 (± 0.08)	

[a, b] Means within the same column with no common superscript differ significantly (P< 0.05)

[A, B] Means within the same row with no common superscript differ significantly (P<0.05)

As shown in Table 4.9, hens that received the higher mineral inclusion levels from the organic mineral source, produced significant heavier egg shells than hens that received the lowest mineral inclusion, Level 1 (Zn 32: Cu 8: Mn 32). At the lowest inclusion level (Zn 32: Cu 8: Mn 32), the inorganic mineral source produced egg shells that were significantly heavier than the eggs from hens that received the organic mineral source at the same inclusion level. There were no significant differences between the mean values for mineral inclusion level or mineral source.

Table 0.9   The effects of different mineral sources and inclusion level on egg shell weight (g) at 70 weeks-of-age (± standard error of the mean)

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	7.91[bB] (± 0.18)	8.53[A] (± 0.18)	8.23 (± 0.13)
Level 2 (Zn 50: Cu 8: Mn 50)	8.17[ab] (± 0.18)	8.26 (± 0.18)	8.22 (± 0.13)
Level 3 (Zn 60: Cu 15: Mn 80)	8.33[a] (± 0.18)	8.75 (± 0.18)	8.55 (± 0.13)
Mean	8.14 (± 0.1)	8.52 (± 0.1)	

[a ,b] Means within the same column with no common superscript differ significantly (P<0.05)

[A,B] Means within the same row with no common superscript differ significantly (P<0.05)

Significant differences were recorded for egg shell weight between the different inclusion levels at 75 weeks-of-age. Irrespective of the mineral source, inclusion Level 3 (Zn 60: Cu 8: Mn 80) resulted in the highest egg shell weight and differed significantly from Level 1 (Zn 32: Cu 8: Mn 32) and Level 2 (Zn 50: Cu 8: Mn 50). A higher egg shell weight was noted for the inorganic mineral source when included at Level 1 (Zn 32: Cu 8: Mn 32) as compared to the organic source at the same inclusion level. Inclusion Level 3 (Zn 60: Cu 8: Mn 80) resulted the highest egg shell weight in the organic mineral source treatment. Within the inorganic treatment group, inclusion Level 3 (Zn 60: Cu 8: Mn 80) produced the highest egg shell weight, which was significantly higher than Level 1(Zn 32: Cu 8: Mn 32).

Table 0.10   The effects of different mineral sources and inclusion level on egg shell weight (g) at 75 weeks-of-age (± standard error of the mean)

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	8.06^{cB} (± 0.18)	8.37^{aA} (± 0.18)	8.22^{b} (± 0.13)
Level 2 (Zn 50: Cu 8: Mn 50)	8.31^{bc} (± 0.18)	7.95^{b} (± 0.18)	8.14^{b} (± 0.13)
Level 3 (Zn 60: Cu 8: Mn 80)	9.12^{a} (± 0.18)	8.73^{a} (± 0. 18)	8.93^{a} (± 0.13)
Mean	8.50 (± 0.1)	8.36 (± 0.1)	

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

[A,B] Means within the same row with no common superscript differ significantly (P< 0.05)

4.2.2 Reject eggs

Neither mineral inclusion level nor the source of mineral showed any significant effect on the number of cracked eggs produced over the production period of the hens (Table 4.11).

Table 0.11   The effects of different mineral sources and inclusion level on the total amount of cracked eggs measured as a percentage of total eggs produced during the production period of the flock (± standard error of the mean)

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	4.12 (± 1.19)	6.14 (± 1.19)	5.12 (± 0.84)
Level 2 (Zn 50: Cu 8: Mn 50)	4.64 (± 1.19)	7.1 (± 1.19)	5.87 (± 0.84)
Level 3 (Zn 60: Cu 15: Mn 80)	5.31 (± 1.19)	4.26 (± 1.19)	4.78 (± 0.84)
Mean	4.69 (± 0.7)	5.83 (± 0.7)	

The inorganic source produced significantly more misshapen eggs than the hens that received the organic mineral source at inclusion Level 3 (Zn 60: Cu 15: Mn 80). No significant differences were recorded between the different inclusion levels in the organic treatment group. The highest inclusion level of inorganic minerals produced significantly more misshapen eggs than the lower levels.

Table 0.12 **The effects of different mineral sources and inclusion level on the total amount of misshapen eggs produced measured as a percentage of total eggs during the life time of the flock (± standard error of the mean)**

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	0.28 (± 0.13)	0.18[b] (± 0.13)	0.23 (± 0.09)
Level 2 (Zn 50: Cu 8: Mn 50)	0.42 (± 0.13)	0.38[b] (± 0.13)	0.40 (± 0.09)
Level 3 (Zn 60: Cu 15: Mn 80)	0.33[B] (± 0.13)	0.61[aA] (± 0.13)	0.40 (± 0.09)
Mean	0.34 (± 0.08)	0.39 (± 0.08)	

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

[A,B] Means within the same row with no common superscript differ significantly (P< 0.05)

The inorganic mineral inclusion Level 2 (Zn 50: Cu 8: Mn 50) resulted in the highest number of soft-shelled eggs and differed significantly from the same inclusion level from the organic mineral source. Amongst hens that received minerals from an inorganic mineral source, inclusion Level 2 (Zn 50: Cu 8: Mn 50) recorded the highest number of soft-shelled eggs.

Table 0.13 **The effects of different mineral sources and inclusion level on the total amount of soft-shell eggs produced measured as a percentage of total eggs produced during the life time of the flock (± standard error of the mean)**

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	3.54 (± 0.7)	2.85[b] (± 0.7)	3.19 (± 0.5)
Level 2 (Zn 50: Cu 8: Mn 50)	2.43[B] (± 0.7)	4.17[aA] (± 0.7)	3.30 (± 0.5)
Level 3 (Zn 60: Cu 15: Mn 80)	3.21 (± 0.7)	2.22[b] (± 0.7)	2.71 (± 0.5)
Mean	3.06 (± 0.4)	3.08 (± 0.4)	

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

[A,B] Means within the same row with no common superscript differ significantly (P< 0.05)

4.2.3 Breaking strength

Within the inorganic mineral treatment group, a significantly higher breaking strength was recorded at inclusion Level 1 (Zn 32: Cu 8: Mn 32) compared to inclusion Level 2 (Zn 50: Cu 8: Mn 50). Eggs from hens that received diets supplemented with organic Zn, Cu, and Mn at 50, 8, and 50 mg per kilogramme, respectively, had a significant higher breaking strength when compared to those from the inorganic group at the same inclusion level.

Table 0.14   The effect of different mineral sources and inclusion level on egg breaking strength at 45 weeks-of-age measured in Newton (± standard error of the mean)

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	49.25 (± 2)	48.46[a] (± 2)	48.86 (± 1.4)
Level 2 (Zn 50: Cu 8: Mn 50)	48.98[A] (± 2)	47.27[bB] (± 2)	45.85 (± 1.4)
Level 3 (Zn 60: Cu 15: Mn 80)	47.19 (± 2)	47.89[ab] (± 2)	47.54 (± 1.4)
Mean	48.47 (± 1.15)	46.36 (± 1.15)	

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

[A,B] Means within the same row with no common superscript differ significantly (P<0.05)

As shown in Table 4.15, neither mineral inclusion level, nor the source of supplemented mineral, had any significant effect on the breaking strength at 70 weeks-of-age.

Table 0.15   The effect of different mineral sources and inclusion level on egg breaking strength at 70 weeks-of-age measured in Newton (± standard error of the mean)

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	48.38 (± 2.35)	45.83 (± 2.35)	47.11 (± 1.7)
Level 2 (Zn 50: Cu 8: Mn 50)	49.06 (± 2.35)	46.10 (± 2.35)	47.58 (± 1.7)
Level 3 (Zn 60: Cu 15: Mn 80)	45.52 (± 2.35)	46.33 (± 2.35)	45.93 (± 1.7)
Mean	47.66 (± 1.4)	46.09 (± 1.4)	

4.3 Internal egg quality

4.3.1 Haugh units of eggs

Neither mineral inclusion level nor the source of mineral showed any significant effect on the Haugh units at 45 weeks-of-age (Table 4.16).

Table 0.16 The effect of different mineral sources and inclusion level on albumin quality measured Haugh units at 45 weeks-of-age (± standard error of the mean)

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	82.44 (± 2.18)	79.47 (± 2.18)	80.96 (± 1.54)
Level 2 (Zn 50: Cu 8: Mn 50)	81.41 (± 2.18)	80.42 (± 2.18)	80.91 (± 1.54)
Level 3 (Zn 60: Cu 15: Mn 80)	79.38 (± 2.18)	81.04 (± 2.18)	80.21 (± 1.54)
Mean	81.08 (± 1.26)	80.31 (± 1.26)	

Mineral inclusion level and mineral source had no effect on Haugh unit measurements at 70 weeks-of-age, as shown in Table 4.17.

