Chapter 1

Review of Literature

1.1 The Australian egg industry and food safety

The Australian egg industry plays an important role in the gross economy of the country. The gross value of production of the Australian Egg Industry in 2010/2011 was $1.428 billion at market (AECL 2010 Annual Report) and $447 million at the farm gate (ABS Cat. No.7503). According to the AECL (Australian Egg Corporation Limited) report released to the media (19, Jan, 2010), egg sales hit a record high in 2010 at more than 122 million dozen, an increase of 9 million dozen from the year 2009. According to the AZTEC report 2010, 58.6% of eggs in the Australian commercial market were from the cage production system, 28.4% from free range, 9.4% from barn laid eggs and 3.6% of organic egg production. It is of utmost importance to take preventive measures to maintain the high value of the egg market and consumer satisfaction.

The egg is one of the most perishable commodities that provide nutrients to the human body in the form of different foodstuffs. The hen's egg contains most of the essential nutrients necessary for the physiological functions of the human body. The egg contains a number of antimicrobial enzymes and other defensive barriers; however, these barriers do not always prevent microbial penetration into the egg.

The Australian egg industry is considered to be relatively safe and free from food borne pathogens such as *Salmonella* Enteritidis. Chousalkar *et al.* (2010), Cox *et al.* (2002) and Kirk *et al.* (2008) stated that the dramatic increase of *Salmonella* Enteritidis infections occurring in other countries has not been observed in Australia and the Australian poultry industry is considered to be free from this serotype. However, recent studies carried out by Ross and Heuzenroeder (2008) have revealed that food-borne *Salmonella*-induced gastroenteritis is often attributed to *Salmonella enterica* serovar Typhimurium and Enteritidis in Australia. They further reported that, in recent years, there has been a significant increase in the number cases of *Salmonella enterica* serovar Infantis in Australia. The dramatic increase of salmonellosis cases caused by *Salmonella* Enteritidis in the European Union countries since the 1980s is on the decline currently (efsa/March, 2012).

The OzFood Network has identified eggs and foods containing eggs as the most common vehicle implicated in human *Salmonella* outbreaks in Australia over the past years. Every state, except the Northern Territory, reported egg-related outbreaks of *Salmonella* Typhimurium in 2006 and 2007 (Robley, 2010). Heat labile food microbes can be destroyed by proper cooking; however it is common practice to eat half fried or half cooked egg or egg product world-wide including Australia. The number of *Salmonella* in infected eggs has little impact on human health risk for well cooked foods but the rate of illness increases if the same eggs are consumed as a raw food after a long storage time at above 30°C (Thomas *et al.,* 2006). Heat stable microorganisms (thermophile) may even remain stable after cooking, and pose a great threat to human health by liberating enterotoxin in the food and *Escherichia coli* is among one of them (Chousalkar *et al.,* 2010). Studies on microbial penetration of the eggshell helps to understand the mechanism of growth of food poisoning bacteria within egg contents and spoilage of table eggs (Al-Natour *et al.,* 2011).

Australia is a country where the probability of incidence and outbreak of certain diseases is relatively low due to strict quarantine policies, proper prophylaxis, effective treatment of clinical cases and extensive research methodologies unveiling unidentified microbial species including their serotyping and biotyping. However, the presence of pathogenic microbes cannot be ruled out completely. In recent years, food safety has become a major societal concern driven by widespread media reporting of *Salmonella*, *Campylobacter* and *Escherichia coli* O157 cases worldwide (Bolton *et al.,* 2011; Knape *et al.,* 1999).

1.2 Hen reproductive system

The reproductive system of the domestic hen (*Gallus gallus*) consists of the ovary and the oviduct which is about 65-76 cm long (Jacob *et al.,* 2011). The oviduct is further divided for simplicity into five distinguishable regions: infundibulum (10cm), magnum (35cm), isthmus (8cm), shell gland (10cm), and vagina which is 4cm long (Hodges, 1974; Jacob *et al.,* 2011; Johnson, 2000; Nys and Guyot, 2011).

Plate 1.2.1 Ovary, oviduct and egg with shell

Source: ulisse.cas.psu.edu/4hembryo/female.html

1.2.1 The ovary

A newly developing embryo of the hen has two ovaries, right and left, but the right ovary does not reach maturity, remains regressed and persists throughout life only as an inconspicuous vestige in the galliforms (Hodges, 1974). The left ovary is an irregularly shaped, pinkish organ which is located on the left side of the abdominal cavity close to the median line (Hodges, 1974; Nys and Guyot, 2011). The left ovary is attached by the mesovarian ligament at the cephalic end of the left kidney (Nys and Guyot, 2011). The ovary consists essentially of an outer cortex containing ova which surrounds a highly vascular medulla composed of connective tissues (Hodges, 1974).

The ovary receives its blood supply from the ovarian artery, which usually arises from the left renolumbar artery but may branch directly from the dorsal aorta (Hodges, 1974; Sturkie and Mueller, 1976). Within the ovary, blood flow is greatest to the five largest preovulatory follicles (Scanes *et al.*, 1982). The organ is drained by large veins that anastomose and converge into an anterior and a posterior ovarian vein, both of which empty into the venue cava (Lofts and Murton, 1973). The hen ovary is well innervated by both adrenergic and cholinergic fibres (releasing epinephrine and nor epinephrine, respectively) of the autonomic nervous system and a greater number of neurons are present within the theca layer as a follicle progressively matures (Bennett and Malmfors, 1970; Dahl, 1971; Freedman, 1968; Gilbert, 1969).

1.2.2 The Oviduct

The oviduct is a highly convoluted, muscular duct which transports the ovum away from the ovary, with fertilization of the ovum and deposition of the albumen, membranes and shell onto the ovum occurring to form the finished egg (Hodges, 1974; Nys and Guyot, 2011). The oviduct of the hen is derived from the left Mullerian duct during embryogenesis (Johnson, 2000), and is suspended from the left side of the abdominal cavity by the dorsal ligament (Hodges, 1974). The structure of the oviduct wall is made up of seven layers: an outer covering of peritoneal epithelium; a double layer of smooth muscle, outer longitudinal and inner circular, with an intervening connective tissue layer in which lie large blood vessels; a layer of connective tissue internal to the circular muscle; a lamina propria containing glands in most regions of the oviduct; and an inner epithelia lining (Hodges, 1974).

1.2.2.1 Infundibulum

The infundibulum, a funnel shaped structure which receives a freshly released ovum (Johnson, 2000), is 10 cm in length (Nys and Guyot, 2011). The funnel shaped portion of the infundibulum consists of a thin walled, funnel shaped opening, flattened in the dorso-ventral direction with its flared lips lying in close proximity to the ovary (Hodges, 1974). The funnel walls converge rapidly to form the infundibular neck, a narrow thin walled tube which rapidly increases in size and thickness to form the magnum (Hodges, 1974; Parto *et al.*, 2011). The mucosa of the infundibulum consists of non secreting ciliated cells, non ciliated, mucous secreting goblet cells and secretory cells other than mucous secreting goblet cells (Aitken and Johnston, 1963; Hodges, 1974). The egg is fertilized in the infundibulum and it is here that the first layer of albumen is produced in the egg (Jacob *et al.*, 2011; Johnson, 2000; Sturkie and Mueller, 1976). The activity of the infundibulum is initiated by the ovum as it is normally quiescent until the ovum is liberated (Sturkie and Mueller, 1976). The infundibulum has the tubules that store sperm and is the site of fertilization (Jacob *et al.*, 2011; Nys and Guyot, 2011). Innervation of the infundibulum is via the aortic plexus (Johnson, 2000). The ovum remains in the infundibulum for approximately 0.5hr (Jacob, *et al.*, 2011; Roberts and Brackpool, 1994).

1.2.2.2 Magnum

The magnum is the longest (35cm) and most conspicuous part of the oviduct (Jacob *et al.,* 2011; Nys and Guyot, 2011; Rose and Hincke, 2009; Sturkie and Mueller, 1976) which is readily distinguished from the infundibulum by its white colour, its greatest diameter and its thicker wall (Solomon, 1991). The overall diameter of the magnum is greater than the infundibular neck (Hodges, 1974). Estrogen stimulates epithelial cells to develop into three morphologically different cell types: tubular gland cells, ciliated cells, and goblet cells where tubular gland cells are responsible for the production of ovalbumin, lysozyme and conalbumin (Johnson, 2000; Jung *et al.,* 2011). Goblet cells synthesize avidin following exposure to progesterone and estrogen (Tuohimaa *et al.,* 1989). Mucosal folds are prominent in the magnum and are carpeted with both ciliated and non ciliated cells (Hodges, 1974; Sturkie and Mueller, 1976). When the egg is in transit, these folds are compressed and flattened laterally (Solomon, 1983). The magnum is considered the "albumen synthesizing machinery" as the majority of the albumen is formed here (Jacob *et al.,* 2011; Nys *et al.,* 2004; Palmiter and Wrenn, 1971; Roberts and Brackpool, 1994; Sturkie and Mueller, 1976; Wyburn *et al.,* 1970). Asmundson *et al.* (1943) suggested that the release of the albumen is probably affected by the mechanical stimulation of the descending egg. Hodges (1974) states that innervation of the magnum is via aortic and renal plexuses. The ovum remains in the magnum for approximately 3 hr (Jacob *et al.,* 2011)

1.2.2.3 Isthmus

The isthmus is distinguishable from the magnum by a thick circular muscle layer where glandular tissue is less developed as compared to the magnum (Hodges, 1974; Johnson, 2000; Solomon 1983, 1975; Sturkie and Mueller, 1976). The isthmus is narrower than the magnum and is approximately 10 cm long (Jacob *et al.,* 2011; Nys and Guyot, 2011; Roberts and Brackpool, 1994). The ridges of the isthmus are angular in appearance, and the apertures of the tubular glands, situated in depressions of the surface epithelium, are much more numerous than in other regions of the oviduct (Hodges, 1974). The surface epithelium is tall, being about 25 µm in height, and consists of alternating ciliated and secretory cells (Hodges, 1974). The tubular glands are similar in appearance to those of the magnum but their secretions, where these are visible in the lumina, tend to form long threads rather than the amorphous masses which are characteristic of the magnum (Solomon, 1983). The egg stays here for approximately 1 to 2 hr, during which the shell membranes (in tubular gland or white isthmus) and

the mammillary cores (in tubular shell gland or red isthmus) are formed (Johnson, 2000; Jonchere *et al.*, 2010; Rose and Hincke, 2009; Sturkie and Mueller, 1976).

1.2.2.4 Shell gland (Uterus)

The shell gland or uterus (10 cm) is an expanded pouch-like part of the oviduct in which the egg remains for a period of approximately 20 hr during which the processes of plumping and shell formation take place (Hodges, 1974; Jacob *et al.*, 2011; Johnston *et al.*, 1963; Nys and Guyot, 2011; Roberts, 1994; Rose and Hincke, 2009; Sturkie and Mueller, 1976). The initial part of the shell gland is short, approx. 2 cm and tube shaped (Richardson, 1935) and the glands of this region are of different structure to those of the isthmus (Hodges, 1974). The shell gland is characterized by a prominent longitudinal muscle layer lined medially with both tubular gland and unicellular goblet cells (Johnson, 2000). The shell gland mucosa consists of a surface epithelium covering the leaf shaped folds and an underlying layer of complex, branched tubular glands contained within the folds (Hodges, 1974). Hodges (1974) further explains that, in cross section, the mucosal folds are of lower height than those of the magnum and are less distended by the tubular gland layer, such that there is some secondary folding of the surface. Calcium salts deposition takes place onto the shell membrane fibres in the shell gland (Hincke *et al.*, 2010; Johnston *et al.*, 1963; Nys *et al.*, 2004; Roberts, 1994; Sturkie and Mueller, 1976). Calcification within the shell gland is associated with stimuli initiated by ovulation or by neuroendocrine factors that control and coordinate both ovulation and calcium secretion (Johnson, 2000). Calcification of the egg first occurs slowly, increases to a rate of up to 300 mg/hr over duration of 15 hr, and then again slows during the last 2 hr before oviposition (Johnson, 2000). The deposition of the calcium salts is specifically targeted at the chemically modified end portions of the membrane fibres, the mammillary cores (Roberts & Brackpool, 1994). Sympathetic innervations of the shell gland are via the hypogastric nerve, which is the direct continuation of the aortic plexus (Johnson, 2000). An extensive mesh of large nerve fibres has been identified in the uterine walls (Gilbert and Lake, 1963).

1.2.2.5 Vagina

The vagina is the last part of the oviduct and contains glands that help in sperm storage before spermatozoa reach the infundibulum where they fertilize the ovum (Johnson, 2000). It is a narrow, relatively short muscular duct, often strongly curved, making artificial insemination difficult (Nys

and Guyot, 2011). The glands are present only near the junction with the shell gland pouch (Solomon, 1983). The lining of the glands is made up of ciliated and non ciliated cells (Johnson, 2000). The vagina is separated from the shell gland by a ring of voluntary muscle called the uterovaginal sphincter. The greater part of the thickness of the vaginal wall is composed of the muscle layers (Hodges, 1974). The outer, longitudinal layer is only moderately developed and consists of bundles of muscle fibres scattered throughout a connective tissue stroma, whilst the inner, circular layer is strongly developed, being thicker here than in any other part of the oviduct (Hodges, 1974). Jacob *et al.* (2011) and Johnson (2000) state that, in the domestic fowl, the spermatozoa remain viable in the specialized sperm storage tubules/ glands for a period of 7–14 days at body temperature. The uterovaginal junction glands are apparently devoid of nerves and contractile tissue, but possess a well developed vascular system (Burke *et al.,* 1972; Gilbert *et al.,* 1968b; Tingari and Lake, 1973). These authors further mention that spermatozoa fill the uterovaginal glands in a sequential fashion without mixing so that, with successive inseminations, sperm from the latest insemination is most likely to fertilize an ovum. The vagina apparently takes no part in the formation of the egg but helps in its expulsion during laying (Jacob *et al.,* 2011; Sturkie and Mueller, 1976).

1.3 The Egg

As uricoletic animals, chickens produce cleidoic eggs, which are a self contained bacteria-resistant biological package for extra-uterine development of the chick embryo (Jonchere *et al.,* 2010). The unfertilized egg is a nutritious foodstuff for human consumption and shell quality is of paramount importance (Hincke *et al.,* 2010; Hunton, 2005). The egg is composed of a central yolk surrounded by the albumen, eggshell membranes, calcified eggshell and cuticle (Roberts, 2004; Rose and Hincke, 2009). The hen’s egg contains approximately 76% water, 12% protein, 10% lipids with the remainder being vitamins, minerals, carbohydrates and calcium salts necessary for the development of the embryo (Jonchere *et al.,* 2010). The egg is a major source of human dietary protein with high biological value and excellent protein efficiency ratio (Gupta, 2010). Several biological activities have now been associated with egg components, such as novel anti-microbial activities, anti-adhesive properties, immunomodulatory, anti-cancer, and anti-hypertensive activities (Mine, 2007).

1.3.1 Egg components; their development and function

1.3.1.1 Yolk

The yolk is a slightly elongated spheroid enclosed within the yolk membrane (Hodges, 1974). A pair of yellow and white bands corresponds to a single day's growth of the ovum within the ovary (Hodges, 1974). Yolk is an excellent source of energy and fat soluble vitamins for the developing embryo as it contains all lipid components of the egg (Burley and Vadehra, 1989). The final composition of yolk in the hen's egg consists of approximately 33% lipid (by wet weight) compared to 17% protein while the greater proportion of yolk is water (48%) with lesser amounts of free carbohydrates (0.2%) and inorganic elements (1%) (Hodges, 1974; Johnson, 2000). Yolk serves to provide lipids and many of the proteins required for embryonic growth (Johnson, 2000). It is generally accepted that yolk protein synthesis takes place in the liver and is regulated primarily by gonadotropin and steroid hormones (Bell and Freeman, 1971; Johnson, 2000). From the chicken yolk, 119 different proteins have been identified among which albumen, vitellogenin, apovitellenins, IgY, and ovalbumin are most abundant (Mann and Mann, 2008). Of the total lipids, about two thirds are true fats, mainly glyceride esters of palmitic, oleic and linoleic acids (Bellairs, 1961; Hodges, 1974).

Following ovulation, the yolk is captured by the infundibulum where the developing egg remains for about 15 minutes during which the perivitelline membranes are formed (Hincke *et al.*, 2010). The yolk is surrounded by the yolk membrane that varies in thickness from 4 to 12 μm, but appears to be somewhat thicker in the fresh state, 24 μm, and is made up of outer and inner fibrous layers (Bellairs, 1961). The yolk membrane is composed of a three dimensional network of fibres running mainly parallel to the yolk surface (Hodges, 1974). The fibres are 0.2-0.6 μm in diameter and appear to have a substructure of fine fibrils each approx. 30 nm in thickness (Bellairs *et al.*, 1963). The yolk membrane also plays an important role in the defensive mechanisms and relative quality of the egg (Roberts, 2010). The decreased integrity of this membrane with flock age allows moisture to move from the albumen into the yolk which leads to the leakage of the yolk components into the albumen. Any leakage of the fat components into the albumen is very detrimental to the foaming functionality (Curtis, Online). With increased hen age, the integrity of the vitelline membrane is decreased while the amount of the yolk solid is increased (Curtis, Online). Immunoglobulin Y (IgY) in the yolk plays an active role in microbial defense.

1.3.1.2 Albumen

Albumen is a clear, viscous substance with a slight yellowish tint. During the 3–4 hr period in which the yolk/ovum complex travels down the largest portion of the oviduct, the magnum, it progressively acquires the albumen (Nys *et al.,* 2004). The albumen is composed of water, salts and proteins (158 different types) (Mann and Mann, 2011) and acts in a nutritional capacity, as a buffer to protect against physical injury, nourish the developing embryo, prevent the growth of microorganisms and as a template for the deposition of the shell membranes (Hincke *et al.,* 2010; Roberts and Brackpool, 1994; Roberts, 2004). Albumen in the magnum is in highly concentrated form and represents only half of its volume present in the freshly laid egg (Roberts and Brackpool, 1994). Albumen contains most of the protein of an egg, holds the yolk in the middle of the egg and consists of 4 distinctive layers namely; chalaziferous layer (2.7%); inner thin layer (16.8%); outer thick layer (55.5%); and outer thin layer which is about 25% (Johnson, 2000). The majority of the albumen proteins is secreted by the tubular gland cells of the magnum except avidin which is synthesized by the goblet cells (Johnson, 2000). The major proteins found in albumen are; ovalbumen (54%); ovotransferrin (13%); ovomucoid (11%); ovoglobulins (8%); lysozyme (3.5%) and ovomucin (1.5-3%) (Hincke *et al.*, 2010; Johnson, 2000). Other characteristic proteins include; serpin, transferrin, protease inhibitors Kazal, glycosyl hydrolases, lipocalin, bactericidal permeability-increasing protein and clusterin (Guerin-Dubiard *et al.,* 2006).

Ovomucin is a protein that helps in the maintenance of the structural integrity of the egg while ovotransferrin, avidin and lysozyme have antimicrobial properties (Johnson, 2000). The alkaline pH of the albumen and the presence of proteins such as ovotransferrin and lysozyme significantly reduce the growth of micro organisms (Deeming 2002, as cited in Hincke *et al.*, 2010). The pH of freshly laid egg albumen (7.6) quickly rises to 9.5 during storage, which provides an unfavourable media for most bacterial growth (Sharp and Powell, 1931). Albumen quality is a standard measure of egg quality that is most often measured at the height of the inner thick albumen or a function of this, the Haugh unit (Silversides and Scott, 2001). The major influences on albumen height are the strain and age of the hen, storage time and conditions (Silversides and Scott, 2001). The albumen height of all eggs is at a maximum when the egg is laid and decreases with increased storage time (Silversides and Budgell, 2004; Silversides and Scott, 2001). One of the parameters that indicate the freshness of the egg is Haugh unit, devised by Haugh in 1937. Haugh unit is an expression relating to egg weight and

the height of the thick albumen (Curtis, Online). The higher the Haugh unit value of the albumen, the better is the internal quality of the egg. As the Haugh unit is a function of the height of the thick albumen and the weight of the egg, it will always be dependent on egg weight (Honkatukia *et al.,* 2005). Thus, especially for heavy eggs, Haugh values could be biased and might not be comparable to eggs of medium weight (Honkatukia *et al.,* 2005). With the advancing age of the hen, the albumen solids decreased while the total egg solids increased (Curtis, Online). CO_2 is released from albumen during storage, which increases the alkalinity and processes of albumen liquefaction leading to the progressive decline in the albumen quality (Karoui *et al.,* 2008).

1.3.1.3 Eggshell

Eggshell quality is of utmost importance to the commercial egg industry as well as in breeder flocks. The eggshell is essential for the propagation of all avian species and its properties reflect its crucial functions in reproduction (Nys *et al.,* 2004). The losses of eggs due to poor shell quality result in a loss of revenue for producers (Bain, 2004; Ingram *et al.,* 2008).