Table 0.17: **The effect of different mineral sources and inclusion level on albumin quality measured Haugh units at 70 weeks-of-age (± standard error of the mean)**

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Means**
Level 1 (Zn 32: Cu 8: Mn 32)	90.94 (± 1.36)	92.78 (± 1.36)	91.86 (± 0.96)
Level 2 (Zn 50: Cu 8: Mn 50)	87.78 (± 1.36)	91.00 (± 1.36)	89.39 (± 0.96)
Level 3 (Zn 60: Cu 15: Mn 80)	91.25 (± 1.36)	91.81 (± 1.36)	91.53 (± 0.96)
Means	89.99 (± 0.78)	91.86 (± 0.78)	

The Haugh units measured at 75 weeks-of-age indicated a significantly higher Haugh unit for inclusion Level 1(Zn 32: Cu 8: Mn 32) and inclusion Level 3 (Zn 60: Cu 15: Mn 80), this was irrespective om the mineral source fed.

Table 0.18: **The effect of different mineral sources and inclusion level on albumin quality measured Haugh units at 75 weeks-of-age.**

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	94.38 (± 1.42)	95.83 (± 1.42)	95.11[ab] (± 1.00)
Level 2 (Zn 50: Cu 8: Mn 50)	94.72 (± 1.42)	93.00 (± 1.42)	93.86[b] (± 1.00)
Level 3 (Zn 60: Cu 15: Mn 80)	97.77 (± 1.42)	96.50 (± 1.42)	97.13[a] (± 1.00)
Mean	95.63 (± 0.82)	95.11 (± 0.82)	

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

4.3.2 Mineral concentration in egg yolks

Egg yolks were analysed for mineral concentration at three different ages during the hens' production period. Tables 4.19 – 4.26 summarise the concentrations of Cu, Zn, and Mn in the egg yolk at 45, 70, and 75 weeks-of-age.

Neither mineral inclusion level nor the source of mineral showed any significant effect on the Cu concentration at 45 weeks of age (Table 4.19).

Table 0.19   **The effect of different mineral sources and levels on Cu levels in egg yolk (ppm)at 45 weeks-of-age (± standard error of the mean)**

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	1.60 (± 1.6)	1.59 (± 1.6)	1.59 (± 0.03)
Level 2 (Zn 50: Cu 8: Mn 50)	1.56 (± 1.6)	1.57 (± 1.6)	1.56 (± 0.03)
Level 3 (Zn 60: Cu 15: Mn 80)	1.61 (± 1.6)	1.58 (± 1.6)	1.59 (± 0.03)
Mean	1.60 (± 0.03)	1.59 (± 0.03)	

Significant differences between mineral concentrations in the yolk were observed at 70 weeks-of-age (Table 4.20). Supplementing the hens' diet with organic minerals resulted in a higher concentration of Cu in the egg yolk when compared to the inorganic mineral source. Eggs from hens that received inclusion Level 1 (Zn 32: Cu 8: Mn 32) and Level 2 (Zn 50: Cu 8: Mn 50) in their feed had significantly higher levels of Cu when compared to Level 3 (Zn 60: Cu 15: Mn 80). Organic mineral source treatment Level 2 (Zn 50: Cu 8: Mn 50) produced a higher concentration of Cu in the yolk when compared to the two other organic mineral treatments. Among the inorganic treatments, Level 1 (Zn 32: Cu 8: Mn 32) and Level 2 (Zn 50: Cu 8: Mn 50) produced a higher Cu concentration in the egg yolk than mineral inclusion Level 3 (Zn 60: Cu 15: Mn 80). When organic minerals were added to the feed at both Level 2 (Zn 50: Cu 8: Mn 50) and Level 3 (Zn 60: Cu 15: Mn 80) the Cu concentration in the yolk was significantly higher compared to the inorganic sources at the same inclusion levels.

Table 0.20:   **The effect of different mineral sources and levels on Cu levels in egg yolk (ppm) at 70 weeks-of-age (± standard error of the mean)**

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	1.46[b] (± 0.04)	1.42[a] (± 0.04)	1.46[a] (± 0.03)

Level 2 (Zn 50: Cu 8: Mn 50)	1.62[aA] (± 0.04)	1.43[aB] (± 0.04)	1.37[a](± 0.03)
Level 3 (Zn 60: Cu 15: Mn 80)	1.33[cA] (± 0.04)	1.06[bB] (± 0.04)	1.59[b](± 0.03)
Mean	1.47[A](± 0.03)	1.30[B](± 0.03)	

[a-c] Means within the same column with no common superscript differ significantly (P<0.05)

[A,B] Means within the same row with no common superscript differ significantly (P<0.05)

Addition of organic minerals to the layer diets resulted in significantly higher concentrations of Cu in the egg yolk at 75 weeks-of-age (Table 4.21). Inclusion of minerals at Level 3 (Zn 60: Cu 15: Mn 80) resulted in the highest concentration of Cu in the yolk, irrespective of source. This differed significantly from treatment Level 2 (Zn 50: Cu 8: Mn 50), but not from treatment Level 1 (Zn 32: Cu 8: Mn 32). Within the inorganic treatment groups, the highest Cu yolk concentration was recorded at inclusion Level 3 (Zn 60: Cu 15: Mn 80), whereas amongst the organic treatment groups, the highest egg yolk concentration for Cu was recorded at inclusion Level 1 (Zn 32: Cu 8: Mn 32) and inclusion Level 3 (Zn 60: Cu 15: Mn 80).

Table 0.21 **The effect of different mineral sources and levels on Cu levels in egg yolk (ppm) at 75 weeks-of-age (± standard error of the mean)**

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	1.61[aA] (± 0.05)	1.30[bB] (± 0.05)	1.46[a] (± 0.03)
Level 2 (Zn 50: Cu 8: Mn 50)	1.39[b] (± 0.05)	1.35[b] (± 0.05)	1.37[b] (± 0.03)
Level 3 (Zn 60: Cu 15: Mn 80)	1.60[a] (± 0.05)	1.59[a] (± 0.05)	1.59[a] (± 0.03)
Mean	1.53[a] (± 0.03)	1.41[b] (± 0.03)	

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

[A,B] Means within the same row with no common superscript differ significantly (P<0.05)

At 45 weeks-of-age, the mean Zn level of treatment Level 3 was significantly higher than that of treatment Level 2 (Zn 50: Cu 8: Mn 50) as shown in Table 4.22. Hens that received feed containing organic mineral treatments inclusion Level 1 (Zn 32: Cu 8: Mn 32) and Level 3 (Zn 60: Cu 8: Mn 80) produced higher concentration of Zn in the yolk than that of treatment Level 2 (Zn 50: Cu 8: Mn 50). Inclusion Level 1 and 3 recorded significantly higher Zn concentrations in the egg yolk than inclusion Level 2 (Zn 50: Cu 8: Mn 50).

Table 0.22 **The effect of different mineral sources and levels on Zn levels in egg yolk (ppm) at 45 weeks of age (± standard error of the mean)**

	Source		

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Mineral inclusion level	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	36.65[a] (± 0.56)	36.23[ab] (±0.56)	36.44[ab] (± 0.39)
Level 2 (Zn 50: Cu 8: Mn 50)	35.55[b] (± 0.56)	35.98[b] (± 0.56)	35.77[b] (± 0.39)
Level 3 (Zn 60: Cu 15: Mn 80)	37.69[a] (± 0.56)	37.12[a] (± 0.56)	37.41[a] (± 0.39)
Mean	36.63 (± 0.32)	36.45 (± 0.32)	

^{a,b} Means within the same column with no common superscript differ significantly (P<0.05)

^{A,B} Means within the same row with no common superscript differ significantly (P<0.05)

Neither mineral inclusion level nor the source of mineral showed any significant effect on the yolk Zn concentration at 70 weeks of age (Table 4.23).

Table 0.23   The effect of different mineral sources and levels on Zn levels in egg yolk (ppm) at 70 weeks of age (± standard error of the mean)

	Source		
Mineral inclusion level	**Organic**	**Inorganic**	**Mean**
Level 1 (Zn 32: Cu 8: Mn 32)	35.54 (± 0.49)	35.83 (± 0.49)	35.69 (± 0.34)
Level 2 (Zn 50: Cu 8: Mn 50)	34.91 (± 0.49)	34.91 (± 0.49)	34.91 (± 0.34)
Level 3 (Zn 60: Cu 15: Mn 80)	35.81 (± 0.49)	35.90 (± 0.49)	35.86 (± 0.34)
Mean	35.42 (± 0.28)	35.55 (± 0.28)	

As shown in Table 4.24, the highest recorded mean Zn value was at inclusion Level 1 (Zn 32: Cu 8: Mn 32). This value differed significantly from the mean values of inclusion Level 2 (Zn 50: Cu 8: Mn 50) and inclusion Level 3 (Zn 60: Cu 15: Mn 80). Within the organic mineral treatment group, hens that were fed inclusion Level 1 (Zn 32: Cu 8: Mn 32) had the highest Zn egg yolk concentration, and differed significantly from the concentration of Zn in the egg yolk from hens fed the organic mineral inclusion Level 2 (Zn 50: Cu 8: Mn 50) and Level 3 (Zn 60: Cu 15: Mn 80). The organic mineral source recorded the highest Zn yolk concentration at inclusion Level 1 (Zn 32: Cu 8: Mn 32). The opposite was seen at

inclusion Level 2 (Zn 50: Cu 8: Mn 50), where the inorganic mineral source recorded the highest Zn yolk concentration.

Table 0.24 **The effect of different mineral sources and inclusion levels on Zn levels in egg yolk (ppm) at 75 weeks of age (± standard error of the mean)**

	Source		
Mineral inclusion level	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	36.51[aA] (± 0.52)	34.21[B] (± 0.52)	35.36[a] (± 0.37)
Level 2 (Zn 50: Cu 8: Mn 50)	32.04[bB] (± 0.52)	34.72[A] (± 0.52)	33.38[b] (± 0.37)
Level 3 (Zn 60: Cu 15: Mn 80)	33.29[b] (± 0.52)	34.33 (± 0.52)	33.81[b] (± 0.37)
Mean	33.95 (± 0.3)	34.42 (± 0.3)	

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

[A,B] Means within the same row with no common superscript differ significantly (P<0.05)

As shown in Table 4.25, organic mineral inclusion Level 3 (Zn 60: Cu 15: Mn 80) produced the highest Mn concentration in the yolk, differing significantly from inclusion Level 1 (Zn 32: Cu 8: Mn 32), but not from inclusion Level 2 (Zn 50: Cu 8: Mn 50).

Table 0.25 **The effect of different mineral sources and inclusion levels on the Mn level in egg yolk (ppm) at 45 weeks-of-age (± standard error of the mean)**

	Source		
Mineral inclusion level	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	1.42[b] (± 0.06)	1.45 (± 0.06)	1.43 (± 0.04)
Level 2 (Zn 50: Cu 8: Mn 50)	1.43[ab] (± 0.06)	1.44 (± 0.06)	1.44 (± 0.04)
Level 3 (Zn 60: Cu 15: Mn 80)	1.59[a] (± 0.06)	1.47 (± 0.06)	1.53 (± 0.04)
Mean	1.48 (± 0.04)	1.45 (± 0.04)	

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

Feed that contained mineral inclusion Level 2 (Zn 50: Cu 8: Mn 50) produced the lowest concentration of Mn in the egg yolk at 70 weeks-of-age but did not differ significantly from Mn concentration recorded from hens that were fed mineral inclusion Level 1 (Zn 32: Cu 8: Mn 32).

Table 0.26 **The effect of different mineral sources and inclusion levels on the Mn level in egg yolk (ppm) at 70 weeks-of-age (± standard error of the mean)**

	Source		
Mineral inclusion level	Organic	Inorganic	Mean
Level 1 (Zn 32: Cu 8: Mn 32)	1.41[ab] (± 0.05)	1.43[ab] (± 0.05)	1.42 (± 0.04)
Level 2 (Zn 50: Cu 8: Mn 50)	1.31[b] (± 0.05)	1.33[b] (± 0.05)	1.32 (± 0.04)
Level 3 (Zn 60: Cu 15: Mn 80)	1.52[a] (± 0.05)	1.54[a] (± 0.05)	1.53 (± 0.04)

Mean	1.41 (± 0.03)	1.43 (± 0.03)

[a,b] Means within the same column with no common superscript differ significantly (P<0.05)

CHAPTER 5

DISCUSSION

5.1 Production performance

In this trial, hens that were supplemented with an organic mineral source had a significantly lower feed intake (FI) and feed conversion ratio (FCR) over the full trial period, in comparison to hens that were fed the inorganic mineral source. However, there was no difference in egg production between these two main groups. These findings are similar to those found in study by El-Hack et al. (2017), who observed that whilst feed conversion ratio did not change in the group of birds that were fed organic minerals, the feed intake was lower. However, in their study, the egg production was significantly higher in the group of birds that received the organic mineral source. The findings of this trial are also consistent with work done by Gheisari et al. (2011) who reported a significant improvement in feed conversion ratio and feed intake ($P<0.001$) in a study conducted on 180 Hy-Line layer hens for 15 weeks from 38 to 52 weeks-of-age. In contrast to these observations, Stefenallo et al. (2014) did not observe any improvement in the feed conversion ratio, feed intake, or egg production, in the treatment groups that received organic sources of Zn, Cu, and Mn. Swiatekiewics et al. (2008) also found no significant improvement in egg production when including organic sources of Zn, Cu and Mn in the diets of commercial hens. There are several other studies that also support the finding that organic minerals do not have a significant effect on egg production, feed intake, and feed conversion (Fernandes et al., 2008; Machiel et al., 2010).

A study conducted by Stefanello et al. (2014) showed that including Zn, Cu, and Mn trace minerals into the laying hens' diet, could increase the egg weight, irrespective of the mineral source. In the present study, an increase in the egg weight was only seen 75 weeks-of-age, and correlated with an increase in the inclusion level of Zn, Cu, and Mn in the feed, however the increase in egg weight showed no relation to the mineral source. These results are similar to the findings of Abedini et al. (2017), who found that egg weight increased in older hens that were fed three different sources of zinc (Zn-oxide nanoparticles, Zn-methionine and Zn oxide). This may indicate that birds may require an increased dietary mineral level in the feed towards the latter part of the production cycle, in order to support the increasing egg weight over time, as hens aged.

Differences in mineral inclusion level and mineral sources did not produce a significant difference in mortality. However, studies done by Stanley et al. (2012) showed that mortalities were significantly lower in a group of hens that received feed that contained an organic Zn source, and Manangi et al. (2012) observed an increased immune response of layer hens that were fed chelated trace minerals. Further to this, in a study done by Virden et al. (2004) on broiler breeder hens, an increase in the liveability of the progeny from hens that were fed organic sources of Zn and Mn was noted.

5.2 Egg shell quality

Egg shell weight varied throughout the trial, and no clear trend was observed. It should however be noted that in the present study, only the egg shell weight was measured, whilst in other studies, the percentage egg shell relative to the weight of the total egg was measured (El-Hack et al., 2017). In our study, the egg shell weights were increased in the group of hens that received feed containing the inorganic treatment source at the lowest inclusion levels. Stefenallo et al. (2014), on the other hand, found a significant increase in egg shell thickness from hens that were fed organic minerals at a lower inclusion rate. El-hack et al. (2017) also recorded higher egg shell weight percentages for the eggs of hens that received feed that contained an organic source of Zn, Cu, and Mn.

In this study, reject eggs were subdivided into three categories: cracked, misshapen, and softshell eggs. No differences were recorded for the total number of cracked eggs. Desai et al. (2012) observed instead a decrease in percentage of broken eggs for hens receiving diets that contained chelated sources of Zn, Cu, and Mn. The Lohmann breed recommendation for Mn feed inclusion rate is 100 mg/kg. In the present study, the misshapen eggs decreased at the highest level of inclusion (Level 3) and were significantly lower in the organic mineral source treatment. This could be due to the higher concentration of Mn at this inclusion level and the subsequent increase in the absorption of the organic mineral source. Mn is concentrated in the eggshell and contributes significantly to the eggshell matrix, mechanical properties and structure (Nys et al., 2001). In support of the hypothesis, Leach and Gross (1983) observed that hens fed Mn-deficient diets produced eggs with thinner shells and translucent areas. These abnormalities were due to changes in eggs' ultra-structure, particularly in the mammillary layer this causes a subsequent increase in the production of misshapen eggs.