The avian eggshell forms in a confined space, the distal segment of the hen oviduct (Gautron *et al.,* Online; Hodges, 1974, Johnson, 2000; Jonchere *et al.,* 2010; Wyburn *et al.,* 1973;), in an acellular uterine fluid that is supersaturated with respect to calcium and bicarbonate and contains organic precursors of the shell matrix (Fernandez *et al.*, 2003; Hincke *et al.*, 2000; Nys *et al.*, 2004; Parsons, 1982). The eggshell's distinctive features, as compared to bone or teeth, are the nature of the mineral deposit - calcium carbonate in the form of calcite, as well as the absence of cells – whose assembly is directed upon organic cores present on the outer surface of the eggshell membranes (Fernandez *et al.,* 2003; Hincke *et al.,* 2000). Thus the eggshell formation process follows a definitive sequential formula (structural organization), after the ovulation of the ovum into the oviduct (Nys *et al.,* 2004).

The eggshell is a natural composite bioceramic containing organic (5% including membranes), and inorganic (calcite) (95%) components (Gautron *et al.,* Online; Hincke *et al.*, 2000; Hunton, 1995; Johnston *et al.*, 1963; Parsons, 1982; Solomon, 1991;), and is composed of a two-layered membrane and calcified extracellular matrix which are sequentially assembled during a 22 hr period (Arias and Fernandez, 2003; Dennis *et al.,* 1996; Gautron *et al.,* 1996; Nys *et al.,* 1999; Nascimento and Solomon, 1991). The eggshell is an envelope surrounding the egg that is 0.4 mm thick and about 5 g in weight in domestic fowl (Simkiss and Taylor, 1971). The organic contents of the shell are

distributed either in a definite concentration, the mammillary cores and the cuticle, or in a more diffuse form, the shell matrix, throughout the greater part of the shell substance (Hodges, 1974; Wyburn *et al.,* 1973). Nys *et al.* (2004) and Panheleux *et al.* (1999) mention that, during the 16-17 hr period of calcification, rapid calcium carbonate deposition continues outward to give rise to the inner mammillary body (cone) layer and outer palisade (calcitic) layers. Mineralization occurs in the uterine fluid, an acellular environment containing ionized calcium and bicarbonate greatly in excess of the solubility product for calcite (Johnston *et al.,* 1963; Nys *et al.,* 1999; Nys *et al.,* 1991). Fernandez *et al. (*1997), Gautron *et al.* (1996) and Nys *et al.* (2004) explain that eggshell calcification is amongst the most rapid mineralization processes known, with a precise temporal and spatial control of its sequential formation. Thus, eggshell formation occurs in an acellular environment, in contrast to other mineralized tissues, and its particular mineral structure results from self organization of mineral and organic precursors that are secreted into the fluid bathing the eggshell during its deposition (Hincke *et al.,* 2010; Nys *et al.,* 2004). This sequential process and high degree of structural organization has been observed by microscopic study of whole shell and partially demineralized eggshell (Dennis *et al.,* 1996; Solomon, 1991). The ionic and organic constituents of the uterine fluid change progressively during eggshell formation and can be subdivided into the successive stages of initiation (5 hr), growth (12 hr) and termination (1.5 hr) of eggshell mineralization (Gautron *et al.,* Online; Hincke *et al.,* 2010; Nys *et al.,* 1999).

Alterations in eggshell properties are directly related to increasing risks of egg contamination and food borne outbreaks for the consumer (Hincke *et al.,* 2010). The dynamic stiffness is a novel eggshell quality measurement, which could be utilized by the poultry breeders as it has a moderate heritability and high genetic and phenotypic correlation with other shell quality traits including eggshell breaking strength (Bain, 2004). Shell thickness is often used synonymously to shell strength, however a thicker shell in not necessarily a stiffer or stronger shell (Bain, 2004).

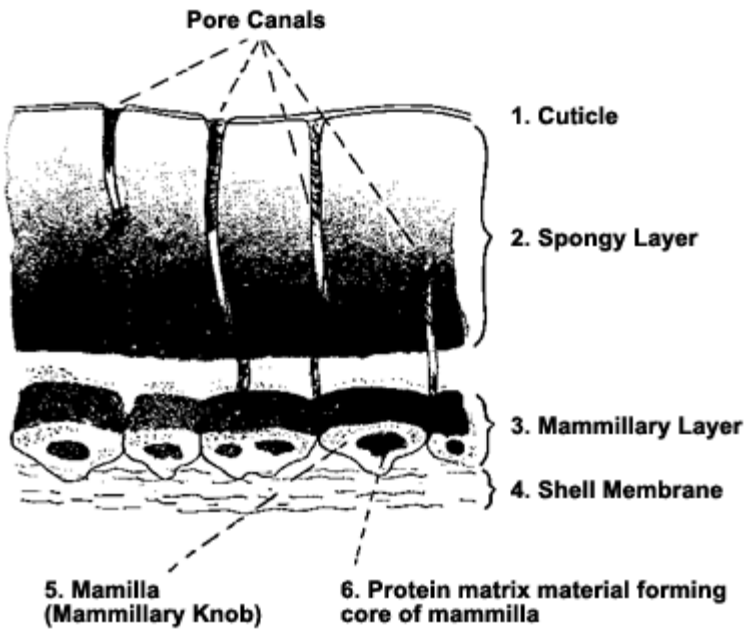

Plate 1.3.1.3.1 Structure of eggshell

Source: http://people.eku.edu/ritchisong/avianreproduction.html

Five different proteins have been identified in the organic matrix of the hen eggshell. Ovocleidin 17 (OC-17) is a soluble matrix protein component which has been purified to homogeneity and has been localized in the palisade and mammillary layer (Hincke *et al.,* 1995). Osteopontin, a bone phosphorylated glycoprotein involved in formation and remodeling of the mineralized tissue (Hincke *et al.,* 2008b; McKee and Nanci, 1996) has also been demonstrated in the hen eggshell (Pines *et al.,* 1994). Ovalbumin, an egg white protein, has also been identified and localized in the mammillary knobs of the eggshell (Hincke, *et al.,* 1995). Ovocalaxin 36 proteins play an important role in keeping the egg free from pathogens as they possess highly similarities in their protein sequence to lipopolysaccharide binding proteins, bactericidal/permeability increasing (BPI) and plunc family proteins (Gantois *et al.,* 2009).

Ultrastructural analysis of the eggs of commercial laying flocks at various periods in their laying year has served to illustrate the complex morphological variations which occur in association with the ageing process (Solomon, 1992). The eggshell constitutes a natural physical barrier against bacterial penetration if it forms correctly and remains intact (Jonchere *et al.,* 2010). Each eggshell has

approximately 10000 – 20000 pores for gaseous exchange with the greatest numbers occurring at the equator or the blunt pole of the egg (Solomon, 1994, 2009).

Calcium is a vital element that plays an important role either in ionic or in conjugated form in most of the physiological functions of the body. It plays a multifunctional role, ranging from triggering nerve impulses (Marks, 2003) to hard tissue formation such as bone (Sharan *et al.,* 2008) and eggshell (Johnson, 2000; Lavelin *et al.,* 2000). The hen's shell gland transports 2-2.5g of calcium within a period of 15 hr for the calcification of a single egg (Johnson, 2000). A hen that lays approximately 280 eggs in a single production year will use a quantity of calcium for the purpose of shell formation corresponding to 30 times the calcium content of the entire body (Johnson, 2000). The blood plays a vital role by providing calcium to the shell gland for eggshell formation through absorption from the chyme in the small intestine. Bone is the secondary source of calcium for eggshell formation and the initial source of calcium is medullary bone with a calcium release from cortical bone occurring when required, especially during calcium deficiency. Absorption of calcium through the intestine is facilitated via 1, 25 dihydroxyvitamin D_3 while resorption from the bone is mainly regulated by parathyroid hormone and 1, 25 dihydroxyvitamin D_3 (Johnson, 2000).

1.3.1.3.1 Organic components of the eggshell

a) Shell membranes

The shell membranes are a double layer of fibrous material surrounding the albumen (Hodges, 1974; Nys *et al.,* 1999; Solomon, 2009) and are about 70 µm in thickness (Gilbert, 1971; Johnson, 2000; Simons and Wiertz, 1963). The fibres are 0.8 to 1 µm thick, having a keratin core surrounded by a less electron dense mantle of about 0.5 µm (Simons and Wiertz, 1963). As the yolk and albumen complex travel through the proximal (white) isthmus, the membranes are acquired over a 1 to 2 hour period (Hincke *et al.,* 2010; Nys *et al.,* 1999). This mesh-work of interwoven fibres is considered to be the innermost component of the eggshell, and is organized into morphologically distinct inner and outer layers (Hincke *et al.,* 2010). The inner membrane (20-22 µm) remain uncalcified; while the fibres of the outer shell membrane (50 µm) penetrate the mammillary cones of the calcified shell, thus providing initial nucleation sites for mammillary cone mineralization (Arias *et al.,* 1991; Nys *et al.,* 2004). The membranes are composed of 10% collagen (types I, V and X) and 70–75% of other proteins and glycoprotein containing lysine derived cross links (Wong *et al.,* 1984; Fernandez *et al.,*

2001). The outer membrane consists of three layers, the outermost of keratin fibres and other two of mucin fibres, and the inner membrane consists of two indistinct layers of keratin and mucin (Baker and Balch, 1962). Each fibre in the membrane consists of a protein core surrounded by a mantle of carbohydrates (Solomon, 2009). The eggshell membranes contain 1.85% ash and, on the ash free basis, 15.54% of nitrogen (Baker and Balch, 1962). The membranes are semipermeable and permit the passage of water and crystalloids but not albumen and there is no relationship between the thickness of the membrane and thickness of the shell but the membrane thickness does decrease with the age of the hen (Johnson, 2000) and indeed fluctuates during the laying period (Solomon, 2009). Shell membranes are thought to play an important role in the formation of an eggshell (Arias and Fernandez, 2003) . Arias and Fernandez (2003) argue that the shell membranes never mineralize, due to an inhibitory effect of type X collagen, and act as a substrate for the deposition of the mammillary knobs. The outer and inner shell membranes are considered one of the most important lines of defense for the egg's internal contents. Board and Halls (1973) and Wang and Slavik (1998) reported that the outer membrane is thicker but more porous than the inner one; therefore it does not provide as much protection as the inner membrane. They further state that the inner shell membrane is very rich in lysozyme, which prevents bacteria from invading the egg contents. Comparing the microbial defense properties of the shell membrane and the eggshell, Lifshitz *et al.* (1964) ranked the inner shell membrane the most important one followed by shell and outer shell membrane. Shell membranes also provide a barrier to prevent inward mineralization (Nys *et al.*, 2004).

b) Mammillary cores

The mammillary cores are the projections from the outer membrane surface and are the initial sites of calcification (Johnson, 2000). Sturkie and Mueller (1976) state that the deposition of the mammillary cores and their initial calcification occur in the isthmus. Simkiss and Tyler (1957) studied the composition of the mammillary cores and reported the presence of protein, carbohydrate and fat.

c) Shell matrix

The eggshell matrix (2-3.5% of the organic matrix) is a series of layers of proteins and acid mucopolysaccharide on which calcification takes place (Johnson 2000; Nys *et al.*, 1999, 2001). The shell matrix functions in the fabrication of eggshell and participate in antimicrobial defense (Hincke *et al.*, 2011). The protein components of shell matrix function to modulate crystal nucleation and

growth, and thus influence the shape and strength of the final eggshell structure (Hincke *et al.,* 1995). Matrix proteins extracted from eggshell demineralization have shown antimicrobial activities against *Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Salmonella* Enteritidis (Mine *et al.,* 2003).

d) Cuticle

The cuticle is the outermost relatively thin non calcified organic layer, which is deposited directly onto the vertical crystal layer of the eggshell (Parsons, 1982). Parsons (1982) and Sparks (1994) described the cuticle as an uneven structure, with many star shaped cracks, fissures and flake-like layers when viewed from above with the electron microscope. The cuticle is composed of spheres which, on the chicken egg are <1 μm in diameter, forming an uneven layer 0.5–2.8 μm thick (Kusuda *et al.,* 2011; Simons and Wiertz, 1963; Wellman-Labadie *et al.,* 2008). The cuticle is of variable thickness and may even be missing, and is composed of glycoprotein (90%), polysaccharides (4%), lipids (3%) and inorganic phosphorus (3%) including hydroxyapatite crystals (Fernandez *et al.,* 2003; Wang and Slavik, 1998). The thickness of the cuticle depends on its location; the thinnest part at the top of the columns and thickest part between the columns of the eggshell (Kusuda *et al.,* 2011). Simons and Wiertz (1963) state that the amount of cuticle present on brown eggs is greater than on white eggs. Ruiz and Lunam (2000) reported that hatching egg viability is independent of the cuticle thickness and that thickness varies with the production cycle of the hen with minimum amounts of cuticle at the start and end of the cycle. This layer, as well as the outer portion of the calcified shell, contains the eggshell pigments responsible for shell colour (Hincke *et al.,* 2010; Miksik *et al.,* 2007; Nys *et al.,* 1991). The cuticle is manufactured in non-ciliated secretary cells lining the uterus (Solomon, 1991) and is secreted as a granular substance (Cooke and Balch, 1970) that contains a thin layer of hydroxyapatite in its inner zone and the bulk (2/3) of the superficial eggshell pigments (Nys *et al.,* 2004). Transmission Electron Microscopy (TEM) has revealed inner and outer layers of the cuticle (Ruiz and Lunam, 2000). The inner layer (vesicular layer) is composed of vesicles each consisting of a core and a mantle showing electron lucent and electron dense properties, respectively (Fraser *et al.,* 1999). The outer cuticle layer is much more compact and homogenous and does not appear to contain any matrix vesicles, hence called the non vesicular cuticle (Fraser *et al.,* 1999). Dennis *et al.* (1996) described a single layered vesicular cuticle, containing a mineral rich inner region and a mineral poor outer region. Cuticular vesicles contain hydroxyapatite, which is believed

to play a role in the shell termination process (Fraser *et al.*, 1999). Kim and Slavik (1996) reported that some egg washing chemicals, such as 0.5% trisodium phosphate and 50 ppm cetylpyridinium chloride can damage the cuticle layer and thereby facilitate microbial penetration. The cuticle is considered to be the first line of defense against microbial invasion (Miksik *et al.*, 2007). In addition to keeping the water and electrolyte levels of the egg contents in balance by preventing evaporation, the cuticle also enhances many fold the antimicrobial defenses of the egg by physically preventing microbial contamination (De Reu *et al.*, 2005; Fraser *et al.*, 1999; Thompson and Goldie, 1990) with some of its chemical components also involved in the antimicrobial defense of the egg (Wellman-Labadie *et al.*, 2008). The cuticle may function as a lubricant that facilitates rotation of the egg during the last 4 hr of the uterine period (Kusuda *et al.*, 2011; Rahman, *et al.*, 2009). The amount of the cuticle present on the eggshell also affects shell thickness which is directly linked to shell strength and the absence of cuticle may decrease shell thickness (Belyavin and Boorman, 1980). Sparks and Board (1984) states that cuticle thickness significantly decreases with increasing age of the hen. Peebles *et al.* (1998) concluded that eggshell cuticle removal in hatching eggs from hens during post peak production may lead to subsequent increases in feed consumption of broiler offspring. Miksik *et al.* (2007) extracted proteins of the insoluble eggshell matrix (ovocalyxin 32, ovocleidin 116, ovocalixin 36 and Ovocleidin 17) from cuticle using the PHLC-MS/MS method.

e) **Pigment**

Present knowledge about shell colour is limited as the published information does not contain much quantitative data or data about variability of pigment content (Miksik *et al.*, 1994). The eggshell colour of brown eggs is a quality aspect that is important for the perception of the consumer (Mertens *et al.*, 2010). The major pigments of avian eggshells are protoporphyrin, zinc porphyrin, biliverdin and zinc biliverdin (Kennedy and Vevers, 1973; Mertens *et al.*, 2010). Porphyrin, which gives most of the colour to an eggshell, is an organic compound composed of four rings of pyrrole. A protein, L-dopachrome tautomerase, is involved in the formation of egg pigments (Jonchere *et al.*, 2010). Kennedy and Vevers (1973) observed that brown or black pigments are associated with protoporphyrin IX, whereas blue or green shades result from biliverdin IX or zinc biliverdin chelate, respectively. With (1973) found, in brown hen eggs, a mixture of porphyrins including protoporphyrin, coproporphyrin, pentacarboxylic porphyrin, uroporphyrin and unidentified porphyrin as well. Sparks (1994) reviewed that the protoporphyrin responsible for the pigmentation of the

chicken egg occurs primarily in the cuticle, yet pigment can be detected in the mineralized shell immediately apposed to the cuticle. Whether the porphyrin compound is synthesized in the liver or locally secreted by the epithelial cells of the uterus is still controversial and different authors have different views. Solomon (1991) states that there is certainly a cyclical build up and release of pigment in and from the surface epithelial cells of the shell gland pouch, as shown by changing patterns in the autofluorescence of these cells and from the biochemical extraction of pigment from the pouch region. Baird *et al.* (1975) argued that the porphyrins are synthesized in the blood and transferred via the surface epithelial cells of the shell gland. However, the earlier view of Giersberg (1921) as cited in Kennedy and Vevers (1973) was quite different and states that, from histological evidence, eggshell pigments are derived from the disintegration of erythrocytes in the mucous layer of the oviduct, and are transported by wandering cells which penetrate the uterine epithelium in the final stages of calcification; at the same time these cells develop dark pigment granules during their migration. Porphyrin has a remarkable affinity for calcium and, in all sites where they are found, calcium is closely associated (Kennedy and Vevers, 1973). Odabasi *et al.* (2007) mention that egg size has a greater effect on shell colour and hens lay lighter colour eggs due to an increase in egg size associated with on proportionate change in the quantity of pigment deposited over the shell surface.

Miksik *et al.* (1996) point out that the function of avian eggshell pigmentation is mainly for cryptic reasons. They explain that the demand for minimum solar heating of the eggs is unlikely to play an important role in colouration as studies on spectral reflectance proved that differently coloured eggs exhibit uniformly high reflectance in the near infrared region, independent of the eggshell colour. These authors further elaborate that the changes in eggshell colouration may reflect physiological conditions such as egg laying and nesting, but they may result from exogenous (environmental) factors as well. Ishikawa *et al.* (2010) have confirmed through their experiments that eggshell pigments have antimicrobial activity particularly against gram positive bacteria. Eggshell pigment is one of the tools for assessing stress and disease conditions in the laying hens. Some feed additives like Nicarbazin (25 ppm), an anti coccidial drug, causes acute depigmentation within 2-4 days of the feed intake (Charlton *et al.*, 2005). These authors state that stress factors such as high cage density, fear and frequent disturbances can change the colour of brown eggs. Enforced molting is also one of the contributing factors to reversible shell depigmentation. Miksik *et al.* (2007) found 70% of the protoporphyrin IX in the cuticle, 27% in the first palisade layer, 2% in the second palisade layer and 1% in the mammillary layer. Jones *et al.* (2010) report that shell colour affects physical qualities of

the egg including shell strength with brown eggs being higher in values than white eggs for shell weight, shell thickness, shell strength (g) and vitelline membrane strength (g). Among the various methods for colour measurement, reflectivity is the most commonly applied technique followed by spectrophotometry based on the L*a*b colour space system. L* represents the clarity of the colour (black= 0 and white= 100), a* indicates the colour position between red and green (a*>0 for more red and a*<0 for more green) and b* represents the colour position between yellow and blue (b*>0 for more yellow and b*<0 for more blue) as described in Mertens *et al.,* (2010).

1.3.1.3.2 Inorganic components of the eggshell

The calcified eggshell consists primarily of calcite, the most stable polymorph of calcium carbonate and is progressively composed of the inner mammillary cone layer, central palisade and the outer vertical crystal layers (Dennis *et al.,* 1996; Fraser *et al.,* 1999; Miksik *et al.,* 2007; Nys *et al.,* 1999, 2004; Parsons, 1982; Solomon, 1991). The true eggshell calcified layer varies in thickness from 200-300 μm covered by a very thin layer of the cuticle which is 2-20 μm (Miksik *et al.,* 2007).

a) Mammillary layer

The mammillary layer comprises about one third to one fifth of the total thickness of the shell (Hodges, 1974). It consists of numerous roughly conical knobs, the mammillae, whose apices are embedded in the outer shell membrane and whose irregular bases are fused together to form the foundation of the palisade layer (Fernandez *et al.,* 2003, Rose and Hincke, 2009). The mammillae are irregularly spaced across the inner surface of the shell (Solomon, 1991). Centrally within the tip of each mammilla is a mass of protein material, the mammillary core, which appears to be the centre where calcification starts during the formation of the shell (Simkiss, 1967, as cited in Hodges 1974).

The mammillary layer is a regular array of cones or knobs (Parsons, 1982), with highly organic cores, into which are embedded the individual fibres of the outer eggshell membranes (Hincke *et al.,* 2010). The mammillary knobs have well defined sub regions (Nys *et al.,* 1999), the calcium reserve assembly, which consists of an association of an organic base plate supporting a matrix embedded in calcium which terminates in a distinct structure, a calcium reserve body and a clearly identified crown (Dennis *et al.,* 1996; Dieckert *et al.,* 1989). Within the mammillary cone layer, microcrystals of calcite (Dennis *et al.,* 1996) are arranged with spherulitic texture which facilitates the propagation

of cracks during piping of hatching eggs as well as the mobilization of calcium for use by the embryo by dissolution of highly reactive calcite micro crystals (Nys *et al.,* 2004).

Solomon (1991) reported a number of variations in the structure of the mammillary layer, some of which are associated with reduced shell quality and some with improved shell quality.

- **Ultrastructural variables that have a positive effect on mammillary layer**

High mammillary density, cuffing, mammillary confluence, good cap formation and early fusion of the mammillary layers are factors resulting in the formation of a good quality shell (Solomon, 1992; Nascimento *et al.,* 1992).

- **Ultrastructural variables that have a negative effect on mammillary layer**

A higher incidence of poor cap quality, late fusion, alignment, Type A mammillary bodies, Type B mammillary bodies, aragonite, cubics, cubic cone formation, changed membranes, depression, erosion, pitting and hole imparts poor quality to the mammillary layer and results in weakening of the shell (Solomon, 1992, 1985; Solomon and Bain, online).

The mammillary caps arise from the deposition of calcium carbonate into the membrane fibres (Solomon and Bain, online) such that the shell membranes are attached to the mammillary caps. Late fusion slows the commencement of growth of the palisade columns and reduces the effective thickness of the shell (Solomon and Bain, online). Mammillary alignment encourages crack growth, and thus increases the incidence of downgrading of the egg and the risk of microbial attack (Nascimento *et al.,* 1992; Solomon, 1992). Type A mammillary bodies are often found in the eggs of young birds and in the eggshells of hens that have been stressed (Solomon, 1985). Type B mammillary bodies are spherical concretions normally located between otherwise normal mammillae and show no obvious points of contact with the membrane fibres (Solomon, 1985). Type B mammillary bodies occur more frequently in the mammillary layer than do the Type A bodies (Solomon, 1991). Type B mammillary bodies make contact with the membrane fibres to varying degrees and they make no contribution to the thickness of the true shell, which is more susceptible to breakage (Solomon, 1992). Extra crystalline cuffs at the junction of the cone and palisade layers (the phenomenon of cuffing) assist in the early fusion of the palisade columns and thereby increase the lateral distribution of stress within the shell (Solomon, 1991). Aragonite, normally found in reptilian

shells, occurs in hen eggshells as a consequence of changes in the rate of mineralization mostly because of stress factors (Solomon, 1992; Solomon and Bain, online). The presence of too much aragonite results in an open framework which leads to weaker shell formation and so the egg is more exposed to microbial attack. The incidence of aragonite was higher in poor quality eggs (Nascimento and Solomon, 1991). The more open framework, with poor membrane attachment associated with a high incidence of aragonite increased permeability of the shell to bacteria and decreased fracture toughness. Pitting of the mammillary layer, often caused by the accumulation of oviducal debris on the shell membranes so obscuring potential nucleation sites, causes patchy thinning of the shell (Solomon, 1992). Nascimento and Solomon (1991) reported that pitting damages the eggshell capacity to prevent microbial entry into the egg. The density of the mammillary knobs in the shell is inversely related to the breaking strength of the shell (Van Toledo *et al.*, 1982).

b) Palisade layer

The palisade layer represents the greatest portion (~300 μm thick) of the shell and is mainly composed of crystalline calcium carbonate in calcite form that arises from the nuclei of mammillary knobs (Johnson, 2000; Rodriguez-Navarro *et al.*, 2002; Rose and Hincke, 2009). The palisade layer is arranged as groups of columns extending for 200 μm (Nys *et al.*, 1999; Rodriguez-Navarro *et al.*, 2002) outwards from the mammillary knobs perpendicular to the surface (Hincke *et al.*, 2010; Silyn-Roberts and Sharp, 1986) and at high magnification presents a faceted appearance (Nys *et al.*, 1999). The appearance of this layer is dominated by fibrils which are maximally 10 μm long and 0.01 μm thick, running parallel to the surface of the eggshell (Simons and Wiertz, 1963). This layer ends at the vertical single crystal layer which has a crystalline structure of higher density than that of the palisade region (Hincke *et al.*, 2010). The outer region of the palisade layer is a tough structure made of large crystals where the external impacts are absorbed by thin intercrystalline organic layers that make intercrystalline crack propagation difficult (Nys *et al.*, 2004). The mineral content of the palisade layer is essentially a continuation of the crystal columns which originate in the mammillary layer (Hodges, 1974).

c) Surface crystal layer

This is the outermost layer of calcification which is 3 to 8 μm thick (Johnson, 2000) between the cuticle and palisade layer (Miksik *et al.*, 2007; Parsons, 1982). Its vertical deposition may result from the perpendicular orientation of the matrix to the surface (Miksik *et al.*, 2007; Parsons, 1982).

1.4 Egg washing/Sanitizing

Disinfecting eggshells is a common and fundamental practice to eliminate or reduce populations of pathogens on the surface of eggshells (Park *et al.*, 2005). Washing of eggs is practiced by some commercial layer farmers in order to remove most of the debris including some microbes from the outer surface of the eggshell. Some countries like Australia, United States, Canada and Japan allow the eggs to be washed and graded before being packaged for retail, while European Union countries are reluctant to allow the washing of grade A eggs (Hutchison *et al.*, 2004; Leleu *et al.*, 2011; Sparks, 1994). In Australia, most commercially produced eggs are washed before marketing (Hutchison *et al.*, 2004). Microbial quality is improved when eggs are washed soon after lay with clean, warm, low iron content water containing approved sanitizer detergents, dried immediately, and stored at a cool temperature after packaging (Musgrove *et al.*, 2008). Egg washing can also decrease the shelf life of eggs as the wet surfaces increase the chances of microbial cross contamination if the egg surfaces are not properly dried (Leleu *et al.*, 2011). Effectiveness of the sanitizer against debris and microbes, safety to workers and environment and economic feasibility are the factors that help in selecting an appropriate disinfectant (Park *et al.*, 2005). Disinfectants commonly used for egg washing contain chlorine, quaternary ammonium and disodium carbonate, peroxidase catalyzed compounds, zinc sulphate solution, formaldehyde and electrolyzed water (Park *et al.*, 2005). Washing eggs with water colder than the egg, with water heavily contaminated with bacteria, in machines whose surfaces are contaminated with large numbers of microorganisms are factors which increase the chances of bacterial cross contamination during egg washing (Moats, 1978; Zeidler, 2002; Hutchison *et al.*, 2003). At the present time, most commercial eggs are washed in a special machine called a "continuous egg washer" that consists of three chambers; egg spraying chamber, sanitizing chamber and drying chamber (Sparks, 1994). Messens *et al.* (2009) concluded that the standard washing procedure utilized in Sweden does not affect the quality of the cuticle of table eggs. However, cuticle damage resulting from egg washing needs to be monitored.

Wang and Slavik (1998) concluded from their experiments that there were significant differences in the microstructure of eggshells washed with different chemicals, e.g. quaternary ammonium compounds (QAC) did not damage cuticle layers but left faecal stains and chemical residues on shell surfaces. Other chemicals used, alkaline solutions, sodium carbonate, and sodium hydroxides produced visually clean eggs but were hard enough to partially or completely remove the cuticle layers. In the experiments of Wang and Slavik (1998), eggs washed with alkaline chemicals were 30 to 70 times more porous than eggs washed with other chemicals. A significant reduction was recorded between control and UV treated eggs for aerobic plate count (Chavez *et al.,* 2002), *Salmonella* Typhimurium and *Escherichia coli* load (Coufal *et al.,* 2003). Similar results ($p < 0.05$) were obtained by Knape *et al.* (2001) by washing eggs with various chemical disinfectants after inoculation of *Salmonella* Typhimurium and *Salmonella* Enteritidis on the eggshell surface.

Musgrove *et al.* (2004) have confirmed that washed eggs have significantly lower numbers of Enterobacteriaceae on the outside of the eggs than unwashed eggs. A signification reduction in the total cfu of *Listeria monocytogenes* and *Salmonella* Enteritidis was recorded when eggs were washed first with deionized and then alkaline water (Park *et al.,* 2005). Washing improves the microbiological quality of eggs, and in a study conducted by Jones *et al.* (2004), significantly fewer bacteria were found on the washed eggs decreasing from 2.5 log cfu/mL on the day of collection to 1.0 log cfu/mL. For the unwashed eggs, aerobic shell counts remained at about 4 log cfu/mL throughout most of the storage time, increasing to 5.3 log cfu/mL at 8 weeks. Similar studies conducted by Musgrove *et al.* (2005) showed that commercial egg processing (including washing) significantly reduced the total bacterial, yeast and mould load on the eggshell surface. Manfreda *et al.* (2010) evaluated the decontamination power of hot air treatment of eggs treated experimentally with *Salmonella enterica*, *Escherichia coli* and *Listeria monocytogenes*, in which the *Salmonella* Enteritidis and *Listeria monocytogenes* populations on the surfaces of treated eggs showed a significant reduction compared with the untreated eggs. Caudill *et al.* (2010) suggested that incorporating cold water into commercial eggshell processing, while maintaining at pH of 10 to 12, lowers post processing egg temperature and allows for more rapid cooling, without causing a decline in egg quality or increasing the presence of aerobic microorganisms and fungi for approximately 5 weeks post processing.

1.5 Eggshell contamination and housing system

Housing plays an important role in the production of quality eggs although proper sanitizing measures can reduce the chances of bacterial contamination of eggs to a large extent. De Reu *et al.* (2008) state that contamination of eggshells with aerobic bacteria is generally higher for nest eggs from non cage systems compared to nest eggs from furnished cages or eggs from conventional cages. Quarles *et al.* (1970) reported that litter floor houses had, on average, approximately nine times more bacteria in the air, and twenty to thirty times more aerobic bacteria on the shell than wire floor houses. A high bacterial load of floor eggs (>6.3 log cfu total aerobic flora/eggshell) compared to cage egg (5.08 log cfu/eggshell) was obtained (De Reu *et al.*, 2006c).

1.6 Hen molting

Molting is a natural physiological process that occurs in chickens, fowl, and other avian species, allowing a cessation of egg production and permitting the reproductive tract and plumage time to regenerate (Kretzschmar-McCluskey *et al.,* 2008). One of the main disadvantages of molting is increased shedding of bacteria in the faeces and their ultimate increased count in the internal organs (Holt, 2003). Molting increases the productive life of a hen and, with proper procedures, it is possible to achieve a productive life from 70 to 105 wk of age with an additional 25 to 30 wk of production if a second molt is used (Butcher and Miles, 1994). Restriction of feed is one of the common methods used in conventional forced molting, however many poultry companies are now using non-fasting programs (Kretzschmar-McCluskey *et al.,* 2008). In the studies of (Kretzschmar-McCluskey *et al.,* 2008), there was a significant effect ($p<0.05$) of molting on the frequency of *Salmonella* positive samples, however it was also concluded that the feed restricted treatment had the lowest frequency of *Salmonella* when compared with the non fasted and non molted groups. Molted hens shed more bacteria (*Salmonella*) than unmolted hens and shedding transmits bacteria horizontally to the healthy hens (Holt, 1995). Holt (1995) concluded that induced molting can have a substantial effect on transmission of *Salmonella* Enteritidis to uninfected hens, which could affect the overall *Salmonella* Enteritidis status of a flock.

1.7 Egg, bacteria and Enterobacteriaceae

Enterobacteriaceae are organisms that are found primarily in the gastrointestinal tract of animals and human, but may invade other organs and cause an infection (Pang *et al.,* 2011). Bacteria of primary concern are *Escherichia coli, Salmonella, Yersinia* and *Shigella*. Some members of this family are opportunistic (Quinn *et al.,* 2002). Enterobacteriaceae are gram negative facultative rod shaped anaerobes, which are oxidase negative as they lack cytochrome oxidase (Quinn *et al.,* 2002). *Escherichia coli* are lactose fermenting bacteria while *Salmonella, Shigella,* and *Yersinia* lack this characteristic (Quinn *et al.,* 2002). Some other species that do not belong to Enterobacteriaceae but can cause food spoilage particularly of egg are; *Acinetobacter* and *Pseudomonas* which are gram negative anaerobes (Jones and Musgrove, 2008). The *Salmonella* are chemoorganotrophic, gram negative rods, which are relatively small bacteria measuring about 0.5µm by 2 to 3µm and most strains are motile with peritrichous flagella (Cox *et al.,* 2000; Alakomi and Saarela, 2009). *Salmonella* can be found within a variety of phagocytic and non phagocytic cells *in vivo* (Ibarra and Steele-Mortimer, 2009). *Salmonella* grows at 7-48°C with an optimum growth temperature at 37°C and at pH 4 to 9.5 with an optimal growth at pH 6.5 to 7.5 (Alakomi and Saarela, 2009). Following intestinal colonization, *Salmonella* enters enterocytes, M cells and dendritic cells in the intestinal epithelium and is then internalized by macrophages from sub mucosa from where it is disseminated through the bloodstream (Ibarra and Steele-Mortimer, 2009).

1.7.1 Microorganisms found on the shell and in the egg

Gram-positive bacteria are the dominant microflora of the egg and eggshell, whereas gram-negative bacteria are better equipped to overcome the antimicrobial defenses of the egg content (De Reu *et al.,* 2002, 2008, 2010) and thus the latter are the principal contaminants of rotten eggs (Clay and Board, 1991; Al-Natour *et al.,* 2011).

1.7.1.1 Bacteria on the shell and pores

Bacteria found on the eggshell and in the pores include: *Micrococcus, Achromobacter, Aerobacter, alcaligenes, Arthrobacter, Bacillus, Cytophaga, Flavobacterium, Pseudomonas, Staphylococcus, Aeromonas, Proteus, Sarcina, Serratia, Streptococcus, Klebsiella* spp., *Campylobacter* spp., *Kluyvera, Providencia, Rahnella, Citrobacter diversus, Enterobacter agglomerans, Enterobacter*

cloacae, Escherichia coli, Escherichia fergusonii, Salmonella spp., *Vibrio* spp., *Proteus* spp., *Tatumella ptyseos* (Musgrove *et al.,* 2008; 2004; De Reu *et al.,* 2008; Stepien-Pysniak, 2010; Mayes and Takeballi, 1983; Cook *et al.,* 2003).

1.7.1.2 In the egg

Bacteria found in the contents of the egg include: *Micrococcus, Achromobacter, alcaligenes, Arthrobacter, Bacillus, Cytophaga, Escherichia, Flavobacterium, Pseudomonas, Aeromonas, Proteus, Streptococcus, Citrobacter freundii, Acinetobacter* spp., *Salmonella* spp., *Vibrio* spp., *Tatumella ptyseos* (De Reu *et al.,* 2008; Stepien-Pysniak, 2010; Cook *et al.,* 2003).

1.7.2 Routes of egg contamination

Increasing consumer awareness of food safety issues has changed the public perception of a "good egg" from shell cleanliness and physical properties to that of microbial integrity (De Reu *et al.,* 2006). Microbial contamination of eggs is a well-established phenomenon and has important economic implications for the poultry industry (Bruce and Drysdale, 1994). Microorganisms can contaminate eggs at different stages, from production through processing to the preparation and consumption (De Reu *et al.,* 2006). Chickens are among the avian species that shed *Salmonella* and a number of other pathogenic bacteria in the faeces. These bacteria, in turn, attach the eggshell surface and make their way to the internal contents of the egg (Humphrey, 1994). Rate of penetration is affected by a number of factors such as bacterial load (De Reu *et al.,* 2006c) and egg shell properties (Solomon, 1992). When such infection occurs in hatching eggs, hatchability is reduced while, in commercial eggs, bacteria pose a serious threat to public health (Williams and Dillard, 1973). Eggs can be contaminated in two ways, namely horizontal (trans-shell) and vertical (transovarian) contamination (De Reu *et al.,* 2008). Some *Salmonella* serovars (e.g. Enteritidis) transmit vertically (Botteldoorn *et al.,* 2010; Gast and Beard, 1990; Miyamoto *et al.,* 1997) while others (e.g. Infantis and Typhimurium) enter through the eggshell (Aabo *et al.,* 2002; Barrow and Lovell, 1991) however these serovars can contaminate the egg through either route depending upon the source of contamination (Clavijo *et al.,* 2006; De Reu *et al.,* 2010; Miyamoto *et al.,* 1997). Miyamoto *et al.* (1997) inoculated hens with *Salmonella* Enteritidis using different routes and found that intravenous inoculation caused colonization of the ovary and contamination of eggs forming in the oviduct. Their experiments also revealed that intra vaginal inoculation led to the colonization of only the lower parts

of the oviduct, but internally contaminated eggs were being produced which suggests that some internal contamination of eggs may be coming from the lower oviduct through penetration of the eggshell in the oviduct.

1.7.3 Factors Affecting Microbial Penetration

The shell and internal contents of a freshly laid egg are mostly free from microbes as their formation and packaging occur in an aseptic environment in the healthy hen (Mayes and Takaballi, 1983), although contamination of an egg passing through the vent cannot always be ruled out (Mallet *et al.*, 2006). Kretzschmar-McCluskey *et al.* (2009) states that the egg is naturally equipped with barriers that help keep microorganisms from penetrating the interior shell, membranes, and egg contents. A number of factors like relative humidity (Gregory, 1948), overall shell quality (Sauter and Petersen, 1974; Solomon, 1991; Roberts, 2004), number of shell pores (Walden *et al.*, 1956; Brown *et al.*, 1965; Kraft *et al.*, 1958), temperature (Graves and Maclaury, 1962), pH (Sauter *et al.*, 1977) and bacterial load (Williams *et al.*, 1968) directly affect microbial penetration across the eggshell.

1.7.3.1 Egg quality

Sauter and Peterson (1974) found that whole eggs with low specific gravity or low shell quality were more likely to be penetrated by *Salmonella*. Berrang *et al.* (1998) reported the influence of egg weight, specific gravity, conductance and flock age on the ability of *Salmonella* to penetrate the shell and membranes. It has been established that the penetration of the eggshell and shell membranes is not a unique characteristic of *Salmonella* Enteritidis as it occurs also with other serotypes (Gantois *et al.*, 2009). In the egg penetration studies of De Reu *et al.* (2006), gram negative, motile and non clustering bacteria were found to penetrate the eggshell most frequently. The yolk of the egg has been shown to be an excellent growth medium for certain bacteria, and *Salmonella* is at the forefront of concern (Kretzschmar-McCluskey *et al.*, 2009). The three stages of microbial penetration are: penetration through the cuticle and shell, colonization of the shell membranes and contamination of the albumen and yolk leading ultimately to whole egg contamination (Lock *et al.*, 1992). Thus, with horizontal transmission, the egg contents are not contaminated until the cuticle, shell and shell membranes fail to prevent microbial invasion and penetration (Berrang *et al.*, 1999).

The part of the egg in which a microorganism is deposited depends largely on whether the microorganism becomes localized in the ovary, oviduct or cloaca (Barrow, 1994). The ability of organisms to become localized in these organs and remain viable depends on their resistance to innate or acquired immunity of the host (Barua and Yoshimura, 2004; Berndt *et al.,* 2007; Keller *et al.,* 1995). Eggs are most vulnerable to bacterial penetration in the first 30 to 60 seconds after laying before the cuticle hardens and effectively caps the pores (Berrang *et al.,* 1999). Shell thickness does not have a significant effect on bacterial penetration but the presence of cuticle plugging the shell pores is more important (William *et al.,* 1968). Cotter *et al.,* (1995) reported that eggs can be infected with *Salmonella* Enteritidis bacteria, with some abnormal shell production, after inoculation through the cloaca. Kraft *et al.* (1958) found that shell porosity appeared to be a useful index for determining susceptibility of eggs to bacterial penetration. Similarly, Vadehra *et al.* (1970) found that infection of eggshells after production is most readily achieved by contaminating the blunt end of the eggs. However, Nascimento and Solomon (1991) reported that bacterial penetration was independent of pore numbers. Cracked eggs are more prone to bacterial penetration and a 1000 times higher level of bacterial contamination was found in cracked eggs as compared to intact eggs in the studies of March (1969). In spite of the eggs barriers to microbes, *Salmonella* can penetrate the shell of the chicken egg (Miyamoto *et al.,* 1998).