In this trial, breaking strength was only significantly improved at 45 weeks-of-age, and not at 70 weeks-of-age. This difference was recorded only at inclusion Level 2, and the breaking strength of the organic treatment was higher than the inorganic source. However, this finding could not be repeated at 70 weeks-of-age. The Lohman breed standard (Lohman manual 2013) for breaking strength of eggs is ≥ 40 Newton. Breaking strength of eggs from all the treatments were higher than the breed standard at both 45 and 70 weeks-of-age. Mabe et al. (2003) fed three groups of hens at a diet containing either organic or inorganic sources of Zn, Cu, and Mn and breaking strength of eggs at 32,60 and 69 weeks of age a consistent improvement in breaking strength of eggs in hens that received a feed that had a higher inclusion level of Zn, Mn, and Cu, irrespective of the source of the mineral. Mabe et al. (2003) also found that the improvement in breaking strength was more pronounced in later in the production period. These results coincide with the findings of Xiao et al. (2014), who recorded that the breaking strength and elastic modulus of eggs improved with the inclusion of organic Mn. This may be due to the fact that Mn is primarily concentrated in the bone and egg shell. Research done by Swiatekiewicz et al. (2008), indicated that the inclusion of organic complexes of Mn could alleviate the negative effect of hen age on eggshell breaking strength.

5.3 Egg internal quality

In the present study, no significant differences were recorded for albumin height, as the Haugh units measured at 45, 70, and 75 weeks-of-age did not differ between the organic and inorganic mineral treatments. This finding is similar to that of Abedini et al. (2017), whose results also showed no differences in Haugh unit measurements between an inorganic and an organic methionine source of Zn. Fernandes et al. (2008) and Steffenalo et al. (2014) also reported no differences in Haugh units of eggs from hens fed diets supplemented with organic trace minerals sources. There was, however, one inexplicable finding on the albumin measured at 75 weeks-of-age, where the lowest inclusion rate (Level 1) and the highest-level inclusion rate (Level 3) recorded the highest Haugh units. This could possibly be due to measurement error.

The majority of the minerals are concentrated in the yolk (Uni et al., 2012). In this study, the highest yolk concentration of Zn was found at the highest level of inclusion, irrespective of the mineral source. Inclusion Level 3 (Zn 60: Cu 15: Mn 80) produced the highest Zn

concentration in the yolk, at both 45 and 75 weeks-of-age. In contrast to these findings, Yenice et al. (2015) recorded a significant increase in concentration of Zn, Cu, and Mn in the egg yolk in birds fed diets with higher mineral concentrations, however, similar to the present study, the mineral source did not affect Zn, Cu, or Mn concentration in the egg yolk. It seems, therefore, that the mineral egg yolk concentration is influenced directly by the dietary inclusion level of Zn, Cu, and Mn in the feed. This theory is supported by the studies of Goa et al. (2018), who recorded that maternal organic zinc supplementation improved zinc deposition in the egg yolk. According to Lim and Paik (2003), Zn liver content was increased by including Zn-methionine chelate in the diet of commercial laying hens. Chantiratikul et al. (2008) observed that by increasing the dietary concentration of zinc-L-selenomethionine, the concentration of Se significantly increased ($P<0.05$) in the egg yolk. Although no significant differences were recorded in Cu concentration in the yolk at 45 weeks-of-age, the Cu concentration in the yolk was significantly increased in the organic treatments at 70 and 75 weeks-of-age. Mabe et al. (2003) also found that the inclusion of Zn, Cu and Mn in combination increased the levels of these minerals in the egg yolk. It can possibly be concluded that the bioavailability of organic Cu is superior to that of the inorganic mineral source, and that the difference is more pronounced in older hens. In our study, the concentration of Mn concentration in the yolk showed no differences between the organic and inorganic sources of Mn, however when it came to the concentration of Mn, a dose response was noted for Mn concentration in the yolk, with the highest concentration coinciding with the highest dietary supplementation of minerals.

CHAPTER 6

Conclusion

The aim of this trial was to examine the effects of dietary supplementation of Zn, Cu, and Mn as chelated hydroxy-analogues of methionine at three different inclusion levels in comparison to conventional inorganic salt mineral sources on egg production and quality. Replacing the conventional salt mineral sources with chelated organic mineral sources provides the nutritionist with a cost-effective tool to increase mineral availability and absorption in the hens' diet, whilst optimising egg production and egg quality without adverse effects on the environment.

In this study, a decrease in feed intake and feed conversion ratio was recorded for hens that received feed that contained an organic mineral source. The implication of this finding is significant and has a considerable cost-saving benefit to the egg producer. The inclusion of organic mineral sources in the feed should have resulted in a direct improvement in egg shell quality. However, in this study the results were inconclusive, and organic minerals could not elicit a repeatable positive response on any of the egg shell quality measurements. Feeding organic minerals to the hens showed no changes in the internal quality of the egg, as there were no differences in the Haugh units of the albumin of the eggs sampled. The organic copper source was able to increase the concentration of copper in the egg yolk towards the latter part of the production period, and the Cu concentration was dose responsive as the Cu concentration in the yolk increased as Cu inclusion rate was increased. This, however, was not the case for Zn or Mn, where neither the mineral source nor the different inclusion rates could illicit a significant effect on the concentration of these minerals in the egg yolks.

In conclusion, the results of this trial indicate that the inclusion of an organic mineral source in the diets of commercial laying hens could not illicit a consistent positive response on any of the egg quality parameters that were measured. Contradictory results require further research to be conducted in this regard. Results based on layer performance suggest that feed conversion ratio and feed intake may be improved with the inclusion of organic minerals in the diet. It would seem that certainly in the case of Cu, there is merit in including organic minerals to increase Cu concentration in the egg.

CHAPTER 7

CRITICAL REVIEW

- The trial started at 16 weeks-of-age when the birds were transferred to the facilities at the University experimental farm. Conducting the trial over such a long period (59 weeks) consumed a considerable amount of man hours. The trial could rather have been run over shorter period, with specific focus on the latter part of lay. The effect of organic mineral inclusion in the feed may be greater in latter stages of the hens' life.

- The feed ought to have been tested for Zn, Mn, and Cu mineral concentration. This would have been a good indication of the formulated versus the as-fed concentration of Zn, Cu, and Mn. It would also have indicated the contribution of the raw materials to the actual mineral concentration in the feed.

- The use of a mineral exhaustion phase prior to the onset of the trial can be considered for similar future trials. These diets are formulated at low levels of mineral inclusion. This forces the bird to exhaust its mineral reserves in the body. Once the trial diets are fed a greater difference between the mineral source and concentration fed may be observed.

- Further studies are warranted, using organic mineral sources in order to observe how these minerals can influence the structural organisation of the eggshell. These studies would establish greater insight into the mechanism of action of the trace minerals and their participation in the eggshell ultrastructure, and subsequent resistance to cracking. These studies can be done using an electron microscope.

- Haugh units were measured one day after the date of lay. It would have useful to have established the initial Haugh unit of the fresh egg and then observed the rate of Haugh degradation at 12 days after the date of lay so as to establish whether higher levels of minerals may assist in preserving shelf-life of eggs.

- Other measurements, such as tibial strength, liver mineral concentration, carbonic anhydrase concentration in serum, and antibody level (immune response) could have been included in this trial.

- A negative control diet with Zn:0 Cu:0 Mn:0 would have given an indication whether the micro minerals derived from raw materials were adequate or not, although such a negative control might not have been ethical.

- Further studies should include the concentration of these minerals in the egg albumin and shell as some minerals may be more important in other parts of the egg.
- The egg shell weight in comparison to the total egg weight should have been measured, this ratio is more representative of egg shell quality than only measuring egg shell weight.

REFERENCES

AAFCO.1998. Association of American Feed Control Officials. Official Publicatio.Assoc.Am.Feed Control Off., Atlanta.GA

AAFCO.1998. Association of American Feed Control Officials. Official Publicatio.Assoc.Am.Feed Control Off., Atlanta.GA

AOAC 2000. Official method of analysis 935.13 (17th Edition) Volume I, Association of Official Analytical Chemists, Inc, Maryland, USA

Abedini, M.F., Shariatmadari, M., Karimi Torshizi, H. and Ahmadi F., 2017. Effects of a dietary supplementation with zinc oxide nanoparticles, compared to zinc oxide and zinc methionine, on the performance, egg quality and zinc status of laying hens. Livestock Science 203:30-36.

Abd El-Hack M.E., Alagawany, M., Amer, S.A., Arif, M., Wahdan, K.M.M., El-Kholy M.S., 2018. Effect of dietary supplementation of organic zinc on laying performance, egg quality and some biochemical parameters of laying hens.J.Anim.Physiol.Anim. Nutr. :542-549

Abd-Elrazig, S.M. and E.A Elzubeir, 1998. Effects of feeding pearl millet on laying hen performance and egg quality. Animal Feed Sci. and Technol. 76:89-94.

Ahmadi F. and F. Rahimi., 2011. Factors affecting quality of egg production in laying hens: A Review. World Applied Sciences Journal 12(3):372-384.

Aksu, D.S., Aksu T., and Ozsoy B.2010. The effects of lower supplementation levels of organically complexed minerals (zinc,copper and manganese) versus inorganic forms on hematological and biochemical parameters in broilers. Kafkas Univ.vet.Fak.Derg. 16:553-559.