Messens *et al.* (2005) concluded that *Salmonella* Enteritidis penetration through thin shelled eggs was greater than for thick shelled eggs while the amount of cuticle deposition did not affect *Salmonella* penetration. These findings are in contrast to the studies of De Reu *et al.* (2010) in which the mean cuticle deposition was lower for penetrated compared to the non penetrated eggshell. Similarly, Williams *et al.* (1968) concluded that shell thickness did not significantly affect the amount of penetration. In the eggshell penetration studies of De Reu *et al.* (2010), gram negative motile and non clustering bacteria penetrated the eggshell most frequently: *Pseudomonas* spp. (60%) and *Alcaligenes* spp. (58%) followed by *Salmonella* Enteritidis. In the same studies, in comparison with the non *Salmonella* strains, *Salmonella* Enteritidis was the primary invader of whole eggs (32%). *Salmonella* are known to secrete their own chelators during extended storage which enables them to compete with ovotransferrin, which in turn favors *Salmonella* survival and proliferation in the egg (Cudjoe *et al.,* 1994). Humphrey *et al.* (1991) stated that the survival of *Salmonella* Enteritidis in the egg albumen and subsequent proliferation depends on the age of the egg and the proximity of the organism to the yolk membrane. Iron (ferrous sulphate) has been shown to promote the proliferation

of gram negative bacteria in eggs (Clay and Board, 1991). Iron is also used successfully at levels of 35 mg/L in a non selective broth to isolate *Salmonella* from raw eggs (Gast, 1993).

1.7.3.2 Environment

Normally the prevalence of *Salmonella* in a positive flock varies with the husbandry conditions and *Salmonella* is not always recovered from eggs produced by positive flocks. Poppe *et al.* (1992) recovered *Salmonella* from only 2 out of 16000 eggs tested. Similarly, Humphrey *et al.* (1991, 1989) found low numbers of *Salmonella* positive eggs from naturally contaminated hens. The shell can already be infected when passing through the vent, but many researchers suggest that the main bacterial contamination occurs within a short period after laying due to contact with dirty surfaces (Quarles *et al.,* 1970; Gentry and Quarles, 1972). In the external contamination of eggshell with viable pathogens, the presence of chicken manure and other moist organic materials facilitates the survival and growth of *Salmonella* by providing the required nutrients and a degree of physical protection (Gantois *et al.,* 2009). The quick proliferation rate of *Salmonella* in eggs after artificial contamination with faeces suggests that faeces can serve as a nutritional reservoir for *Salmonella* (Schoeni *et al.,* 1995). There has been little systematic investigation of *Salmonella* contamination of eggshells from different production systems or on the effects of the production system on the internal bacterial contamination of eggs; thus shells contaminated with faecal and environmental *Salmonella* are an important potential source of this organism (Holt *et al.,* 2011).

Berrang *et al.* (1999) argued that eggshells can be penetrated by bacteria when water or some other liquid is present, especially if there is a temperature difference between the egg and the liquid. Due to the temperature difference between the hen and the environment, the freshly laid warm egg cools rapidly, resulting in egg contents contraction (Berrang *et al.,* 1999). These authors further explain that such contraction tends to form a negative pressure within the egg, and bacteria present in the environment or on the egg surface can then be pulled into and through the eggshell and its membranes. Vadehra *et al.* (1970) found that the air cell end is most prone to penetration (*Pseudomonas aeruginosa*) when challenged by a temperature differential immersion. Temperature and number of viable *Salmonella* play a vital role in the growth of bacteria and the growth rate of *Salmonella* markedly increases as the temperature increases above 4°C (Kim *et al.,* 1989) but declined rapidly at temperature above 42°C (Guan *et al.,* 2006). Eggshell penetration studies at 9°C,

25°C and 35°C showed maximum bacterial activity at 25°C (Stadelman, 1994). Jones *et al.* (2002) stated that the rate of cooling of an egg plays an important role in the microbial integrity of the egg by changing the physical properties of the vitelline membranes. The cryogenic egg treatment also increased the strength of the vitelline membrane, which was more prominent in poorer quality eggs (older flock) than the highest quality eggs (Jones *et al.,* 2002). Control of the proliferation of *Salmonella* within eggs may be achieved by their storage at lower than ambient temperature, which slows down both bacterial growth rates and changes to egg contents which facilitate *Salmonella* multiplication (Cogan *et al.,* 2004). William *et al.* (1968) found that eggs are penetrated almost immediately when challenged with moist *Salmonella*-contaminated chicken faeces. Relative humidity (50-65%) encourages survival of bacteria in the environment and on the eggshell surface (Messens *et al.,* 2006; Radkowski, 2002).

Globally, many cases of human gastroenteritis are directly linked with the consumption of eggs contaminated with *Salmonella* (Hald *et al.,* 2004). De Reu *et al.* (2008) concluded that, generally, aerobic bacterial counts on eggshells are lower from caged (conventional and furnished) than from non caged (aviary and floor) flocks, and this difference is very marked when eggs laid outside of the nest boxes in the non cage flocks are included, but the same difference could be seen when the investigation was done for Enterobacteriaceae. Stress factors like rehousing, thermal extremes, transport, initiation of egg lay and molting have all been shown to exacerbate infection susceptibility in poultry (Holt *et al.,* 2011). *Salmonella enterica* serovar Typhimurium is one of the most frequent causes of food borne gastroenteritis in humans, and is also an important pathogen of food producing animals including cattle, pigs and chickens (Ibarra and Steele-Mortimer, 2009). In -bred chickens were found to be more resistant to *Salmonella* Typhimurium strain versus *Salmonella* Enteritidis and Infantis in the oral inoculation experiment of Barrow *et al.* (2004). The prevalence of *Salmonella* Typhimurium in Australian egg industry is quite high and every year outbreaks of human salmonellosis linked to the poultry meat and egg consumptions have been recorded. Salmonellosis was the second most notified enteric disease in Western Australia in 2011 (Anonymous, 2012). In Western Australia, *Salmonella* Typhimurium was the most commonly notified *Salmonella* serotype, comprising 428 notifications linked to egg and poultry meat consumption, approximately 30% higher than the mean number for the previous 5 years (Anonymous, 2012). In Australia, *Salmonella* Typhimurium is the most frequently notified *Salmonella* serotype associated with foodborne outbreaks linked to poultry meat and egg consumptions (Dyda *et al.,* 2009, Reynolds *et al.,* 2010;

Roberts-Witteveen *et al.*, 2009) . The most common *Salmonella* serotype notified in Australia during 2007 was *Salmonella* Typhimurium, and the most common phage type was *Salmonella* Typhimurium 135 (Fullerton, 2008). *Salmonella* Typhimurium was accounted for 73% of outbreaks of human salmonellosis linked to egg consumption in 2004 (Daughtry *et al.*, 2005). More than 80 different phage types associated with human salmonellosis in Australia have been differentiated (Slinko *et al.*, 2009).

Salmonella enterica subspecies *enterica* serovar Infantis has been widely isolated from chicken (meat and eggs), pig, cattle, dog and human globally. *Salmonella* Infantis have been identified as a dominant source of dog salmonellosis which is believed to be primarily transmitted by infected eggs (Sato and Kuwamoto, 1999). Most of the *Salmonella* serovars including *Salmonella* Enteritidis and *Salmonella* Infantis are not serious pathogens in the chicken but they pose a potential threat to public health globally due to their implication in human salmonellosis (Lapuz *et al.*, 2012. *Salmonella* Infantis has been reported to more intensely colonize the chicken alimentary tract compared to other serovars (Smith and Tucker, 1980) but its presence in the reproductive tract and vertical transmission to the egg is still debatable.

1.8 Introduction to present study

The main objective of the present study was to evaluate the importance of egg quality to food safety in table eggs. The study tested the hypothesis that good egg quality results in an egg which is microbiologically safer for the consumer. The present study examined the effect of flock age, of the same flock over time and of different flocks of the same strain, on overall egg quality, extent of cuticle cover, the incidence of mammillary layer shell ultrastructural variations and total microbial load on the eggshell and in eggshell pores. The effect of two different production systems (cage and free range) was also studied. The amount of shell pigment (Protoporphyrin IX) was quantified from the cuticle and true eggshell of brown eggs and correlated with measures of shell colour, shell reflectivity and shell colour as measured by spectrophotometry. Egg washing, as well as shell characteristics including egg translucency and the amount of cuticle, were correlated with the ease of microbial penetration by *Salmonella* Infantis in artificially infected egg experiments.

Chapter 2

General Materials and Methods

Eggs were collected as described in each of Chapters 3, 4 and 6 and brought to the egg laboratory of the University of New England, Armidale. Fresh and unprocessed eggs were collected directly from cages and nest boxes wearing sterile gloves. Out of 150 eggs collected each time from each farm, 90 eggs were processed for determination of traditional egg quality, cuticle estimation and shell mammillary layer ultrastructural scoring while 60 eggs were processed for egg microbiology at the University of Adelaide, Roseworthy Campus, South Australia.

2.1 Traditional eggshell and egg internal quality measurements

2.1.1 Eggshell translucency scoring

Eggs were scored for the incidence of translucency by placing the intact eggs over a light source in an egg candling box. The translucency scores were from“0” to “5” representing the least and highest incidence of translucency, respectively. Some eggs were photographed on the candler to illustrate the scoring system used.

2.1.2 Eggshell quality

Specialized equipment supplied by Technical Services and Supply (TSS) (UK) was used for egg quality measurements. Eggshell measurements conducted were:

- egg weight (TSS equipment balance) Egg weight is the weight of the egg in grams.
- shell colour (measured by TSS reflectivity meter). Shell reflectivity, expressed as a percentage, is the amount of light that is reflected from the surface of an egg. It is an indication of shell colour lightness – the higher the value, the lighter the colour of the eggshell.
- shell breaking strength (measured by quasi-static compression using TSS equipment). Shell breaking strength, in Newtons, is the force which must be applied to the egg before it fails.

- deformation (TSS shell breaking strength machine). Deformation is the distance in micrometres that the egg is depressed by the eggshell breaking strength machine before the egg fails. It is an indicator of the elasticity of the eggshell.
- shell weight (TSS equipment balance). Shell weight is the weight, in grams, of the shell with intact shell membranes which has been carefully washed out and dried.
- shell thickness (UNE shell thickness gauge). Shell thickness is measured in micrometres, using a custom-built gauge, based on a Mitutoyo Dial Comparator Gauge. Three pieces of shell with intact shell membranes were taken from around the equator of the egg for shell thickness measurement.
- shell weight : egg weight ratio (calculated). This ratio is also called the percentage shell and is shell weight divided by egg weight, multiplied by 100 to obtain a percentage.

2.1.3 Egg internal quality

The egg internal quality measurements conducted using TSS equipment were:

- albumen height (TSS automatic Haugh unit gauge). Albumen height is the height that the albumen or white of the egg stands up when an egg is broken out onto a flat surface, measured 1 cm from the edge of the yolk. The TSS equipment measures the albumen height via a probe which detects, electrically, when the surface of the albumen is reached.
- Haugh unit (calculated by the TSS software from egg weight and albumen height). Haugh unit is calculated from albumen height and egg weight by the formula developed by Raymond Haugh (1937). The Haugh unit takes into account the size of the egg. Albumen height and Haugh unit are used as an indicator of internal egg quality or freshness. The equation for calculation of Haugh unit is:

$$H.U. = 100 \log \left[H - \frac{\sqrt{G}\,(30W^{0.37} - 100)}{100} + 1.9 \right]$$

H. U. = Haugh unit

H = albumen height in mm

G = 32. 2

W = weight of whole egg in grams

- Yolk colour score (TSS automatic yolk colourimeter). Yolk colour is determined on the Roche (now DSM) Scale. The TSS yolk colourimeter measures the colour by measuring the wavelength of light reflected from the yolk. There is no user-error associated with this measurement.

2.2 Estimation of the amount of cuticle

2.2.1 MST Cuticle blue stain preparation

MST cuticle stain was prepared according to the manufacturer's instructions (see Appendix A).

2.2.2 Measurement of shell reflectivity and spectrophotometry prior to staining

For the amount of cuticle estimation, shell reflectivity (%) was measured using the TSS shell reflectivity meter. Similarly, eggshell colour (L*a*b) of each individual egg prior to staining was measured with a hand held Konica Minolta spectrophotometer (CM-2600d). The reading was taken 3 times per location at three locations around the equator of each egg and an average recorded.

2.2.3 Cuticle staining

Eggs were soaked in the MST cuticle blue stain for 1 minute, rinsed in distilled water to wash away excessive stain and allowed to dry thoroughly.

2.2.4 Measurement of shell reflectivity and spectrophotometry following staining

Shell reflectivity (%) and shell colour (L*a*b) were again measured using the same procedures described earlier. The Konica Minolta spectrophotometer functions on the SCI (Specular Component Included) and SCE (Specular Component Excluded) L*a*b space system where L* represents the grading between white (100) and black (0). The higher the value for L*, the lighter the shell colour and vice versa. The value for a* represents the colour grading between green and red where green is towards the negative end of the scale and red towards the positive end. More negative values for a*

mean the eggs acquired more stain and thus the amount of cuticle is greater and vice versa. The b* component of the L*a*b space system is the grading between yellow and blue. For “b” blue is towards the negative end and yellow towards the positive end of the scale.

Among all the three components of the L*a*b colour space system, a* is the most important one that shows the amount of cuticle present on the stained eggs.

2.2.5 Light microscopic and scanning electron microscopic verification of cuticle staining

For confirmation of the reliability of the MST cuticle blue stain as an indicator of the amount of cuticle present, egg internal contents were emptied by making a small hole at the blunt end of the eggs using a Dremel High Speed rotary tool 300 series. The egg contents were removed and then internal walls were rinsed with water using a 10 mL syringe with attached tubing. Care was taken not to wash away stain from the cuticle surface. After drying thoroughly, small pieces were cut out from the different areas representing various amounts of stain, mounted on aluminium stubs and photographed under a dissecting microscope at a magnification of 10x1.2. Specimens were sputter coated for 5 minutes in the Neocoater (MP-19020NCTR) and viewed under a scanning electron microscope (JCM-5000 NeoScope) at different magnifications. A scoring method for the SEM of the cuticle, modified from Leleu *et al.* (2011), was used for the quantification of the amount of the cuticle present as shown in Table 2.2.5.1.

Table 2.2.5.1 Scoring sheet for cuticle quantification by SEM

Good intact cuticle (91-100%)	Less patchy cuticle (61-90%)	More patchy cuticle (11-60%)	Negligible or no cuticle (1-10%)
Score= 1	Score= 2	Score= 3	Score= 4

SEM- Scanning electron microscope

2.3 Ultrastructural scoring of the shell mammillary layer

The ultrastructural features of the mammillary layer were scored using a scanning electron microscope (SEM) (JCM-5000 NeoScope). Pieces of shell approximately 1 cm square were cut out from around the equator of the eggshell using a Dremel tool and soaked overnight in small containers of tap water. Shell membranes were manually peeled off to remove as much membrane as possible and the shell pieces were allowed to dry. The dried pieces were then plasma etched in a BioRAD RF Plasma Barrel Etcher PT 7150 for 4 hours following the method of Reid (1983). In the plasma etcher, oxygen gas is ionized by the application of radio frequency power under carefully controlled pressure conditions to form a highly reactive plasma at relatively low temperature. The reaction between the plasma and the carbon in the sample removes the organic component while leaving the crystalline material intact. After plasma etching, each specimen was air dusted in order to remove the ash particles and mounted on a 9 mm diameter aluminium stub using I005Aqueous conductive silver liquid SEM adhesive (ProSciTech). The specimens were sputter coated in a Neocoater for 5 minutes, and viewed under the SEM at various magnifications. Eggshell ultrastructural features of the mammillary layer were scored using the score sheet (see Appendix) developed by Solomon (1991).

Mammillary cap size was scored as 1 (similar), 2 (variable), 3 (highly variable). Mammillary caps were scored according to their quality which was assessed as both the size of the cap in relation to its cone and the degree of membrane attachment from 1 (best) to 5 (worst). Confluence, early fusion, late fusion, alignment, Type A bodies, Type B bodies, aragonite, cubics, cubic cone formations, changed membrane (membrane not removed by plasma ashing) and cuffing were each ranked for incidence from 1 (none) to 4 (extensive). The same was done for the incidence of depressions, erosion and holes although these are rarely observed.

2.4 Egg and eggshell microbiology

2.4.1 Media preparations

Media was prepared according to the manufacturer's instructions as explained in the appendix.

2.4.2 Sampling of eggs

All eggs were scored for translucency between "0" and "3" representing the least and highest incidence of translucency, respectively. For each egg collection, out of the 150 eggs, 60 eggs were

processed for bacterial enumeration from the surface of eggshell and shell crush. Six eggs were pooled for shell wash and shell crush while egg contents from 3 eggs were pooled for further processing of bacteria particularly *Salmonella* recovery.

2.4.2.1 Eggshell microbial enumeration

In order to enumerate the total bacterial count (TBC) and total Enterobacteriaceae count (TEC) on the surface of the eggshell, pooled samples were washed in 60 mL of phosphate buffered saline (PBS, pH 7.4) for 30 sec to 1 minute under strict aseptic conditions. Sterilized gloves were used after washing every pooled sample in order to avoid cross contamination. From the shell rinsate, 0.1mL (100µL) was plated by automatic dispenser onto each of MacConkey (without salt, Oxoid Australia) and Violet Red Bile Glucose Agar (VRBGA, Oxoid Australia). From the same rinsate, 1mL (1000 µL) was inoculated into 4 mL buffered peptone water (BPW). Inoculated plates and BPW vials were placed in the incubator overnight at 37°C. After incubation, the plates were examined for cfu (colony forming unit) and the colonies were counted by using a magnifying glass. VRBGA plates were used for counting total Enterobacteriaceae while MacConkey agar plates showed the total bacterial count on the shell surface. For the *Salmonella* isolation, 0.1 mL (100µL) of incubated Buffered Peptone Water (BPW) was inoculated into 10 mL of Rappaport–Vissiliadis Soya Peptone broth (RVS, Oxoid Australia) and all the tubes were incubated at 42°C overnight. The tubes were examined for *Salmonella* growth based on turbidity. The suspected positive cultures were further streaked onto Bismuth Sulphate agar (BSA Oxoid Australia) or Xylose Lysine Deoxycholate (XLD, Oxoid Australia) agar plates. Plates were incubated at 37°C overnight and were observed for *Salmonella* colony morphology. The suspected colonies were further inoculated into Triple Sugar Iron (TSI, Oxoid Australia) agar. A single suspected to be positive colony was picked up by sterilized loop and inoculated into the TSI slant (see Appendix) tubes. After incubation, all the tubes were checked for colony growth of *Salmonella* which changed the agar colour to a blackish colour. For further serotyping of the suspected cultures, colonies were picked up from XLD plates and inoculated in nutrient agar (Oxoid Australia), incubated overnight at 37°C and sent to the *Salmonella* reference laboratory in South Australia.

2.4.2.2 Eggshell crush microbial enumeration

The eggs used for shell wash (n=30) were dipped for approximately 30 sec in 70% ethanol (C_2H_5OH) to kill shell surface bacteria and remove any possible external contamination (De Reu *et al.,* 2006). Eggs were allowed to dry in a biosafety cabinet. Egg internal contents were removed into a sterile container (200 mL) by breaking the egg at the edge of sterile container into two equal halves near the burner flame. Eggshell internal walls were washed with PBS in order to remove all the adherent albumen which could possibly prevent the growth of bacteria (if any). Eggshells (n=6) with intact shell membranes were transferred into a sterile bag (Nasco Whirl Pak Bags, USA) and crushed in order to expose all the shell pores. 60 mL of the PBS was added into each bag. The shell crush was processed for bacterial enumeration and *Salmonella* isolation as mentioned in section 2.4.2.1.

2.4.2.3 Processing egg internal contents for microbial enumeration

One mL of the homogeneously mixed pooled sample (n=3) in a sterile container was added to 4 mL of BPW, mixed thoroughly and incubated at 37°C. A 0.1 mL sample of the incubated BPW enrichment was inoculated into each of the MacConkey and VRBGA plates under aseptic conditions. The plates were incubated at 37°C overnight. After incubation, all the plates were examined with a magnifying glass to observe the growth of bacterial colonies.

2.4.3 Swabs sampling and culturing for recovery of *Salmonella*

Swabs were collected as described in Chapters 3, 4 and 6. Swabs from poultry manure and egg belts were soaked into 4 mL BPW and incubated overnight at 37°C. A 0.1 mL of BPW enrichment was added into 10 mL Rapapport-Vissaliadis Soya Peptone (RVS) broth and processed for isolation of *Salmonella* as explained in section 2.4.2.1.

2.5 Statistical Analysis

Data were analyzed using Statview Software (SAS Institute Inc., Version 5.0.1.0). A one way (chapters 3 and 4) or two way (chapter 5) analysis of variance used flock age or production system as the independent variables and egg quality and egg microbiology variables as dependent variables. Level of significance was indicated by probability of less than 5%. The Fishers LSD test was used to differentiate levels of significance between mean values.

Chapter 3

Longitudinal Study - Conventional Cage Production System

3.1 Introduction

The domestic hen is thought to be descended from wild jungle fowl but is, at the present time, reared in confined spaces for human benefit in the form of egg and meat production. It is estimated that 70 to 80% of the world egg production comes from conventional cage laying hens (De Reu *et al.*, 2005). Although more layer farmers are changing to the free range production system, conventional cage production is still the most common production system in Australia. In the commercial egg industry, egg quality not only plays an important role for the producers but also for the consumers. Defective shell eggs are a loss of revenue for producers and a health hazard for consumers as a small deterioration of shell quality may enhance microbial entrance into the egg. Infected eggs may carry a number of zoonotic pathogens that not only deteriorate their nutritive quality, but also cause disease if consumed. The egg, which is nature is an incubation chamber for a developing chick, protects its contents by the presence of the cuticle and the architectural organization and effective thickness of palisade and mammillary layers, shell membranes and albumen. Egg translucency is thought to affect shell quality and egg internal quality but how it influences microbial penetration is not clear. Previous research has identified the cuticle as a microbial barrier, and how the amount of cuticle changes with hen age was one of the objectives of this chapter. The evaluation of egg quality parameters is important to both the layer and broiler industries. Good eggshell quality ensures the supply of pathogen-free nutrients to the consumer while at the same time as providing for the hatching of defect-free new chickens if fertile eggs are brooded properly. Genotype is one of the most important factors affecting overall eggshell quality parameters and brown hens lay heavier eggs than white hens (Heil and Hartmann, 1997).

In the present chapter, a longitudinal study was conducted on a cage production flock to evaluate the effect of flock age on eggshell and egg internal quality parameters, amount of cuticle on the shell, mammillary ultrastructural variables and total bacterial load on shell surface and shell crush including shell membranes. Egg internal contents were also tested for the presence of *Salmonella*. In addition,

to evaluate the microbiological status of the flock, swabs from the egg belts and poultry manure were processed for recovery of bacteria including *Salmonella*.

3.2 Materials and methods

In this longitudinal study, one flock of Hy Line brown commercial layers, housed in conventional cages (CC), was followed throughout its production cycle. The flock was located in an environmentally-controlled conventional cage system in the region of Tamworth, NSW. At each egg collection, 150 eggs were directly collected from the cage front at the flock ages of 25, 35, 45, 55, 65, 75 and 85 wk. Of the 150 eggs, 30 eggs were processed for each of the following procedures: traditional shell quality and egg internal quality, estimation of the amount of cuticle and scoring of shell mammillary layer ultrastructural features. The remaining 60 eggs were processed for recovery of bacteria from the shell surface, shell crush and egg internal contents. Thus for a total of 7 collections, 1050 eggs were collected of which 630 were processed for egg quality and 420 for the egg microbiology study. At the time of each collection, 3 swabs were collected from egg belts and 3 from poultry manure. Swabs were collected by dragging a sterile swab on the egg belts and in the manure. All the swabs were processed for the recovery of *Salmonella* as described in Chapter 2 section 2.4.3.

The flock was provided with locally formulated feed containing wheat (or wheat plus sorghum), soybean meal, meat meal, vegetable oil, limestone and yolk colour pigment as major components.

Data were analyzed using one way analysis of variance (ANOVA) as described in Chapter 2 section 2.5.

3.3 Results

3.3.1 Eggshell and egg internal quality measurements

A statistically significant effect ($P \leq 0.05$) of flock age was recorded for all the traditional eggshell quality variables including translucency score. Translucency score was significantly higher in the 25 and 55wk old birds than for the other bird ages, as shown in Figure 3.3.1.1. Shell reflectivity (%) was lowest in the 25 wk old birds and significantly different from all other ages except 45 wk (Figure 3.3.1.2). Egg weight (g) generally increased up to 75 wk of age but tended to be lower at 85 wk of

age (Figure 3.3.1.3). Egg weight at 25 wk of age was significantly different from all other ages. The 35 and 65 wk old egg weights were different only from 25 and 75 wk birds. Similarly, 45 and 55 wk old egg weights were significantly different from 25, 75 and 85 wk old birds and 65 wk was only significantly different from 75 wk. Shell breaking shell strength (N) generally decreased with increasing bird age with breaking strength being lowest at 85 weeks (Figure 3.3.1.4). Shell deformation (μm) fluctuated to some extent but was lowest at 85 wk of age (Figure 3.3.1.5). Shell weight (g) increased slightly (Figure 3.3.1.6) while percentage shell (Figure 3.3.1.7) and shell thickness (μm) (Figure 3.3.1.8) decreased with increased flock age.

A statistically significant effect ($P \leq 0.05$) of flock age was recorded for all the egg internal quality variables. Albumen height (mm) decreased until 45 wk, then increased slightly at 55 and 65 wk and was lowest in 85 wk old flock eggs (Fig. 3.3.1.9). Albumen height (mm) was statistically significantly different between 25 wk and all other age groups except the 35 wk age flock, while 35wk eggs were significantly different from 55 wk, 65 wk, 75wk and 85 wk. Haugh unit paralleled albumen height and decreased with increasing flock age except in 65 wk and 75 wk old eggs (Fig. 3.3.1.10). Yolk score increased slightly with increasing flock age (Fig. 3.3.1.11). All the eggshell quality and egg internal quality parameters are shown in the graphs. Superscripts ($^{a, b, c, d, e, f}$) on the graph bars show significant differences between different age groups for respective variables. Values with different superscripts are significantly different.

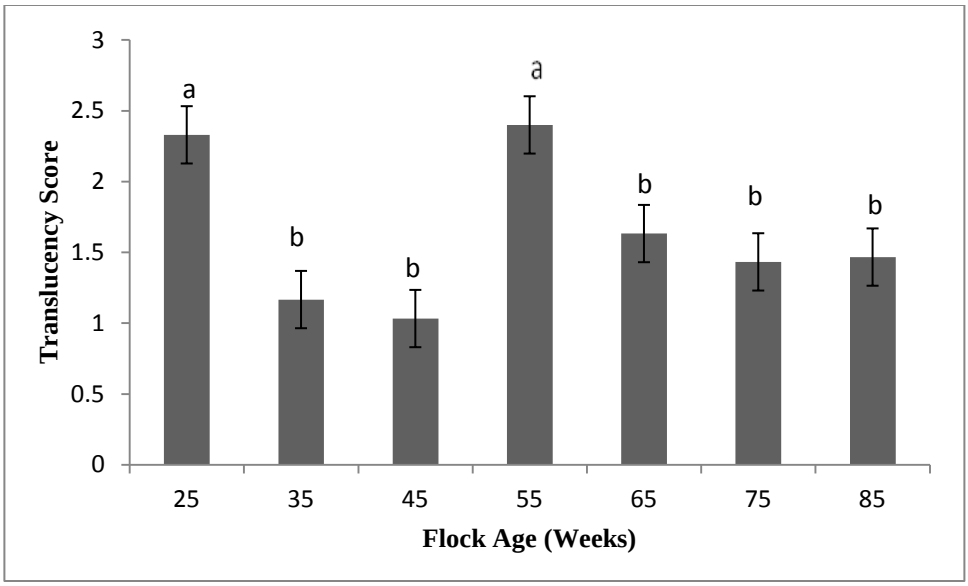

Figure 3.3.1.1 Translucency score at different ages (Mean±SE)

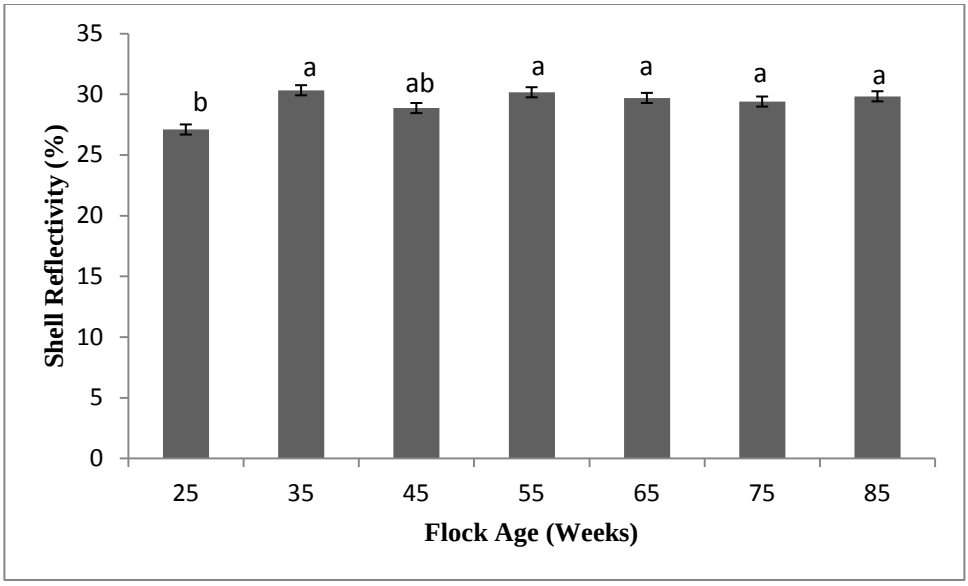

Figure 3.3.1.2 Shell reflectivity (%) at different ages (Mean±SE)

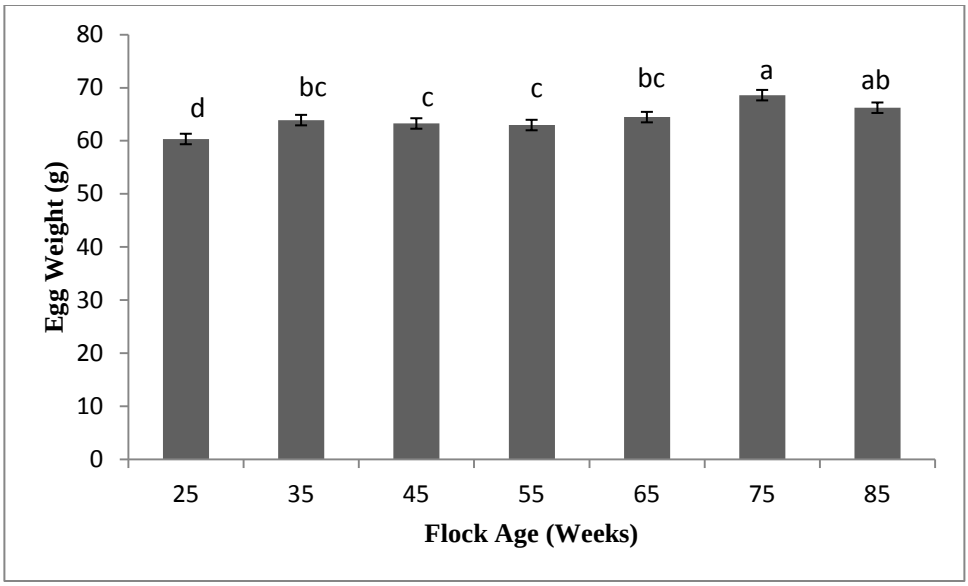

Figure 3.3.1.3 Egg weight (g) at different ages (Mean±SE)

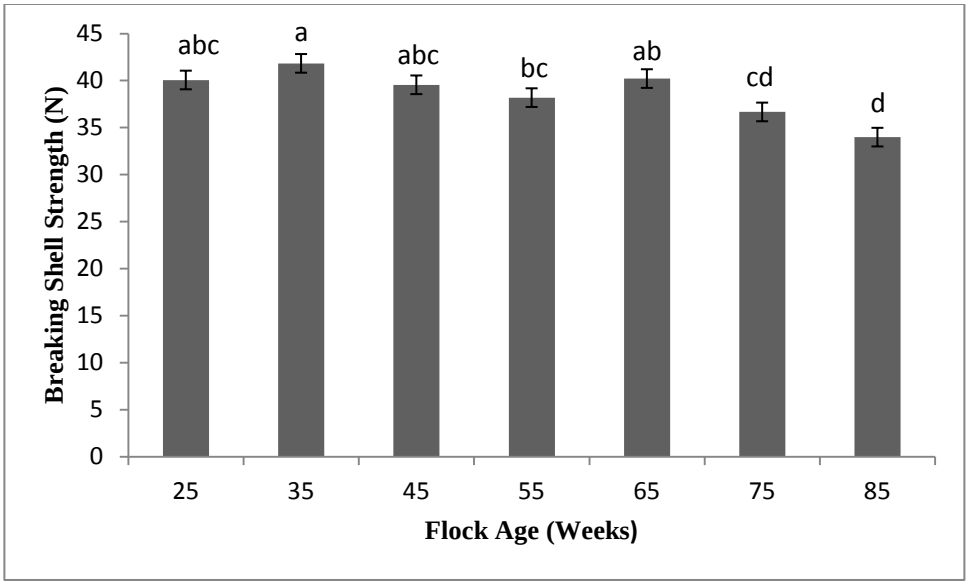

Figure 3.3.1.4 Breaking shell strength (N) at different ages (Mean±SE)

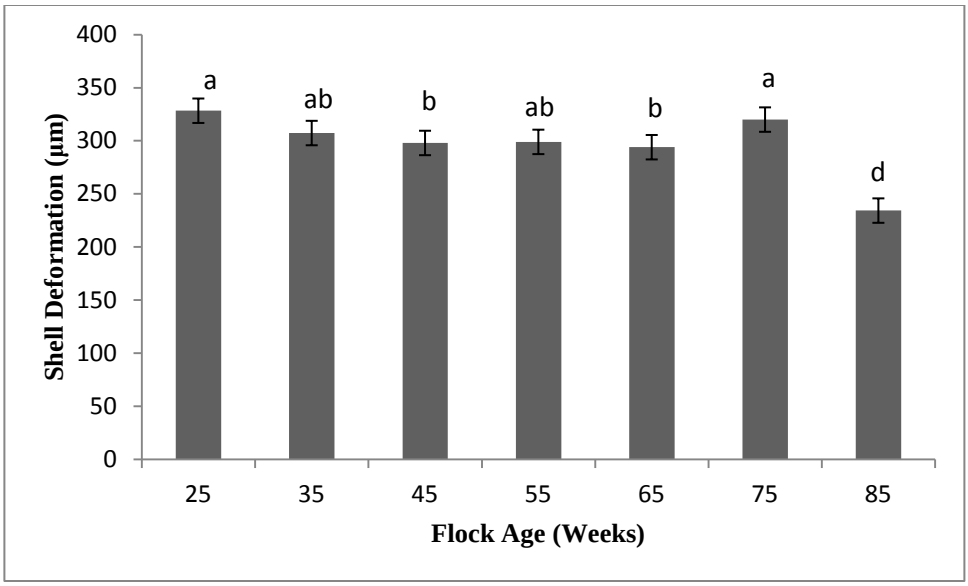

Figure 3.3.1.5 Shell deformation (µm) at different ages (Mean±SE)

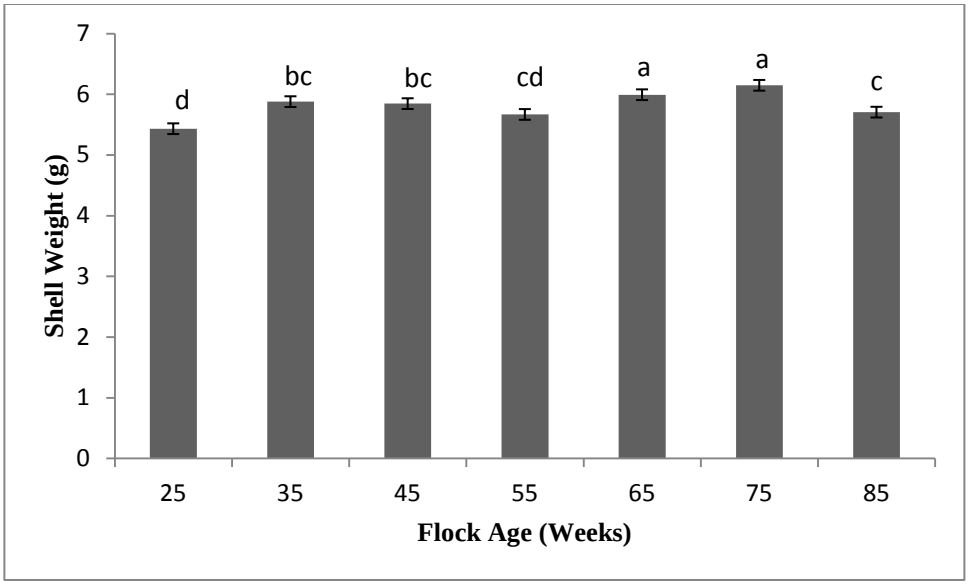

Figure 3.3.1.6 Shell weight (g) of same flock eggs at different ages (Mean±SE)

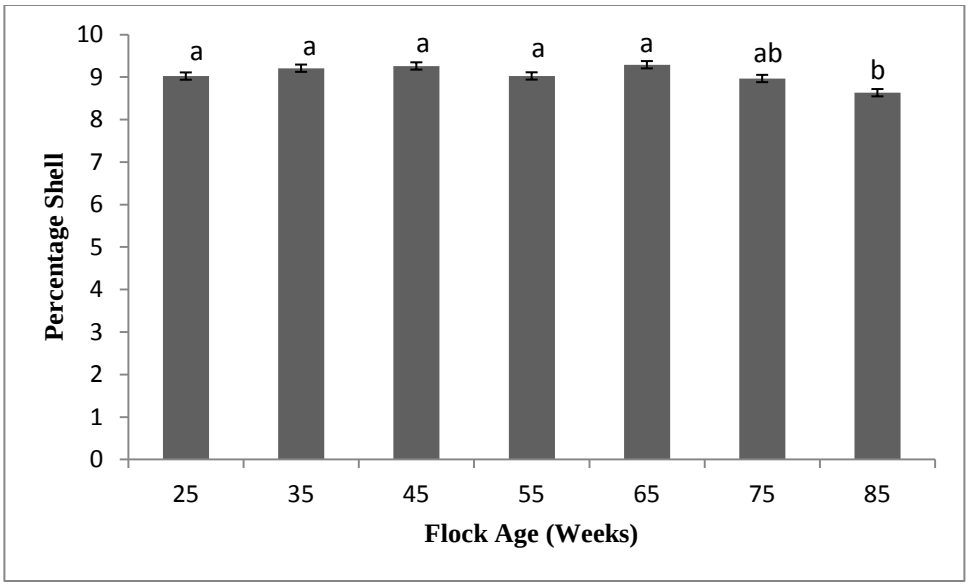

Figure 3.3.1.7 Percentage shell at different ages (Mean±SE)

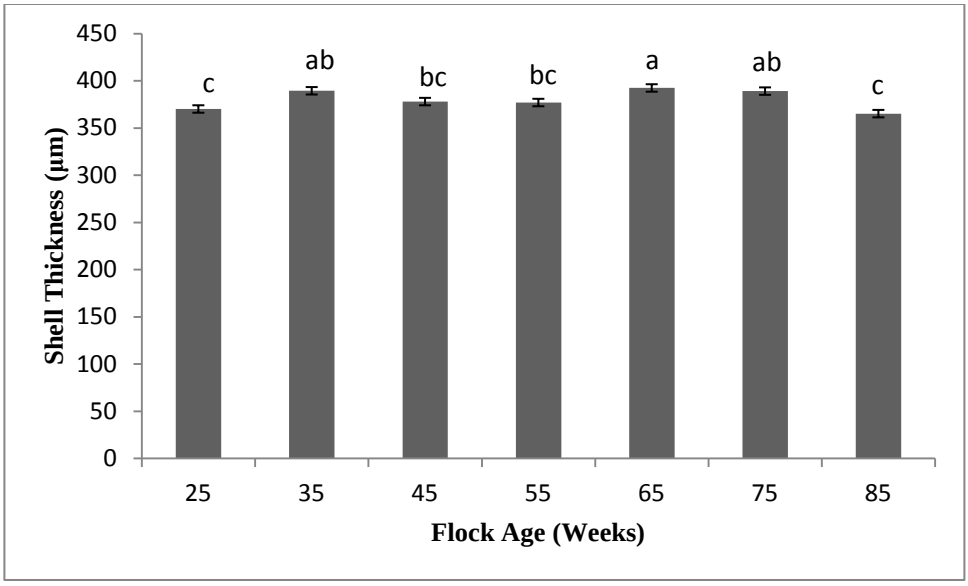

Figure 3.3.1.8 Shell thickness (µm) at different ages (Mean±SE)