Altan, O., A. Altan A., M. Cabuk M., and H. Bayraktar H. 2000. Effects of heat stress on some blood parameters in broilers. Turkish Journal of Veterinary and Animal Sciences 24:145-148.

Ammerman, C.B., Parker, D.P. and Lewis A.J.1995.Bioavailability of nutrients for animals:Amino acids,minerals,vitamins. Academic Press.

Aviagen, Lohmann Tierzucht 2013. Lohmann brown lite layer management guide caged layers. In house publication, Global.

Balnave, D. and Yoselewitz,I. 1997. The relation between sodium chloride concentration in drinking water and egg-shell damage. British J. Nutrition 58:588-593.

Baltaci A.K., and Yuce.K. 2017. Zinc Transporter proteins: A Review: Neurochemical Research https://doi.org/10.1007/s11064-017-2454-y

Bao,Y., M.Choct, M., P.Iji, P. and Bruerton K.2007. Effect of organically complexed copper, iron, manganese, and zinc on broiler performance, mineral excretion, and accumulation in tissues. J.App.poult.Res.16:448-455.

Bao Y.M., Choct M., Iji P.A., Bruerton K. 2010. Trace mineral interactions in broiler chicken diets. Br Poult Sci 51:109-117,doi:10.1080/000716600903571904.

Barnes H.J., Nolan L.K. and Vaillancourt J.P (2008), 'Colibacillosis', in Saif YM Diseases of Poultry 12th Edition, Ames, Iowa, Blackwell Publishing, 691–737.

Baumgartner, S., Brown D.J., Salevsky, E.and R.M Leach R.M 1978. Copper deficiency in laying hen. J.Nutr.108:804-811.

Beal RK, Powers C., Wigley P., Barrow P.A, Kaiser P and Smith A.L. 2005. A strong antigenspecific T-cell response is associated with age and genetically dependent resistance to avian enteric salmonellosis, Infection and Immunity, 73, 7509–7516.

Burley, R.W. and Vedehra, D.V. 1989. The Avian Egg. Wiley. New York.

Butchner, G.D. and Miles R.D 2003. Factors causing poor pigmentation of brown shelled eggs. University of Florida. http://edis.ifas.ufl.edu/pdffiles NMNM04700 pdf.

Butler, E.J., Curtis M.J, A.W. Pearson A.W and McDougall J.S,1972. Effect of infectious bronchitis on the structure and composition of egg albumen. The Science of Food and Agric., 23:359-369.

Chantiratikul, A., O. Cinrasri, and P. Chantiratikul. 2008. Effect of Sodium selenite and zinc-L-selenomethionine on performance and selenium concentrations in eggs of laying hens. Asian-Aust J.Anim.Sci. 21:1048-1052.

Chousalkar kk and roberts jr (2007a), Ultrastructural study of infectious bronchitis virus infection in infundibulum and magnum of commercial laying hens, Veterinary Microbiology, **122**, 223–236.

Chousalkar kk and roberts jr (2007b), 'Ultrastructural observations on effects of infectious bronchitis virus in eggshell-forming regions of the oviduct of the commercial laying hen', Poultry Science, **86**, 1915–1919.

Chousalkar kk and roberts jr (2009), 'Effects of Australian strains of infectious bronchitis virus on internal and external quality of hen eggs', Animal Production Science, **49**, 162–169.

Chowdhury, S.D 1990. Shell membrane system in relation to lathrogen toxicity and copper deficiency.World's Poult.Sci J.46:153-169.

Chowdhury, I.K. Paik, H. Namkung and H.S. Lim. 2005.Responses of Broiler chickens to organic copper fed in the form of copper-methionine chelate. Animal Feed Science and Technology 115:281-293.

Coto, C., F. Yan, S. Cerrate, Z. Wang, P. Sacakli, P. Waldroup, J. Halley, C. Wiernusz, and A. Martinez. 2008. Effects of dietary levels of calcium and nonphytate P in broiler starter diets on live performance, bone development, and growth plate conditions in male broilers fed a corn-based diet. Int. J. Poult. Sci. 7:638-645.

Curtis, P.A., F.A. Gardner and D.B. Mellor, 1995. A comparison of selected quality and compositional characteristics of brown and white shell eggs. Poult.Sci. 64:297-301.

Dale, N. and C.F. Strong 1998. Inability to demonstrate an effect eggshell quality on older hens. J Applied Poultry Res. 7:219-224.

Dao Thi H and Swick R. 2017 nutritional factors affecting egg production and eggshell quality in laying hens. Journal of Animal Husbandary Sciences and technics 223:15-20.

De Ketelaere, B., T. Govaerts, P. Coucke, E. Dewil, J. Visdcher, E. Decuypere and J. de Baerdemaeker, 2002. Measuring the eggshell strength of different genetic strains of laying hens: techniques and comparisons. British Poultry Sci. 43:238-244.

Depersio S., Utterback P.L., Utterback C.W., Rochell S.J., O'Sullivan N., Bregendahl K., Arango J., Parsons C.M. and Koelkebeck K.W (2015), Effects of feeding diets varying in energy and nutrient density to Hy-Line W-36 laying hens on production performance and economics,Poultry Science, 94:195-206.

Desai, D. N., B.G. Gangurde, A.S. Ranade, P.E. Avari, M. Manangi, M. Vazquez-Anon, and D. Joardar. 2012. Studies on egg production, egg quality, tissue mineral profile and immunity during post peak phase of layers fed with HMTBa chelated organic trace minerals. Poult. Sci. 91 (Suppl. 1): 144 (Abstr. P425).

Dobrzanski, Z., D.Jamroz, H,Gorecka, and S.Opalinski.2003Bioavailability of selenium and zinc supplied to the feed for laying hens in organic and inorganic form. EJPAU Anim.Hus.6

Domermuth ch, gross wb and dubose rt 1967. *Mycoplasma salpingitis* of chickens and turkeys, Avian Diseases, **11**, 393–398.

Doyon, G.M. Bernier-Cardou, R.M.G. Hamilton, F. Castaigne and C.J. Randall, 1986. Egg quality 2 Albumen quality of eggs from five commercial strains of White Leghorn hens during one year of lay. Poult. Sci., 78:591-594.

Ewing,H.P.,G.M Pesti,R.I bakalli, and J.Menten.1998.Studies on the feeding of cupric sulfate pentahydrate,cupric citrate, and copper oxychloride to the broiler chickens.Poult.Sci 77:445-448.

Ekweozor, I.K.E., A.W. Granville, E.E. Nkanga and O.K. Ogbalu. 2002. The effects of crude oil contaminated feeds on the yield and quality of eggs of poultry birds. J.Acriculture in the Tropics and Subtropics,103:89-97.

El-Manylawi M.A.F, Omara I.I and Abd-Elsamee M.O (2012), Effect of different levels of dietary metabolizable energy and crude protein on laying hens performance and egg quality,Egyptian poultry science journal,30:679-697.

Gao.J., Nie.W, Xing, K., and Y. Guo 2018. Comparative study of different maternal zinc resource supplementation on performance and breast development of their offspring.Biological Trace Element Research
https://doi.org/10.1007/s12011-018-1513-9

Gast R.K.2008.Salmonella infections, in Saif YM *Diseases of Poultry* 12th Edition,Ames, Iowa, Blackwell Publishing, 619–689.

Gheisari A.A., A. Sanei, A.Samie. M.M. Gheisari, M. Toghyani 2011. Effects of diets supplemented with different levels of manganese, zinc and copper from organic or inorganic sources on egg production and quality characteristics in laying hens. Biol. Trace. Elem. Res 142:557-571.

Gough re and jones rc (2008), 'Avian metapneumovirus', in Saif YM *Diseases of Poultry* 12th Edition, Ames, Iowa, Blackwell Publishing, 100–110.

Guy rk and garcia m (2008), 'Laryngotracheitis', in Saif YM *Diseases of Poultry* 12th Edition, Ames, Iowa, Blackwell Publishing, 137–152.

Farghly, M. F. A., K. M. Mahrose, and Z. U. Rehman. 2019. Intermittent lighting regime as a tool to enhance egg production and eggshell thickness in Rhode Island Red laying hens. Poult. Sci. 98:2459−2465.

Favero,A 2013.Reproductive performance of Cobb 500 breeder hens fed diets supplemented with zinc,manganese, and copper from inorganic and amino acid-complexed sources.J.App.Poult.Res.22:80-91.

feberwee a, de vries ts and landman wjm (2008), 'Seroprevalence of *Mycoplasma synoviae* in Dutch commercial poultry farms', *Avian Pathology*, **37**, 629–633.