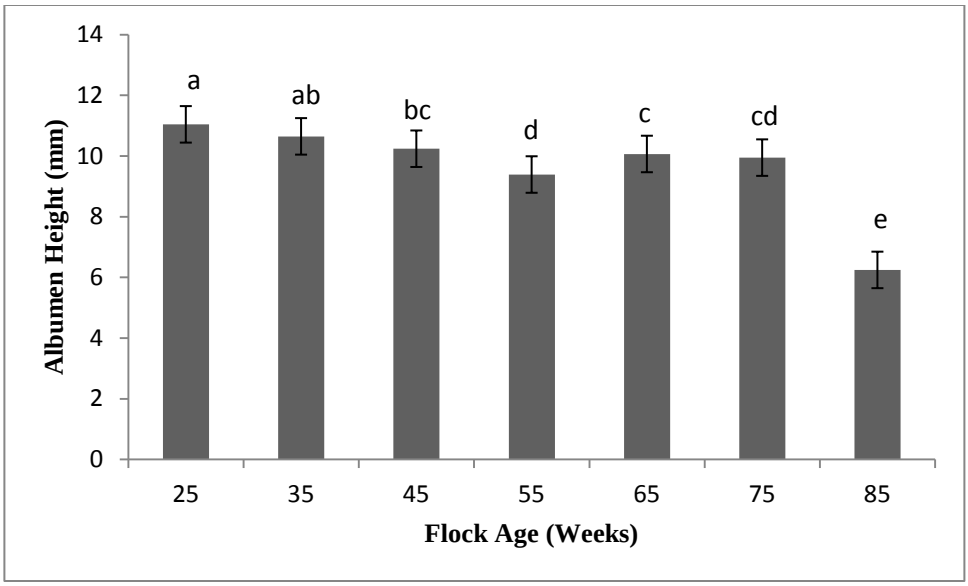

Figure 3.3.1.9 Albumen height (mm) at different ages (Mean±SE)

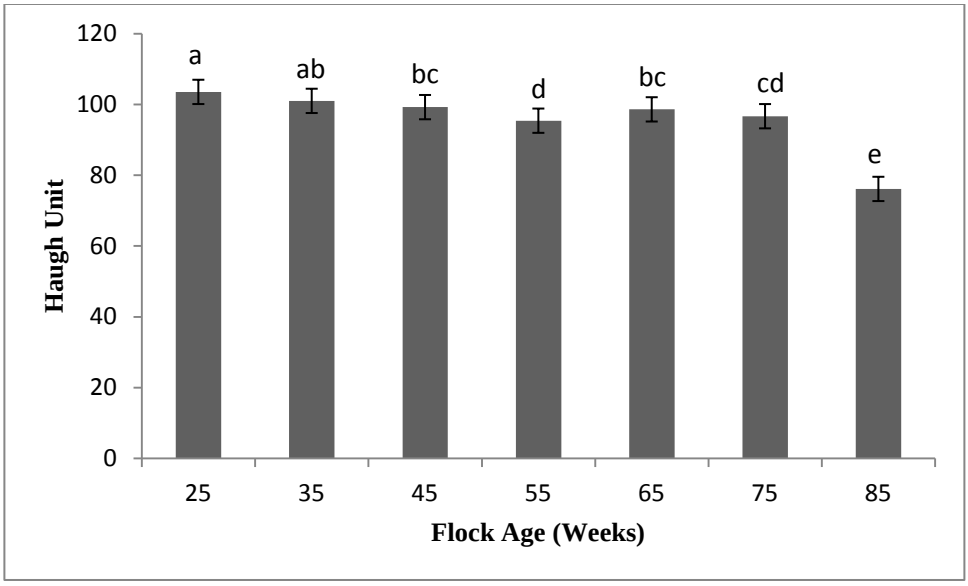

Figure 3.3.1.10 Haugh unit at different ages (Mean±SE)

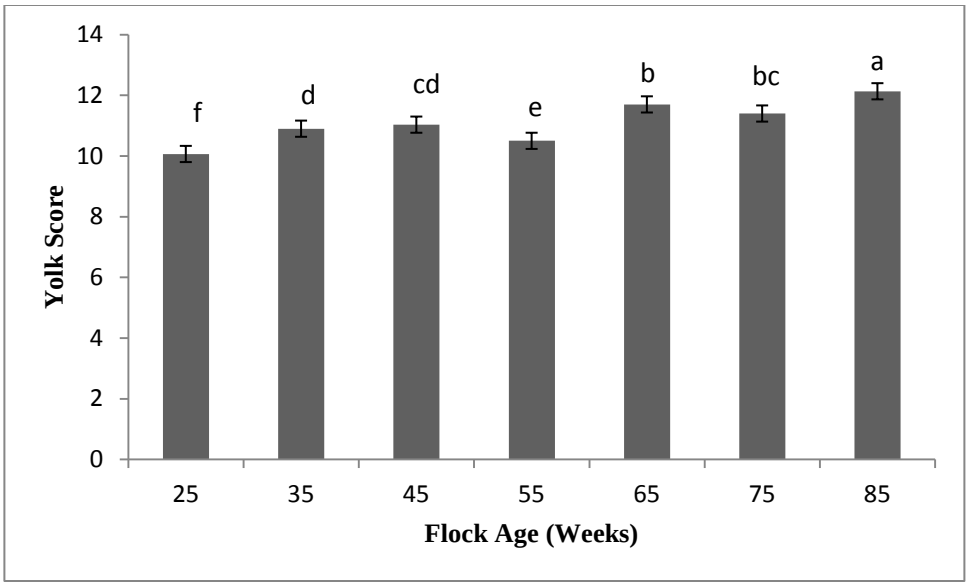

Figure 3.3.1.11 Yolk score at different ages (Mean±SE)

3.3.2 Estimation of the amount of cuticle

3.3.2.1 Shell reflectivity (%) and Spectrophotometry (L*a*b) measurements

Table 3.3.2.1.1 summarizes the results for shell reflectivity and spectrophotometry of eggshells following staining with MST cuticle blue dye. There was a statistically significant effect ($P \leq 0.05$) of hen age for shell reflectivity (%) and all values of the L*a*b colour space system. Shell reflectivity increased slightly to 45 wk of flock age, was slightly lower at 55 wk and then tended to increase in 65 wk eggs. Shell reflectivity at 85 wk tended to be higher than for 75 wk flock eggs. Shell reflectivity (%) values were in parallel with the values of the L* component of L*a*b. Specular Component Included L (SCI L*) and Specular Component Excluded L (SCE L*) values were very similar to each other. SCI a* and SCE a* values were negative for all the collections except the 35 wk eggs. SCI b* and SCE b* values decreased slightly at 35 & 45 wk and 75 & 85 wk periods.

Table 3.3.2.1.1 Shell reflectivity and L*a*b values of stained eggshells

Variables	Flock age (weeks)							P value
	25	35	45	55	65	75	85	
Shell Reflectivity	19.27±0.45^{d}	24.53±0.65^{ab}	25.83±1.35^{a}	23.57±0.83^{bc}	24.40±0.55^{ab}	21.97±0.80^{c}	22.67±0.70^{bc}	<0.0001
SCI L	51.06±0.49^{c}	54.76±0.61^{ab}	56.47±1.22^{a}	54.28±0.89^{ab}	55.06±0.53^{ab}	53.09±0.86^{bc}	54.35±0.74^{ab}	0.0002
SCI a	-2.70±0.75^{bcd}	0.26±0.78^{a}	-3.84±1.20^{cd}	-4.83±1.14^{d}	-1.05±0.66^{ab}	-4.61±1.01^{d}	-1.53±1.18^{abc}	0.0012
SCI b	32.31±0.30^{a}	32.40±0.41^{a}	28.46±0.64^{b}	29.44±0.29^{b}	31.92±0.43^{a}	28.97±0.42^{b}	28.96±0.22^{b}	0.0001
SCE L	50.79±0.50^{c}	54.57±0.62^{ab}	56.37±1.22^{a}	54.07±0.89^{b}	54.95±0.54^{ab}	52.93±0.87^{bc}	54.23±0.76^{ab}	0.0002
SCE a	-2.71±0.76^{bcd}	0.27±0.76^{a}	-3.84±1.21^{cd}	-4.85±1.14^{d}	-1.04±0.67^{ab}	-4.63±1.01^{d}	-1.52±1.19^{abc}	0.0012
SCE b	32.65±0.30^{a}	32.63±0.42^{a}	28.57±0.65^{c}	29.63±0.29^{bc}	32.11±0.44^{a}	29.19±0.42^{c}	29.08±0.21^{c}	<0.0001

Values are Mean ± SE; SCI- Specular Component Included; SCE- Specular Component Excluded

$^{a, b, c, d}$ Values with different superscripts are significantly different from each other

3.3.2.2 Scanning Electron Microscopy of the cuticle surface

The total amount of cuticle cover scored on a scale of 1 (most cuticle) to 4 (least cuticle) by scanning electron microscopy (SEM) varied significantly (P=0.0157) with hen age as shown in Table 3.3.2.2.1. The amount of cuticle cover was highest at 55 wk followed by 75 wk.

Table 3.3.2.2.1 Scanning electron microscopy (SEM) values of cuticle cover

Variable	Flock age (weeks)							P value
	25	35	45	55	65	75	85	
Cuticle cover	2.07±0.14^{ab}	2.45±0.16^{a}	2.17±0.15^{a}	1.80±0.19^{b}	2.20±0.15^{a}	1.87±0.16^{b}	2.47±0.17^{a}	0.0157

Values are Mean ± SE

$^{a, b}$ Values with different superscripts are significantly different from each other

3.3.3 Ultrastructural scoring of the shell mammillary layer

The results of the scoring of ultrastructural features of the mammillary layer are shown in Figures 3.3.3.1-3.3.3.11 and in Table 3.3.3.1. For the ultrastructural scoring of the mammillary layer, a significant main effect ($P \leq 0.05$) of hen age was recorded for variables for which a higher incidence positively affects mammillary layer quality. The variability of mammillary cap size increased to 45 wk, decreased with flock age (55, 65, 75 wk) and was highest in 85 wk flock eggs. Overall, the incidence of confluence, early fusion and cuffing decreased with hen age (Figs. 3.3.3.2, 3.3.3.3, 3.3.3.4). A statistically significant effect of flock age was recorded for all those variables for which a higher incidence negatively affects mammillary layer quality: mammillary cap quality, late fusion, alignment, Type A bodies, Type B bodies, cubic cone formation and changed membrane. Generally, the incidence of these negative features increased with increasing flock age except for cubic cone formation and changed membranes. The incidence of cubic cone formation was higher at 45 and 65 wk compared to other ages (Fig. 3.3.3.10). The incidence of changed membrane was higher in 25 wk flock eggs and remained relatively constant in subsequent ages (Fig. 3.3.3.11). Variables not

significantly affected by flock age were the incidence of aragonite, cubics, depression, erosion and holes (Table 3.3.3.1). A higher incidence of these features is thought to negatively affect mammillary layer quality.

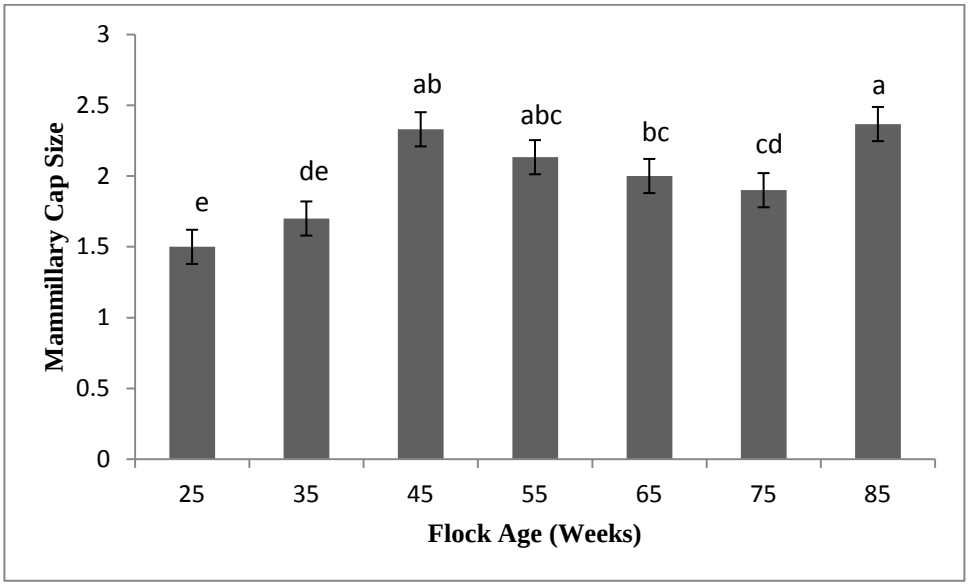

Figure 3.3.3.1 Incidence of mammillary cap size variability at different ages (Mean±SE)

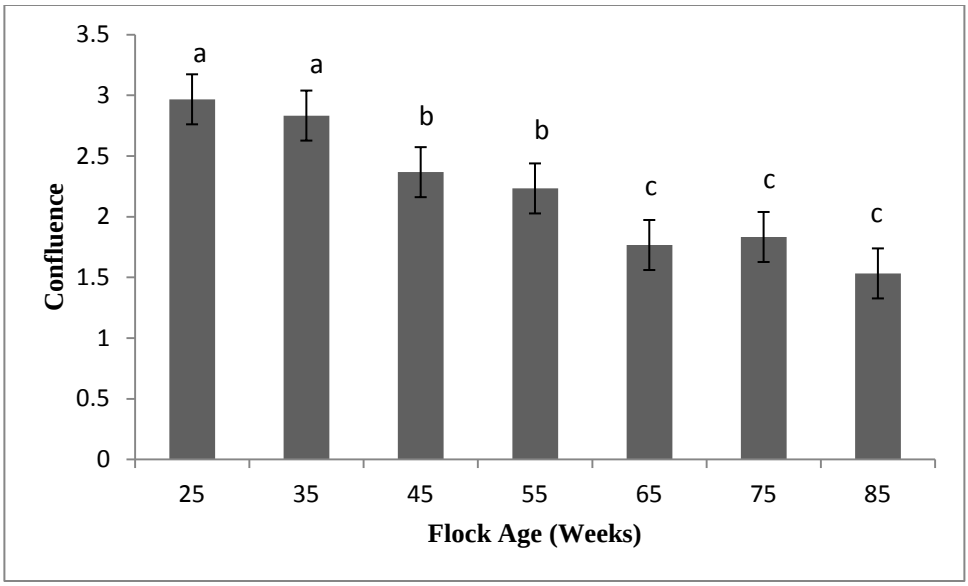

Figure 3.3.3.2 Incidence of confluence at different ages (Mean±SE)

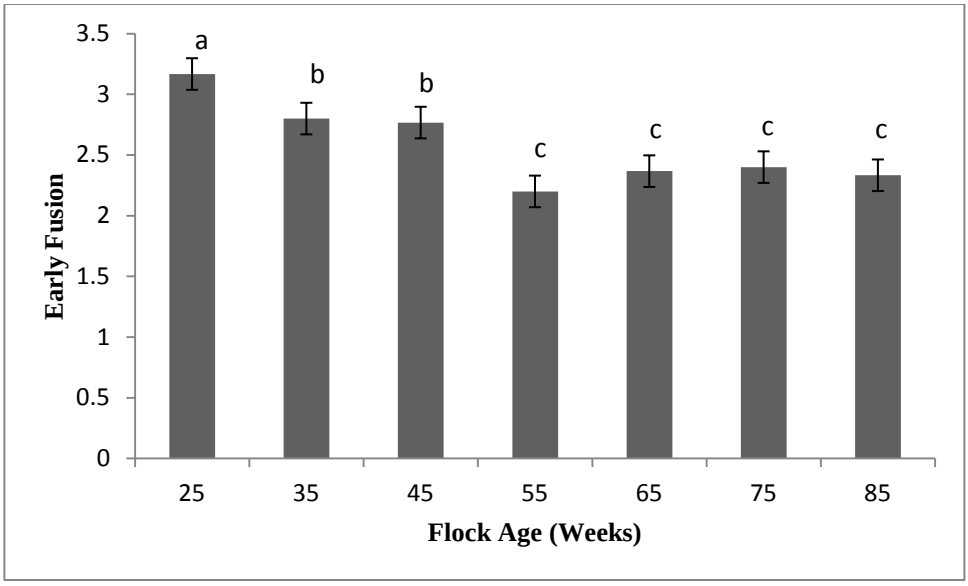

Figure 3.3.3.3 Incidence of early fusion at different ages (Mean±SE)

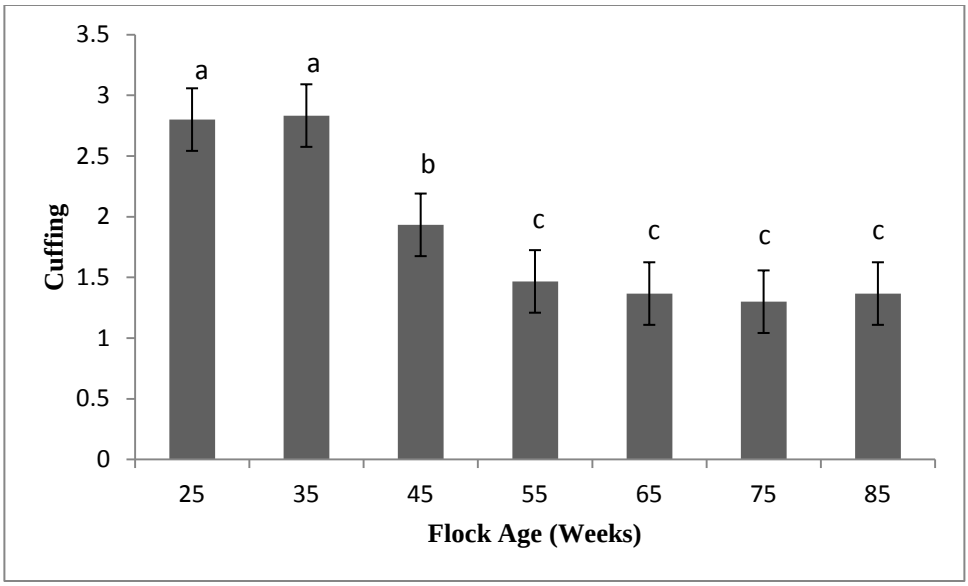

Figure 3.3.3.4 Incidence of cuffing at different ages (Mean±SE)

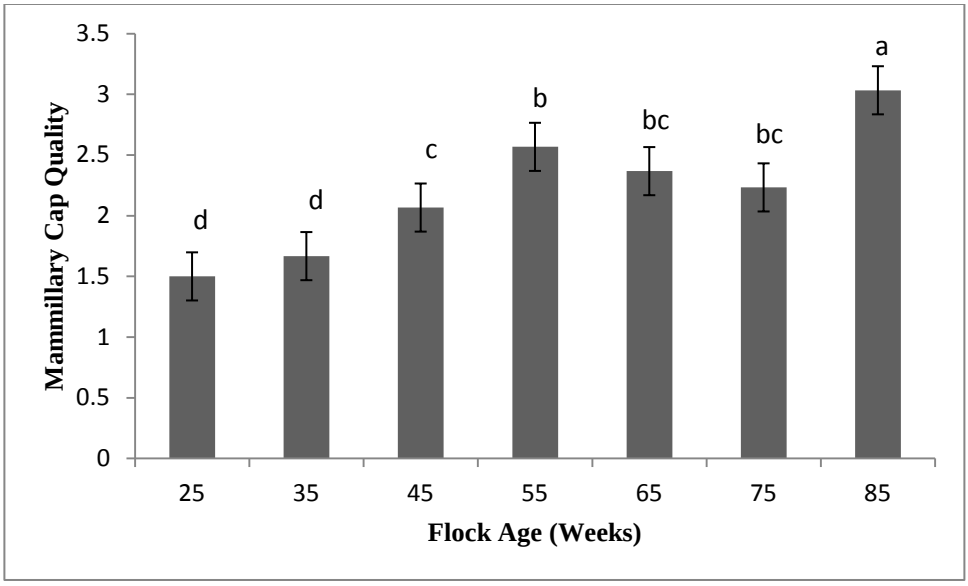

Figure 3.3.3.5 Incidence of mammillary cap quality at different ages (Mean±SE)