Fernandes, J. I. M., A. E. Murakami, M. I. Sakamoto, L. M. G. Souza, A. Malaguido, and E. N. Martins. 2008. Effects of organic mineral dietary supplementation on production performance and egg quality of white layers. Braz. J. Poult. Sci. 10:9–65.

Hartmann,C and M. Wilhelmson, 2001. The hen's egg yolk: A source of biologically active substances. World's Poult. Sci. J. 57:13-28.

Hojyo S., and T. Fukoda., 2016. Zinc transporters and signaling in physiology and pathogenesis. Arch. Biochem. Biophys 5:1-8.

hughes cs, gaskell rm, jones rc, bradbury jm and jordan ftw (1989), 'Effects of certain stress factors on the re-excretion of infectious laryngotracheitis virus from latently infected carrier birds', *Research in Veterinary Science*, 46, 274–276.

Hudson, B.P., W.A. Dozier, III, B.D. Fairchild, J.L. Wilson, J.E. Sander, and T.L. Ward. 2004. Reproductive performance and immune status of caged broiler breeder hens provided diets supplemented with either inorganic or organic sources of zinc from hatching to 65 weeks of age. J. Appl. Poult. Res. 13:349-359.

Hudson B.P., W.A. Dozier, III, B.D. Fairchild, J.L. Wilson, J.E. Sander, and T.L. Ward. 2004. Live performance and immune responses of straight-run broilers: Influence of Zinc source in broilers breeder hen and progeny diets and ambient temperature during the broiler production period. J. Appl. Poult. Res. 13:291-301

Hudson, B.P., W.B.D. Fairchild, J.L. Wilson, A. Dozier, III, and R.J. Buhr. 2004 Breeder age and zinc source in broiler breeder hen diets on progeny characteristics at hatching. J. Appl. Poultry. Res.13:55-64.

Johnson A.L. Sturkie's Avian Physiology 5th edition Chapter 22: 569-591.

Julian rj (2005), 'Production and growth related disorders and other metabolic diseases of poultry – a review', *Veterinary Journal*, **169**, 350–369

Katanbaf, M. N., E. A. Dunnington, and P. B. Siegel. 1989. Restricted feeding in early and late-feathering chickens. 2. Reproductive responses. Poult. Sci. 68:352−358.

Kimura T., and T. Kambe., 2016 The functions of metallothionein and ZIP and ZnT transporters: An overview and perspective. Int. J. Mol. Sci 17:336.

Klecker,D.,L.Zeman,P.Jelinek, and A.Busenova. 2002. Effect of manganese and zinc chelates on the quality of eggs. Acta Univ Agricult.Sylvic.Mend.Brunensis

Kleven sh (2008), 'Mycoplasma synoviae infection', in Saif YM *Diseases of Poultry*
12th Edition, Ames, Iowa, Blackwell Publishing, 845–856.

Kleyn, R. 2013. Chicken Nutrition: A guide for nutritionist and poultry professionals. Context Products Ltd, Leicestershire, UK.

Kucukersan K.,yesilbag D. and Kucukersan S. (2010) Influence of different dietary oil sources on performance and cholesterol content of egg yolk in laying hens, Journal of Biological and Environmental Sciences ,4 (12):117-122

Leach, R.M. 1983. Biochemistry of the organic matrix of the egg-shell. Poult. Sci. 61:2040-2047.

landman wjm and cornelissen ra (2006), '*Escherichia coli* salpingitis and peritonitis in layer chickens: an overview', *Tijdschrift Voor Diergeneeskunde*, **131**, 814–822.

Leach, R., and J. Gross. 1983. The effect of manganese deficiency upon ultrastructure of the eggshell. Poult. Sci. 62: 499-504.

Leeson,S.,J.D Summers, and M.L Scott.2001.Nutrition of the chicken

Leeson,S.,J. Taylor-Pickard, and L.Tucker.2005.Trace mineral requirements of poultry-validity of the NRC recommendations.Re.Min.Nut.:107-117

Ley D.H (2008), '*Mycoplasma gallisepticum* infection', in Saif YM *Diseases of Poultry*
12th Edition, Ames, Iowa, Blackwell Publishing, 807–834.

Li,S et al 2004. Use of chemical characteristics to predict the relative bioavailability of supplemental organic and manganese sources for broilers.Journal of .Animal .Science .82:2352-2363.

Li F., Zhang L.M.,Wu X.H.,Li C.Y.,Yang J., Dong Y., Lemme A., Han J.C. and Yao J.H (2013), Effects of metabolizable energy and balanced protein on egg production, quality and components of Lohmann brown laying hens, ournal of applied poultry research,22:36-46

Lim, H.S. and I.K. Paik. 2003. Effects of supplementary mineral methionine chelated (Zn,Cu,Mn) on the performance and eggshell quality of laying hens. Asian-Aust. J. Anim. Sci.Vol. 16:1804-1808.

Lim, H.S., Namkung, H. and Paik. I.K. 2003. Effects of phytase supplementation on the performance, egg quality, and phosphorous excretion of laying hens fed different levels of dietary calcium and non-phytate phosphorous, 82: 92-99.

Lima, F.R., Mabe,I., & Albuquerque, R. (2000). Zinc and manganese additional supplementations may benefit egg production and quality. Poultry Science, 79 (Suppl 1),46.

Linder,M.C.2013.biochemistry of copper.Springer Science & Business Media

Lonnerdal B., 1996. Bioavailability of Copper. Am. J. Clin. Nutr. 63:8218-8295.

Lui, Y.P., and Jie, H. 2011. Breeding for disease resistance in poultry:opportunities with challenges. World's Poultry Science Journal,Vol 67,Issue 4:687-696

Mabe, I., Rapp, M. Bain, M. and Y. Nys. 2003. Supplementation of a con-soybean meal diet with Manganese, Copper and Zinc from organic or inorganic sources improves eggshell quality in aged laying hens. Poul. Sci. 82:1903-1913.

MACIEL, M.P., SARAIVA, E.P., AGUIAR, E.D.F., RIBEIRO P.A.P., PASSOS, D.P. and SILGA, J.B. (2010) Effect of using organic microminerals on performance and external quality of eggs of commercial laying hens at the end of laying. *Revista Brasileira de Zootecnia* 39: 344-348.

Manangi, M.K. Vazques-Anon, M., Richards, J.D. Carter, S. and C.D. Knight, 2015. The impact of feeding chelated trace minerals on shell quality, tibia strength, and immune response in laying hens. J. Appl. Poult. Res. 24:316-326.

McDowell,L.R.2003. Minerals in an animal and human nutrition.Elsevier Science BV.

mcferran jb and smyth ja (2000), 'Avian adenoviroses', *OIE Revue Scientifique et Technique*, **19**, 589–601.

McNamara,J.2006 Mechanistic modelling at the metabolic level: a model of metabolism in the sow as an example.CAB internt.:282-304. Miles,R.,S.O'Keefe,P.Henry,C.Ammerman, and X.Luo.1998. The effect of dietary supplementation with copper sulfate or tribasic copper chloride on broiler performance,relative copper bioavailability, and dietary prooxidant activity. Poult.Sci 77:416-425.

meroz m, elkin n, hadash d and abrams m (1990), 'Egg drop associated with avian encephalomyelitis virus', *Veterinary Record*, **127**, 532.

Moghaddam H.N., Kazemi Fard M., Agah M.J., Hosseini S.J and Mirakzehi M.T (2012), Effect of different levels of methionine , protein and tallow on the productive performance and egg quality of laying hens in the late-phase production, Brazilian Journal of Poultry Science 14:149-158.

muneer ma, halvorson da, sivandan v, newman ja and coon cn (1986), 'Effects of infectious bronchitis virus (Arkansas strain) on laying chickens', *Avian Diseases*, **30**, 644–647.

Natalie, G. 2009. Factors affecting egg quality in commercial laying hens:A review. http://www.eggfarmers.co.nz/upload/A369F_Factors_affecting egg_quality PDF.

Nollet, L., G.Huyghebaert, and P. Spring. 2008. Effect of different levels of dietary organic (Bioplex) tace minerals in broiler diets on productive performance and mineral excretion. J.App.Poult.Res. 17: 109 -115.

Nollet,L., J.van der Klis,M.Lensing, and P.Spring.2007. The effect of replacing inorganic with organic trace minerals in broiler diets on productive performance and mineral excretion.J.App.Poult.Res. 16:592-597.

Nollet,l.,J van der Klis,M.lensing, and P.Spring.2007. The effect of replacing inorganic with organic trace minerals in broiler diets on productive performance and mineral excretion. J. App. Poult.Sci 16:592-597.

NRC. 1994.Nutrient requirements of poultry.National Research Council.National Academy Press Washington.USA

Nicodemus N., Callejo A., Calle A., Gutierrez del Alamo A., Villamide M.J., Perez de Ayla P. and Buxade C. (2011) , Optimal digestible methionine and cysteine /lysine relationship for ISA brown laying hens from 34 to 42 weeks of age, Proceedings of the XIV Jornadas sobre Produccion Animal, Zaragoza , Espana,pp. 168-170.

Nunoya t, kanai k, yagihashi t, hoshi s, shibuya k and tajima m (1997), 'Natural case of salpingitis apparently caused by *Mycoplasma gallisepticum* in chickens', *Avian Pathology*, **26**, 391–398.

Nys, Y. 1999. Nutritional factors affecting egg quality. Czech J. Animal Sc, 44:135-143.

Nys Y.T., M.T Hincke, J.L. Arias, J.M. Garcia-Ruiz and S.E Solomon.2001. Avian eggshell mineralization.Poultry and Avian Biology Reviews 10:143-166.

Okeudo, N.J., Onwuchekwa, C.I. and Okoli, I.C. 2003. Effect of oil treatment and length of storage on the internal quality, organoleptic attributes and microbial profile of chicken eggs. Tropical Anim. Prod. Investigations, 6:63-70.

Oliveira D.D ., Baiao N.C., Cancado S.V., Figueiredo T.C., Lara L.J.C and Lana A.M.Q. (2010), Lipid sources of layer hen diet:performance and egg quality, Arquivo brasileiro de Medicina veterinaria e Zootecnia, 62:718-724.

Pandey i and chauhan ss (2007), 'Studies on production performance and toxin residues in tissues and eggs of layer chickens fed on diets with various concentrations of aflatoxin AFB1', *British Poultry Science*, **48**, 713–723.

Parker, D. 2013. Layer Health and Performance: Feed science. AFMA Matrix 22(2): 31-35.

Peric,L.,L.Nollet,N.Milosevic, and D.Zikic.2007. Effect of Bioplex and Sel-Plex substituting inorganic trace mineral sources on the performance of broilers.Archiv.Geflugelkunde 71:122-129.

Qiujuan Sun et al. 2012. Effects of methionine hydroxy analog chelated Cu/Mn/Zn on laying performance, egg quality. Enzyme activity and mineral retention of laying hens. J. Poult. Sci, 49:20-23.

Qiujuan Sun et al., 2012. Dietary mineral sources altered lipid and antioxidant profiles in broiler breeders and post hatch growth of their offspring. Biol Trace Elem 145:318-324.

Rahn, H., Ar, A., and C.V. Paganelli 1990. Gas fluxes in avian eggs: Driving forces and the pathway exchange. Comp. Biochem. Physiol. A 95:1-15.

Rajkumar, D., R.P. Sharma, K.S. Rajaravindra, M. Niranjan and B.L.N. Reddy, T.K. Bhattacharya and R.N. Chatterjee 2009. Effect of genotype and age on egg quality traits in naked neck chicken under tropical climate from India. Int. J. Poult. Sci 8:105-115.

Rao S.V.R, Raju M.V.L.N., Ravindran V. and panda A.K (2013), Influence of different concentrations of metabolizable energy at constant ratio to dietary protein,lysine,methionine, calcium and phosphorous on the performance of White Leghorn layers in the tropics, Animal Production Science, 53:523 – 530.

Ravindran, V.W.L. Bryden and E.T. Cornegay, 1995. Phytates occurrence, bioavailability and implications in poultry nutrition. Poult Avian Biol.Rev. 125-143.

Renema, R. A., F. E. Robinson, and M. J. Zuidhof. 2007a. Reproductiveefficiency and metabolism of female broiler breeders as
affected by genotype, feed allocation, and age at photostimulation.
2. Sexual maturation. Poult. Sci. 86:2267–2277.

Renema, R. A., M. E. Rustad, and F. E. Robinson. 2007b. Implications
of changes to commercial broiler and broiler breeder body
weight targets over the past 30 years.Worlds Poult. Sci. J. 63:457–
472.

Robineau b and moalic p y (2009), 'A clinical aspect of infectious bronchitis: the false layers, changes in the coronaviruses involved in France', *Bull. Acad. Vét. France*, **162**, 155–160. Available from: http://www.academie–veterinaire–defrance.org/bulletin/ pdf/2009/numero02/155.pdf [Accessed March 19 2008].

Rocha T.C da,Gomes p.C., Donzelle J.L., Rostagno H.S., Mello H.H. de C.,Ribeiro C.L.N and Troni A.R (2013) Digestible threonine to lysine ratio in diets for laying hens aged 24-40 weeks, Revista Brasileira de Zootecnia 42:879-884.

Roland, D.A. and M. Bryant 1994. Feed consumption, energy consumption, shell quality egg production and egg weight as influenced by pre-peak production calcium levels of commercial leghorns. J. Appl. Poultry Sci. 3:184-189.

Roberts, J. R. 2004. Factors affecting egg internal quality and egg
shell quality in laying hens. Jpn. Poult. Sci. 41:161–177.

Saif, Y.M. 2008. Diseases of Poultry. Blackwell Publishing. London

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

Sauer A.K., Pfaender, S., Hagmeyern S., Torana, L., Mattes, A.K., Briel, F., Kury, S., Boechers T.M., Grabrucher, A.M. 2017. Characterization of Zn amino acids complexes for zinc delivery in vitro Caco-2 cells and enterocytes from hiPSC. Biometals, 30(5):643-661.

Salevsky, E., and Leach, R.M. 1980.Studies on the organic components of shell gland fluid and the hen's eggs shell. Poult. Sci. 59:438-443.

Sevoian m and levine pp (1957), 'Effects of infectious bronchitis on the reproductive tracts,
egg production, and egg quality of laying chickens', *Avian Diseases*, **1**, 136–164.

Sharp, P. J. 1993. Photoperiodic control of reproduction in the domestic
hen. Poult. Sci. 72:897−905.

Shi, L., Y. Y. Sun, H. Xu, Y. F. Liu, Y. L. Li, Z. Y. Huang, A. X.
Ni, C. Chen, P. L. Wang, J. H. Ye, H. Ma, D. L. Li, and J. L.
Chen. 2019. Effect of age at photostimulation on reproductive
performance of Beijing-You Chicken breeders. Poult. Sci. pez267.

shivaprasad hl (2000), 'Fowl typhoid and pullorum disease', *Revue Scientifique et Technique de L'Office International Des Epizooties*, **19**, 405–424.

Silversides, F. G., D. R. Korver, and K. L. Budgell. 2006. Effect of
strain of layer and age at photostimulation on egg production,
egg quality, and bone strength. Poult. Sci. 85:1136–1144.

Silyn-Roberts, H., and R.M. Sharp, 1986. Crystal growth and the role of the organic network in the eggshell biomineralization. Proc. R. Soc. London B 227:307-324.

Sloan DR Harms RH Abdullah AG Kuchinski KK, 2000Variation in egg content density makes egg specific gravity a poor indicator of shell weight. J Appl.Res 18:121-128

Sohail, S.S. and D.A. Roland, 2002. Influence of dietary phosporous on performance of Hy-line W-O hens. Poult.Sci. 81:75-83.

Sohail, S.S., Roland Sr., D.A., 1999. Influence of supplemental phytase on performance of broilers four to six
weeks of age. Poultry Sci. 78, 550–555.

Solomon SE (1991) Egg and Eggshell Quality. The Veterinary Press Iowa State University

Press, Ames

Solomon, S. E. 2010. The eggshell: Strength, structure and function.
Br. Poult. Sci. 51:52–59.

South African Poultry Association, 2016 Industry profile, Wild Fig tree business park, Block

C 1494, cranberry street, Honey dew ext 19,2170.

South African Poultry Association, 2018 Industry profile, Wild Fig tree business park, Block

C 1494, cranberry street, Honey dew ext 19,2170.

Souza H.R.B.D., Faria D.E.D., Caetano V.C., Santos A.L.D., Araujo R.B. and Sakamoto M.I (2014), Digestible lysine levels from brown layers, Acta Scientiarum – Animal Sciences,35:369-372.

STANLEY, V.G., SHANKLYN, P., DALEY, M., GRAY, C., VAUGHAN, V., HINTON, A. Jr and HUME,
M. (2012) Effects of organic selenium and zinc on the aging process of laying hens.
Agrotechnology 1: 103,
doi:10.4172/2168-9881.1000103.

Statistical Analysis Systems, 2017. SAS Users Guide: Statistics Version 9.3 SAS Institute

Inc. Cary, NC, USA

Stefanello C., Santos, T.C., Murakomi, A.E., Martins, E.N.< and T.C. Carneiro 2014.