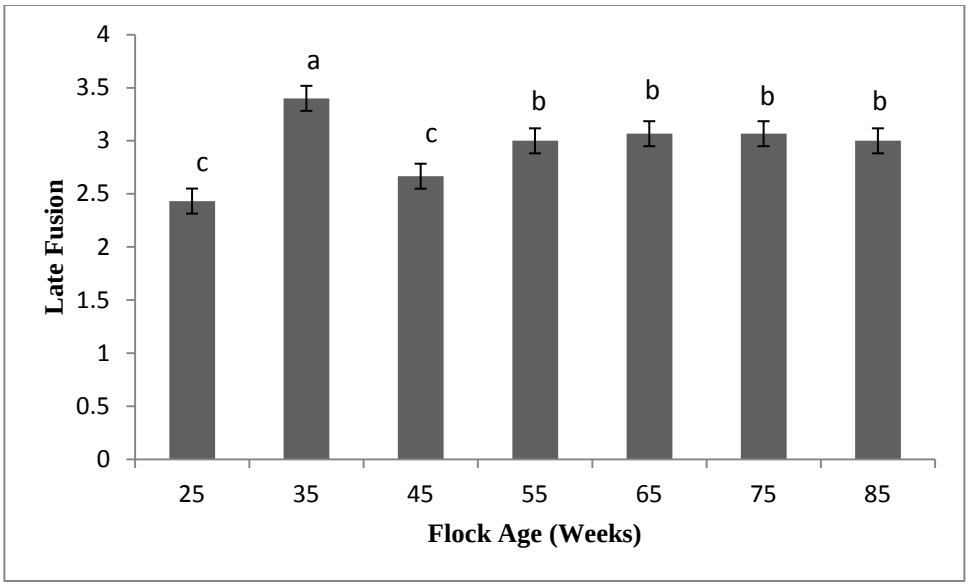

Figure 3.3.3.6 Incidence of late fusion at different ages (Mean±SE)

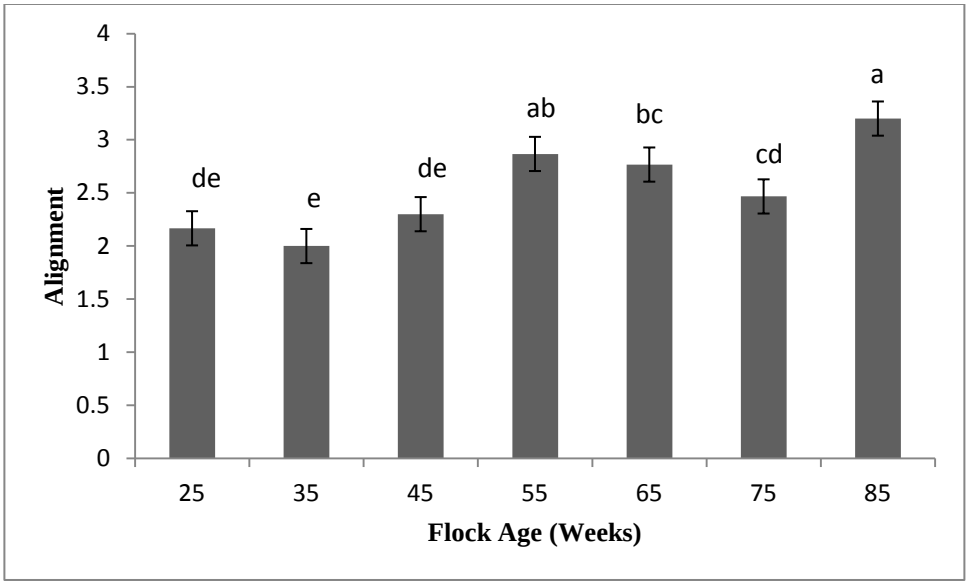

Figure 3.3.3.7 Incidence of alignment at different ages (Mean±SE)

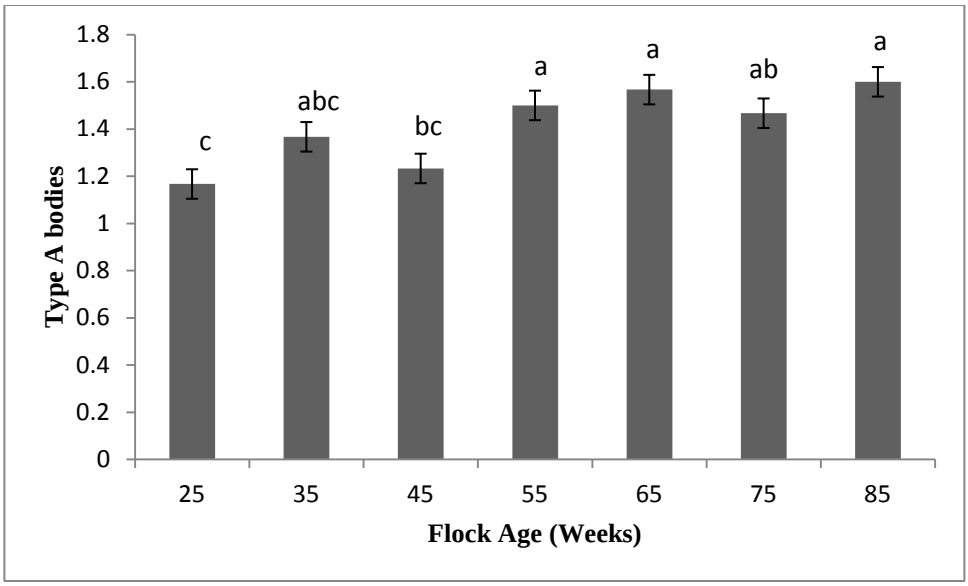

Figure 3.3.3.8 Incidence of Type A bodies at different ages (Mean±SE)

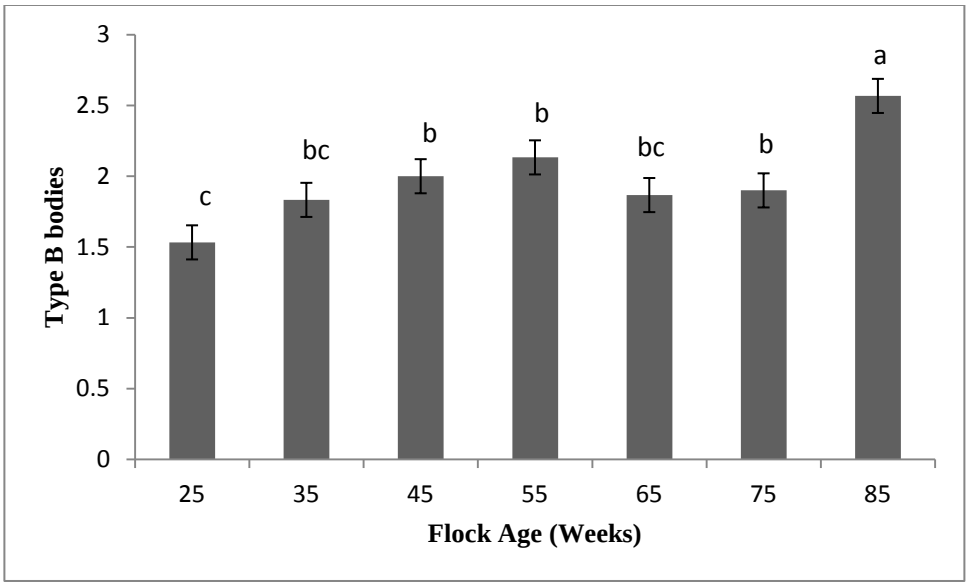

Figure 3.3.3.9 Incidence of Type B bodies at different ages (Mean±SE)

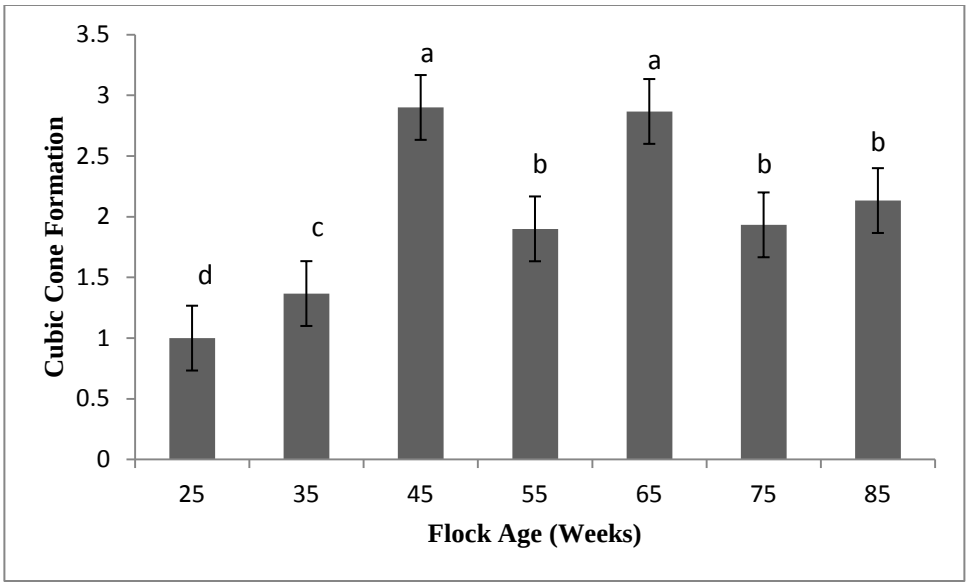

Figure 3.3.3.10 Incidence of cubic cone formation at different ages (Mean±SE)

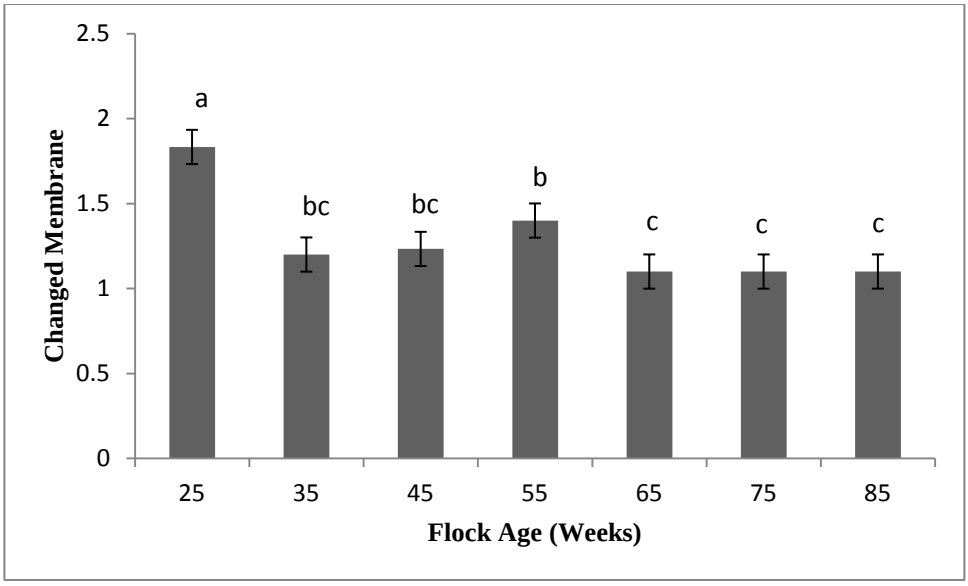

Figure 3.3.3.11 Incidence of changed membrane at different ages (Mean±SE)

Table 3.3.3.1 Shell ultrastructural variables not significantly affected by hen age

Variables	Flock age (weeks)							P value
	25	35	45	55	65	75	85	
Aragonite	1.00±0.00	1.10±0.56	1.03±0.03	1.13±0.09	1.07±0.05	1.00±0.00	1.17±0.08	0.2388
Cubics	1.10±0.06	1.13±0.06	1.17±0.08	1.13±0.08	1.13±0.06	1.07±0.05	1.07±0.05	0.9105
Depression	1.07±0.05	1.03±0.03	1.00±0.00	1.10±0.06	1.13±0.06	1.00±0.00	1.10±0.07	0.2958
Erosion	1.00±0.00	1.00±0.00	1.07±0.05	1.07±0.05	1.10±0.06	1.03±0.06	1.13±0.06	0.2187
Holes	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.03±0.03	1.00±0.00	1.00±0.00	0.4265

Values are Mean ± SE

3.3.4 Egg Microbiology

There was a significant effect of flock age on total bacterial count (TBC) on eggshells, total Enterobacteriaceae count (TEC) on the shell and in the shell crush as shown in Table 3.3.4.1. The TBC on shell surface was lower at 35 wk, increased slightly with flock age before decreasing at 85 wk. The TBC in shell crush did not vary significantly with flock age but tended to be higher at 65 wk. The TEC on eggshell surface was significantly higher at 55 wk but did not vary significantly among other ages. The TEC in shell crush was significantly higher at 35 and 45 wk compared to the other ages. The overall count of TEC in shell crush was very low. Egg internal contents were negative for bacteria. *Salmonella* was not isolated from any of the egg belts and manure swabs.

Table 3.3.4.1 TBC and TEC (10 log cfu) on eggshell and in shell crush

Variables	Flock Age (weeks)							P Value
	25	35	45	55	65	75	85	
TBC on eggshell	3.42±0.08^{ab}	1.55±0.52^{c}	3.09±0.35^{b}	3.48±0.59^{ab}	3.29±0.22^{ab}	4.07±0.11^{a}	3.93±0.06^{ab}	<0.0001
TBC in shell crush	0.35±0.35	1.56±0.53	1.31±0.45	1.19±0.41	1.96±0.55	0.86±0.48	1.39±0.39	0.2832
TEC on eggshell	1.63±0.45^{b}	0.58±0.39^{b}	1.79±0.52^{b}	3.08±0.36^{a}	1.57±0.44^{b}	1.68±0.47^{b}	1.28±0.44^{b}	0.0159
TEC in shell crush	0.00±0.00^{c}	1.51±0.41^{a}	1.71±0.38^{b}	0.20±0.20^{c}	0.20±0.20^{c}	0.43±0.29^{c}	0.00±0.00^{c}	<0.0001

TBC- total bacterial count; TEC- total Enterobacteriaceae count; Values are Mean ± SE

$^{a, b, c}$ Values with different superscripts are significantly different from each other

3.4 Discussion

3.4.1 Eggshell and egg internal quality measurements

The translucency score varied significantly ($P<0.0001$) with flock age. The highest incidence of translucency was at 25 wk and 55 wk which were scored 2 days after egg collection. The translucency score is a relatively subjective measurement of the incidence and extent of light patches in the eggshell when the egg is placed on the candling light source. Translucency develops when moisture escapes from the egg albumen through the shell membranes into the ultrastructure of the mammillary layer (Solomon, 1986). Most freshly laid eggs show relatively few translucent spots, and translucency develops within the first 24 hr after laying. The incidence of translucency increases with the passage of time until 6 to 7 days after the egg is laid (Roberts, unpublished data). The incidence of translucency varies from egg to egg and is affected by both storage time and other factors such as flock age. It ranges from small pin points to hair like lines and circular spots of approximately 1 mm^2. Some thin shelled eggs show maximum translucency. A relatively high rate of bacterial penetration has been recorded in the eggs having a maximum translucency score in the studies of Chousalkar *et al.* (2010). The current findings can be compared to the findings of Solomon (1986) who reported low translucency in young bird eggs compared to older birds.

Shell reflectivity (%) varies with the amount of pigment (Protoporphyrin IX) deposited in the eggshell. The lighter the shell colour, the higher the shell reflectivity and vice versa. Brown egg markets dominate in many countries including Australia, USA, Europe and New Zealand. Colour is genetically linked to breed as the White Leghorn produces white eggs and Hy Line Brown and Rhode Island Red birds lay brown eggs. In the current study, shell reflectivity (%) at 25 wk was significantly different from all other age groups except 45 wk. There was no statistically significant difference among the other age groups and shell reflectivity remained relatively stable after 55 wk of flock age. Similarly, Zita *et al.* (2009) and Tumova and Ledvinka (2009) found a decrease in eggshell colour intensity with flock age in conventional cages. Odabasi *et al.* (2007) mentioned increased egg size as a possible factor for lower shell colour. These authors suggested that the same amount of colour distributes on a larger shell surface. Darker brown eggs are reported to have higher specific gravity which is an indicator of good shell quality (Hooge, 2007). In contrast to the findings of Odabasi *et al.* (2007), Sekeroglu and Altuntas (2009) found that jumbo eggs were darker than smaller eggs but

unlike the current study the eggs were from one age group (33 wk). Ingram *et al.* (2008) and Mertens *et al.* (2010) explained that shell colour is related to egg quality but it is not a true measurement of shell quality in the same way as shell gravity or shell thickness. In the current study, shell reflectivity was not significantly correlated with egg weight.

In most countries, eggs are marketed based on egg weight and it is one of the most important economic factors that generally increases with the flock age. In the present study, egg weight increased between 25 and 35 wk of age and then again between 65 and 75 weeks of age although egg weight tended to decrease slightly at 85 weeks. Australian producers aim to keep egg weight relatively constant once birds reach peak lay although, in the current study, egg weight increased in the later stages of production. These results correspond to the findings of many authors including Silversides, 1994; Silversides and Budgell, 2004; Silversides and Scott, 2001; Tumova and Ledvinka, 2009; Rayan *et al.*, 2010; Rossi and Pompei, 1995; Johnston and Gous, 2007; Van Den Brand *et al.*, 2004; Novo *et al.*,1997; Rizzi and Chiericato, 2005; Garlich *et al.*,1984; Izat *et al.*,1986; Ronald, 1979; Ledvinka *et al.*, 2011; Ferrante *et al.*, 2009; Berrang *et al.*,1998; Roland, Sr. *et al.*,1975; Odabasi *et al.*, 2007 and Peebles *et al.*, 2000, who recorded an increase in egg weight with flock age. However, Ahmed *et al.* (2005) reported no significant difference in egg weight after molting. Messens *et al.* (2005) and Nys (1986) evaluated the effect of age on egg weight and reported a steady increase and then decrease in egg weight with the flock age, which is similar to the current findings. Egg weight is reported as having a direct linear correlation with shell thickness and egg size (Zita *et al.*, 2009). In the current study, there was no significant correlation between egg weight and shell breaking strength ($P=0.5529$), and albumen height ($P=0.1529$) while it was positively correlated with shell thickness ($P<0.0001$), shell weight ($P<0.0001$) and yolk score ($P<0.0001$). Sekeroglu and Altuntas (2009) recorded a positive correlation between egg weight and yolk colour and a negative correlation with shell thickness.

Breaking shell strength (BSN) determines the shell resistance to breakage when force is applied to the surface of the eggshell. In the current study, BSN was slightly higher in the 2nd collection and then declined until 55 wk of age. Higher BSN values in the mid lay period suggest that shell quality is greatest at this age. Breaking strength tended to be higher in the 65 wk age eggs then declined as the flock advanced in age. Molting is a possible contributing factor to the higher BSN value at 65 wk as the flock was molted at 62 wk. Ahmed *et al.* (2005) reported a significant effect of molting on shell

breaking strength (28.09 vs. 33.71 N) and some feed additives have shown a positive effect on eggshell breaking strength (Tumova *et al.,* 2011). Our results agree with the findings of Rodriguez-Navarro *et al.* (2002); Arpasova *et al.* (2010) and Rayan *et al.* (2010) who state that breaking shell strength decreases with flock age. The current results differ from the findings of Zita *et al.* (2009) who reported higher BSN at 54-60 wk versus 20-26 wk. Rodriguez-Navarro *et al.* (2002) correlated BSN with the orientation of calcite crystals in the eggshell and, in their studies, eggs from older hens had lower BSN values compared to younger hen eggs. Change in components of the organic matrix with hen age might be a contributing factor to poor shell strength (Fraser *et al.,* 1998; Gautron *et al.,* Online; Panheleux *et al.,* 2000). Lower BSN eggs were frequently penetrated by *Salmonella* in the studies of Messens *et al.* (2007).

Shell deformation (μm) generally decreased with flock age except for being higher at 75 wk of age. A decrease in shell deformation is indicative of poor quality of eggs and our findings are similar to the results of Zita *et al.* (2009) in which shell deformation decreased with the flock age (20-60 wk) for birds reared in cages. A higher shell deformation at 75 wk might reflect an improvement in egg shell quality following the induced molt at 62 wk. A relatively weak correlation (r^2=0.102) was recorded between shell deformation and breaking shell strength which might indicate that a stronger eggshell is not necessarily more elastic.

Shell weight (g) generally increases with flock age. Shell weight was correlated with egg weight (r^2=0.357). An increase in shell weight and then decrease with flock age has been recorded by Silversides (1994) and Odabasi *et al.* (2007). A slight increase was recorded in shell weight with increasing flock age by Nys (1986) and Silversides and Budgell (2004) to whom our results can be compared. An increase in post molt shell weight followed by a decline has also been recorded by Garlich *et al.* (1984). Ahmed *et al.* (2005) recorded no significant difference (P>0.05) between pre molt (6.24g) and post molt (6.22g) eggshell weight of ISA brown cage eggs. In the studies of Roland *et al.* (1975) shell weight remained constant with increasing bird age.

A slight increase followed by a decrease in eggshell thickness (μm) with flock age can be compared to the findings of Messens *et al.* (2005) in which there was a slight increase in the shell thickness followed by a decrease at the end of the lay of the same flock. Shell thickness decreased with flock age in the findings of Rayan *et al.* (2010). Shell thickness is directly related to eggshell strength

(Roberts, 2010). In the study of Tumova and Ledvinka (2009), shell thickness increased slightly with increasing flock age (20-60 wk) in birds housed in conventional cages. Roland *et al.* (1975) also reported an increase in the shell thickness with flock age (38-90 wk).