Productive performance , eggshell quality and eggshell ultrastructure of laying hens fed diets

supplemented with organic trace minerals. Poult. Sci. 93:104-113.

suksupath s cole ea, cole rj and bryden wl (1989), 'Toxicity of cyclopiazonic acid in the laying hen', *Proceedings of the Australian Poultry Science Symposium*, Sydney, 94.

Swiatkiewics, S. and J. Koreleski. 2008. The effect of zinc and managanese source in the diet for laying hens on eggshell and bones quality. Veterina Medicina. 53: 555-563.

Tang,P.,P.Patterson, and V.Puri.2006.Effect of feed segregation on the commercial hen and egg quality.J.App.Res.15564-573

Tangere, E.S., Bhandari, B. and J.G. Dingle, 2001. Eggshell quality of caged laying hens given drinking water maintained at in normal and high temperatures and given feed without or with grit or eggs shell. Proceedings of the Australian Poultry Science Symposium, 13:228-231.

Teuling M.(2015), less protein results in more eggs, World Poultry, 31:22 -24.

Torres, C.A. and D.R. Korver. 2011. Maternal broiler breeder flock age and dietary Cu, Zn and Mn form affect embryonic bone development. Poult. Sci. 90:(Suppl 1):83-84 (Abstr. 309).

Trampel dw, wannemuehler and nolan lk (2007), 'Characterization of *Escherichia coli* isolates from peritonitis lesions in commercial laying hens', *Avian Diseases*, **51**, 840–844.

Tuahimaa,P., Joensuu, T., Isola J., Keinanen, R., Kunnas,T., Niemala, A., Pekki, A., Wallen, M. Ylikomi, and M. Kulomaa 1989. Development of progestin-specific response in the chicken oviduct. Int. J. Dev. Biol. 33,125-134.

Uni, Z., L.Yadgary, and R.Yair. 2012. Nutritional limitations during poultry embryonic development. J. App. Poult. Res. 21: 175 – 184

USDA. 2016. Layers and Eggs: Production by State, US.
https://www.nass.usda.gov/Charts and Maps/Poultry/eggmap.php

van der Klein, S. A. S., G. Y. Bédécarrats, F. E. Robinson, and
M. J. Zuidhof. 2018a. Early photostimulation at the recommended
body weight reduced broiler breeder performance. Poult.
Sci. 97:3736–3745.

Vieira, S.L.2008.chelated minerals for poultry.Rev.bras.Cienc.Avic.10:73-79

Viguet-Carrin, S., P. Garnero, and P. D. Delmas. 2006. The role of collagen in bone strength. Osteoporos. Int. 17:319-336.

Virden et al., 2003. Hen mineral impacts progeny liveability. J. Appl. Poult. Res., 12:411-416.

Virden,W.S., J.B.Yeatman, S.J.Barber, K.O.Willeford, T.LWard, T.M.Fakler, R.F.Wideman, and M.T.Kidd. 2004, Immune system and cardiac function of progeny chciks from dams fed diets differing in zinc and manganese level and source. Poultry Science 83:344-351.

Wang X., Y.Wu., Zhou.B., 2009 Dietary Zn absorption is mediated by Zn T in *Drosphila melanogaster*. FASER J 23:2650-2661.

Wang,H.,C.Zhang,Y.Mi, and M.kidd.2014.Copper and lysine amino acid density responses in commercial broilers.J.App.poult.Res:japr959

Watts,D.L. 1990. Nutrient interrelationships:minerals, vitamins, endocrines.J.Orthomol.med5 :11-19

Williams G.T., and Smith, P.J. 1993. Molecular regulation of apoptosis genetic controls of cell death. Cell 74:777-778.

whitehead cc and fleming rh (2000), 'Osteoporosis in cage layers', *Poultry Science*, **79**, 1033–1041.

Wolc A., Stricker C., Arango J., Settar P., Fulton J.E.O'Sullivan N., Habier D., Fernando R., Garrick D.J., Lamont S.J., Dekkers J.C.M. (2011) Breeding value prediction for production traits in layers using pedigree and marker based methods. Genet. Sel. Evol., 43, 5.

J.F. Xiao et al. 2014. Manganese supplementation enhances the synthesis of glycosaminoglycan in egg shell membrane: A strategy to improve eggshell quality in laying hens. Poultry Science 93:380-388.

J.F. Xiao et al., 2015. Bioefficiency comparison of organic managanese with inorganic managanese for eggshell quality in Hy-Line Brown lying hens. Poultry Science 94:1871-1878.

Yenice, E., Mizrak, C. Gultekin, M., Atik, Z., and M.Tunca. 2015. Effects of organic and Inorganic forms of Manganes, Zinc, Copper and chromium on bioavailability of these minerals and calcium in the late-phase laying hens. Biol. Tra. Elem.Res. 167: 300- 307.

Yildiz,A.,Y cufadar, and O.Olgun.2011.Effects of dietary organic and inorganic manganese supplementation on performance, egg quality and bone minerilisation in laying hens.Rev.med.vet.162:482-488.

Zamani, M.A., S.Ahmed and B.C Sutradhar,2005. Study on the egg quality of a breed and three crossbreds at various ages under semi scavenging system of management. Pak. J>Biol, 8:211-214.

Zanella A., Alborali G.L., Bardotti M., Candotti P., Guadagnini P.F., Martino P.A and Stonfer M. 2000. Severe *Escherichia coli* septicaemia and polyserositis in hens at the start of lay, Avian Pathology, **29**, 311–317.

Zavaleta, D., and Ogasawara, F. 1987. A review of the mechanism of the release of spermatozoa from storage tubules in the fowl and turkey oviduct. World's Poult. Sci. J. 43:132-139.

1. APPENDICES

1.1 APPENDIX 1: Vaccination program rearing and laying period

Age	Disease	Product	Application
Hatchery	Marek's	Rismavac	0.2ml Subcutaneous
	Marek's HVT Newcastle	Vectormune HVT NDV	0.2ml Subcutaneous
	Newcastle Infectious Bronchitis	VH + H120	Coarse Spray
(5 Days)		Enrovet	Drinking water 4 ml/1000 chicks in 4-6h per day
14 Days	Newcastle Infectious Bronchitis	VH + H120	STIHL fogger
21 Days	Gumboro	BUR 706	Drinking water
4 Weeks	Newcastle Infectious Bronchitis	Nobilis Ma5 + Clone 30	STIHL fogger (mix)
	E.coli	Poulvac E coli	STIHL fogger (mix)
Day 35	Transfer and split		
6 Weeks	Fowl Pox Laryngotracheitis Avian encephalomyelitis	Vectormune FP ILT AE	Wing-web stab
	Mycoplasmax gallisepticum	TS-11	Eye drop (Right)
	Mycoplasma synoviae	Vaxsafe MS	Eye drop (Left)
8 Weeks	Newcastle Infectious Bronchitis	Nobilis Clone 30 + Nobilis IB 4/91	STIHL fogger (mix)
8-9 weeks	16 x blood samples for ELISA: NCD, IB, MG		
10 weeks	Samonella enteritidis	Talovac 109 SE	0.5ml Intramuscular

Age	Disease	Product	Application
	Mycoplasma gallisepticum	MG Bac	0.5ml Intramuscular
12 Weeks	Coryza	Corvac 4	0.5ml Intramuscular
	Infectious Bronchitis Newcastle EDS	Nobilis IB+ND+EDS	0.5ml Intramuscular
14 Weeks	Coryza	Avivac Coryza	0.5ml Intramuscular
	Newcastle	Nobilis Broiler ND	0.5ml Intramuscular
15 Weeks	Newcastle Infectious Bronchitis	Nobilis Ma5 + Clone 30	Drinking water
	Ecoli	Poulvac E coli	Drinking water
21 weeks and every 5 weeks thereafter until depletion	New Castle	Avinew	Eye drop

1.2   APPENDIX 2: Light program used as per breed standard.

Age (Weeks)	Hours of Light (Standard)	Light Intensity (Lux)
Day 1 – 2	24	20 – 40
Day 3 – 6	18	20 – 30
2	16	10 – 20
3	14	10 – 20
4	12	4 – 6
5	11	4 – 6
6	10	4 – 6
7	9	4 – 6
8	9	4 – 6
9	9	4 – 6
10	9	4 – 6
11	9	4 – 6
12	9	4 – 6
13	9	4 – 6
14	9	4 – 6
15	9	4 – 6
16	9	4 – 6
17	10	5 – 7
18	11	5 – 7
19	12	5 – 7
20	13	10 – 15
21	14	10 – 15
22	14	10 – 15
23	14	10 – 15
24	14	10 – 15
25 onwards	14	10 – 15