Percentage shell remained relatively constant until 65 wk and then declined with flock age. The current results are in comparison to the results of Rayan *et al.* (2010) and Zita *et al.* (2009) who recorded a decrease in percentage shell with age in flocks kept in conventional cages. Silversides and Scott (2001) recorded a decrease in percentage shell with flock age in ISA brown laying hens. In the current study, the effect of molting on percentage shell cannot be clearly explained as the induced molt did not prevent the decline in percentage shell at 85 wk of age. Molting did not affect the amount of shell in the studies of Ahmed *et al.* (2005). However, Roland *et al.* (1975) reported an increase in the percentage shell with increasing flock age.

For egg internal quality, albumen height (mm) is regarded as one of the most reliable parameters for measuring egg freshness. Viscosity of albumen decreases towards the end of the production cycle which, in turn, decreases albumen height. Storage temperature also greatly affects albumen quality. A linear decrease in albumen height until 55 wk indicates that albumen quality was affected by flock age. A slightly higher albumen height at 65 wk might be due to the regenerative effect of molting. The relatively lower value of the 85 wk old age eggs was due to a longer time delay between egg collection and processing which was caused by a technical problem with laboratory equipment. Silversides and Scott (2001) and Karoui *et al.* (2008) and Silversides (1994) mentioned that the height of the inner thick albumen decreases in a logarithmic fashion with increasing storage time. Silversides and Scott (2001), Silversides (1994) and William (1992) mentioned age and strain as important factors affecting albumen height and albumen height decreased with flock age in their studies. In the present study, albumen height was not significantly correlated (r^2=0.004) with egg weight. The work of the Silversides and Scott (2001) further confirms that there is no inherent relationship between egg weight and albumen height. Albumen height heritability (%) from the sire plus dam is 0.48 ± 0.07 (Poggenpoel, 1986). In the studies of Petek *et al.* (2008), there was no significant difference for egg internal and external quality variables between pre molt and post molt flocks.

Haugh unit (HU), which indicates the freshness of an egg, is calculated from albumen height and egg weight. The higher the HU value, the better is the internal quality of an egg. In the current study, the HU was strongly correlated (r^2=0.965) with albumen height and decreased with flock age. Haugh unit's heritability (%) from the sire plus dam is 0.40 ± 0.06 (Poggenpoel, 1986). The current results for HU are similar to the work of Zita *et al.* (2009); Curtis *et al.* (1985) and Izat *et al.* (1986).

Yolk colour score depends upon the composition of feed offered to the flock. From the present study it can be concluded that yolk colour gets darker with the flock age. In the studies of Tumova *et al.* (2011), it was concluded that yolk colour is more related to housing rather than the genotype of the hen. Jacob *et al.* (2011) have mentioned the importance of plant pigment xanthophylls in poultry diets which result in the deep yolk colour egg yolk.

The variation in some of the egg quality parameters as shown in the graphs might be explained, at least in part by the limited number of eggs sampled in the study.

3.4.2 Estimation of the amount of cuticle

3.4.2.1 Shell reflectivity (%) and Spectrophotometry (L*a*b) measurements

The purpose of measuring the shell reflectivity (%) of stained eggs was to correlate reflectivity with the SCI L* values. Shell reflectivity values were partly affected by the underlying shell colour and partly by the cuticle blue stain taken up by the shell cuticle. Consistent with the shell reflectivity values of unstained eggs (Fig. 3.3.1.2), the shell reflectivity values of stained eggs (Table 3.4.2.1.1) also increased with flock age. A slightly higher shell reflectivity value at 65 wk (molted flock) compared to 55 and 75 wk was due to less stain acquired by the eggs as indicated by the SCI a* value. Similarly, shell reflectivity at 55 wk which was expected to be higher than 45 wk, was lower, but the SCI a* values for 55 wk indicated that these eggs stained well compared to 45 wk and thus shell reflectivity remained lower. Shell reflectivity of stained eggs was always lower compared to unstained eggs (data not included here). There was a positive correlation between shell reflectivity and the SCI L* component of the L*a*b space system. From the present experiments it can also be concluded that the amount of cuticle present can be assessed from the eggshell reflectivity and L*a*b measurements of eggs stained with cuticle blue dye. The lower shell reflectivity values for stained

eggs, the higher is the amount of cuticle. Shell reflectivity measurements are less time consuming compared to L*a*b measurements and the equipment required is less expensive.

The L*a*b system measures the grading between white and black. L* is similar to shell reflectivity and, in the current study, there was a high degree of correlation (r^2=0.933) between L* values and shell reflectivity. The a* value was the most important component of the L*a*b colour space system for the present study as it is the grading between red and green. A more negative value indicates a more green colour and therefore a greater affinity of the cuticle for MST blue stain and vice versa. The amount of cuticle deposited on the egg varied with flock age. The 55 wk and 75 wk old eggs showed maximum cuticle deposition followed by the 45 wk eggs. The 35 wk old eggs showed the least amount of cuticle. Cuticle deposition was generally higher in the middle of the lay and declined as the flock aged although there was an increase at the end of lay. Similar results have been previously observed by Messens *et al.* (2005); Sparks and Board (1984); and Bruce and Johnson (1978). Ruiz and Lunam (2000) recorded a thick cuticle in the peak production period compared to the beginning and end of lay. The mean cuticle deposition declined with the flock age in the study of Messens *et al.* (2005). The b* and value showed significant differences among age groups, decreasing slightly in the middle of the lay (45, 55 wk) then increasing (65 wk) before decreasing towards the end of the lay. Based on the present experiments, clear conclusions cannot be drawn concerning the effect of molting on the amount of cuticle present on the eggshell. The amount of cuticle in 65 wk (post molt) was lower compared to 55 wk (pre molt) but cuticle cover then increased at 75 wk before declining again at 85 wk.

Using Konica Minolta spectrophotometer (CM-2600d) for measurements of the eggs stained with MST cuticle blue, the most important component (L*a*b) of interest is a* which shows the amount of stain acquired by the eggshell cuticle. Leleu *et al.* (2011) have converted all three components in order to get one value which gives a very similar pattern to the SCI a* value.

3.4.2.2 Scanning Electron Microscopy (SEM) of the cuticle surface

The two different methods used for the cuticle quantification (Spectrophotometry and SEM) provided different insights into the extent of cuticle cover on the eggshells. The piece of shell viewed under SEM was far smaller than the area of the stained egg measured by the spectrophotometer and was scored for the extent of the patchiness of cuticle cover. The amount of cuticle quantified by SEM was

less significantly affected by flock age and tended to fluctuate with flock age, with the best cuticle cover being recorded at 55 and 75 wk of age and lowest at 35 and 85 wk. It can be concluded that the patchiness of cuticle laid on the eggshell varies less significantly with flock age. Through SEM scoring, it was confirmed that very few eggs had 100% cuticle cover and most of the eggs had cuticle ranging from 50-60% of the total surface. Similarly, Solomon (1992) and Nascimento and Solomon (1991) found that the cuticular layer is rarely present as an even covering and is not affected by flock age (Nascimento *et al.,* 1992). It can be concluded from Spectrophotometry (L*a*b*) and SEM observations that the amount of cuticle is generally best at peak production.

3.4.3 Ultrastructural scoring of the shell mammillary layer

From the Scanning Electron Microscopic (SEM) observations, it has been concluded that no two eggs from the same flock at the same age possess the same ultrastructural characteristics which, in turn, reflects a high rate of variation in the secretion of eggshell precursors in the shell gland of the hen's reproductive system. Thus, one of the possible factors for breaching the shell's inherent defense mechanism is an oviducal malfunction (Nascimento *et al.,* 1992). Analysis of the uterine fluid proteins (Gautron *et al.,* 1997) at various stages of shell formation and *in vitro* crystallization experiments (Gautron *et al.,* 1996) have unveiled the function of egg specific proteins that influence the process of calcification by modifying and modulating crystal growth (Nys *et al.,* 2004). Solomon (1992) concluded that shell quality decreases with increased flock age and old hen eggs are more prone to microbial penetration compared to eggs from younger flocks.

In the current study, variability of mammillary cap size increased to 45 wk of age, declined at 75 weeks but then increased at 85 wk. The lower variability of cap size at 65-75 weeks may be due to the induced molt conducted at 62 weeks of age. Less variability of cap size is thought to be associated with better egg quality.

Confluence results from the attachment of the mammillary caps to each other thus forming a smooth blanket on the surface of the mammillary caps. It is a desirable character and a high incidence of confluence may hinder the entrance of bacteria by obstructing its entrance to the egg. It also impedes crack propagation by decreasing the alignment in the shell. In the current study, confluence decreased with increased flock age and similar findings have been recorded by Solomon (2009). Nascimento (1992) explains that too much confluence may disrupt the pattern of pore distribution and in "setting"

eggs could have deleterious consequences. Good confluence also leads to the firm attachment of the shell membranes to the mammillary cones. Confluence alters pore distribution and influences the formation of the palisade layer (Solomon and Bain, online). A higher incidence of confluence increases resistance of the shell to microbial penetration (Solomon, 1992).

A high incidence of early fusion increases the bonding strength between mammillary cones and has a positive effect on mammillary layer strength (Parsons, 1982). In the current study, early fusion decreased as flock age increased indicating that this aspect of shell quality becomes poor with increased age. A significant difference ($P<0.0001$) was recorded between 25 wk and all other collections. Early fusion enhances the effective thickness of the palisade column (Solomon and Bain, online).

Cuffing is the deposition of extra calcium between the bases of the mammillary cores. An increased incidence of cuffing gives strength to the shell ultrastructure, as cuffing provides extra support to the base of the mammillary layer. With increased flock age, the incidence of cuffing decreased. Extra crystalline cuffs at the junction of the cones and palisade layers assist in early fusion of the palisade columns and thereby decrease the lateral distribution of stress within the shell (Solomon and Bain, online).

The quality of mammillary caps decreased (score increased) with flock age to 55 wk and increased slightly at 75 wk before decreasing again at 85 weeks of age. The incidence of poor cap quality was highest at 85 wk. The current results can be compared to the findings of Nascimento *et al.* (1992) who reported a decrease in cap quality as flock age increased. Good cap formation increases shell resistance to microbial attack (Solomon, 1992; Nascimento and Solomon, 1991).

Late fusion which is considered by Solomon (1991, 1992) as a negative factor on the overall shell ultrastructural quality was slightly increased with the flock age in the current study, the reverse of the pattern for early fusion.

Alignment is a microscopic line passing between the mammillary cones in specific directions where the spaces between the mammillary cones “line up”. A high incidence of alignment encourages micro cracks and penetration of bacteria. The incidence of alignment generally increased with flock age. A slight decrease was recorded in 65 and 75 wk old eggs, which reflects a possible positive effect of

molting. However, Nascimento *et al.* (1992) recorded no significant difference in the incidence of alignment between different age groups. Solomon (1992) and Nascimento and Solomon (1991) have positively correlated alignment with shell cracking and bacterial penetration.

Type A bodies are mammillary cones lying deeply in between the mammillary cones. They have no caps and therefore lack the affinity for attachment to the shell membranes. A high incidence of Type A bodies leads to weakening of the shell ultrastructure. The increased incidence of Type A bodies with flock age indicated that shell quality decreased as the flock gets older. Similar results were obtained by Nascimento *et al.* (1992) who mentioned stress as a contributing factor to the high incidence of Type A bodies. In the study of Rayan *et al.* (2010), the incidence of Type A bodies was higher in the older flock.

A higher incidence of Type B bodies always leads to poor shell quality. Type B bodies are small cones with either no or poor attachment to the nearby mammillary cones and with a poor affinity for the shell membranes. Type B bodies are generally smaller than Type A bodies and occur singly or in bunch form. In cases of high incidence of Type B bodies, the mammillary caps will have less affinity for attachment to the shell membranes. Generally they occur more in poor quality shells and, with increased age, their incidence increases. In the current study, a linear increase was recorded in the incidence of Type B bodies until 55 wk old then a slight decrease in the 65 and 75 wk flocks (eggs were collected post molting) followed by an increase at 85 weeks. The incidence of Type B bodies increased with age in the study of Nascimento *et al.* (1992) which was attributed to environmental stress. A high incidence of Type B bodies may decrease the shell fracture toughness (Bain, 1992). In the studies of Rayan *et al.* (2010), the incidence of Type B bodies was higher in the older flock. However, Type B bodies are often found in the shells of young birds (Solomon and Bain, online).

The high incidence of cubic cone formation may lead to poor mammillary layer quality. Slight increases in the cubic cone formation with the flock age in the current study suggest that cubic cone formation increases with flock age. Under conditions of stress and when the formation of normal palisade columns is impaired, cubic crystals frequently occupy inter mammillary sites (Solomon and Bain, online).

The reason for the higher incidence of changed membrane at 25 wk compared to the rest of all collections might have been due to inefficiency of peeling of shell membranes during specimen

preparations for plasma etching. A slight increase until 55 wk might indicate that the incidence of changed membrane is higher in young hens. A high incidence of changed membrane is thought to negatively affect mammillary layer quality. In the studies of Nascimento *et al.* (1992) the incidence of changed membranes did not significantly change with flock age.

The incidence of aragonite was not statistically significant in the present study. Aragonite occurs in the grape bunches like shapes between the mammillary cones. Aragonite has no attachment to the shell membranes and thus a higher incidence of aragonite leads to poor shell quality. The incidence of aragonite generally increases during the laying cycle (Nascimento, 1992).

Cubics occur mostly singly and scattered throughout the mammillary layer. They are fine specialized structures of calcite and with no attachment to the shell membranes. In the current study, the incidence of cubics did not change significantly with flock age. The incidence of depression, erosion and the hole was not statistically significantly affected by flock age in the current study.

Overall, Nascimento and Solomon (1991) have recorded a significant correlation between bacterial penetration and a high incidence of all ultrastructural variables that negatively affect eggshell quality.

3.4.4 Egg microbiology

The total bacterial count (TBC) on the eggshell surface increased with increasing flock age except for 35 wk. Bruce and Johnson (1978) reported increased contamination of eggshells with increasing flock age as the older hen had more microorganisms in faeces which in turn contaminated eggs passing through the vent. The higher total Enterobacteriaceae count (TEC) on the eggshell surface at 55 wk suggested that eggshell contamination was higher in the mid lay period and remained relatively low as the flock aged. Wall *et al.* (2008) reported no significant variation in eggshell Enterobacteriaceae load with increasing flock age in a conventional cage production system. In the studies of De Reu *et al.* (2005b; 2006c), there was no significant effect of hen age on bacterial eggshell contamination by using the shell wash method for bacterial enumeration.

In this study, the shell rinse and shell crush methodology was followed for recovery of bacterial load from eggshell pores. This methodology has been successfully used previously for the recovery of *Salmonella* bacteria from eggshell pores by Chousalkar *et al.* (2010), Kawasaki *et al.* (2008), Musgrove *et al.* (2005) and Berrang *et al.* (1991).

Based on the egg internal contents negative for *Salmonella*, a clear statement can not be made as the sample size was very small in the current study and also there are selected *Salmonella* serovars which have the ability of vertical transmission. Similarly Chousalkar *et al.* (2010) could not recover *Salmonella* from commercial eggs processed for the recovery of *Salmonella* from eggshell surface, crush and internal contents. The current results of the overall total bacterial load compare with the findings of Chousalkar and Roberts (2012) who reported low recovery of total bacterial load from shell surface and shell crush. In the studies of Daughtry *et al.* (2005), *Salmonella* was not isolated from the internal contents of any of the 11,036 eggs sampled in a pilot test in Australia. In the present study, all the swabs from egg belts and manure were negative for *Salmonella* on the farm in the current study, however intensive sampling is required to rule out the possibility of *Salmonella* free status of the selected flock. It can be concluded that the overall low bacterial load on eggshells, in shell crush, were due to the effectiveness of sanitation procedures on the farm which was sampled during this study. Processing facilities or other layer farms were not included/sampled during this study.

Briefly it can be concluded that flock age does affect overall egg quality, which decreased towards the end of the production cycle. An induced moult does, however, results in rejuvenation of some measures of egg quality. Microbiologically, eggs tend to carry higher microbial loads towards the end of the production period.

Chapter 4

Longitudinal Study- Free Range Production System

4.1 Introduction

Free range egg production is one of the alternatives to the commercial cage system for poultry birds, where layers can roam freely in a defined area with limited access to natural nutrients from the surroundings. In Australia, consumer preference for free range eggs has been increasing and some smaller producers converted to free range production in preference to replacing old poultry sheds and cages with ones which meet the new code of practice. As the result of these factors, the demand for free range poultry eggs has been steadily increasing (Bejaei *et al.,* 2011). Although there are advantages of free range production in terms of freedom of movement of the birds, free range poultry birds have a higher risk of disease and parasites than caged birds (Permin *et al.,* 1999; Maurer, 2009; Lay Jr. *et al.,* 2011) which, in turn, reduces egg production and increases mortality rate (Permin *et al.,* 2006). Factors such as health, nutritional value, environmental issues and animal welfare are important issues in egg type selection for consumers (Bejaei *et al.,* 2011). Research has shown a higher bacterial load (4.82±0.51 log cfu/eggshell) on eggs from floor systems as compared to the cage system (4.97±0.58 log cfu/eggshell) (Huneau-Salaun, *et al.,* 2010). Naturally, the egg is well equipped to withstand microbial penetration but a small breach in the defensive layers of the shell can enhance microbial ingress with resultant deterioration in its properties.

There is limited literature available on the effect of free range production on overall egg quality. The incidence of eggshell translucency is an interesting area of research as this phenomenon might affect egg quality, in particular microbial penetration. The relationship of cuticle cover to the egg microbial defense mechanism has been studied extensively but changes in cuticle cover with flock age have not been clear to date. Ruiz and Lunam (2000) recorded a thick cuticle layer in peak production compared to the beginning and end lay periods in broiler breeder hens. A lesser amount of cuticle deposition at the end of the lay period has been described previously by Messens *et al.* (2005), Sparks and Board (1984) and Bruce and Johnson (1978).

4.2 Materials and methods

For each egg collection, 150 fresh eggs were collected directly from the nests and brought to the egg laboratory of the University of New England. The free range production system was located in the Upper Hunter region of Sydney NSW. The age of the flock at the first collection was 25 wk and the same flock was sampled every 10 wk until 75 wk of age (the age at the end of production). During this study, a total of 900 eggs were collected, of which 540 were processed for determination of eggshell and egg internal quality (180 eggs), amount of cuticle estimation (180 eggs) and shell ultrastructural feature scoring (180 eggs) while the remaining 360 eggs were processed for the recovery of bacteria from eggshell surface, eggshell crush and internal contents. The eggs were processed as described in Chapter 2. At each collection, 3 swabs were collected from egg belts by dragging sterile swabs on the egg belts and 3 swabs from poultry manure by dragging swabs in the manure. Thus a total of 18 swabs was collected from each of egg belts and poultry manure. Swabs were processed for the recovery of *Salmonella* as described in Chapter 2, section 2.4.3.

The flock received locally formulated feed “Ingham Omega” which contained wheat, sorghum, millrun, meat meal, soya meal, canola meal, lysine, limestone, methionine, choline chloride, omega oils, enzymes, salt, sodium bicarb potassium carbonate, yolk colour pigment, vitamin and trace mineral premix.

Data were analyzed using one way analysis of variance (ANOVA) as described in Chapter 2 section 2.5.

4.3 Results

4.3.1 Eggshell and egg internal quality measurements

All eggshell quality measurements were significantly ($P<0.05$) affected by bird age, including the eggshell translucency score. Translucency score was highest at 45 wk and lowest at 35 wk (Fig. 4.3.1.1). Shell reflectivity increased with increasing flock age to 65 wk and was slightly lower at the last collection (75 wk) (Fig. 4.3.1.2). Egg weight increased from 25 to 35 wk of age then remained relatively stable, as shown in Figure 4.3.1.3. Shell breaking strength (BSN) was lower at 65 weeks and highest at 45 weeks with the other sampling ages intermediate between the two (Fig. 4.3.1.4). Shell deformation (μm) remained relatively constant until towards the end of lay, being significantly

lower at 75 wk than for all other ages, as shown in Figure 4.3.1.5. Shell weight (g) increased with flock age until 45 wk, then remained relatively constant (Fig. 4.3.1.6). Percentage shell was relatively constant throughout the production except for 35 weeks (Fig. 4.3.1.7). Shell thickness (µm) was lower at 25 and 35 wk than at the later ages, as shown in Figure 4.3.1.8. There was a significant main effect of flock age for all the egg internal quality measurements. Albumen height (mm) generally declined with bird age, particularly from 55 week onwards (Fig. 4.3.1.9) and a similar pattern was seen for Haugh unit (Fig. 4.3.1.10). Yolk colour increased between 25 and 35 wk, remained relatively constant and then increased at 75 wk, as shown in Figure 4.3.1.11.

All the eggshell quality and egg internal quality variables are shown individually in the graphs below. Superscripts ($^{a, b, c, d}$) on the graph bars indicate significant differences between different age groups for respective variables.

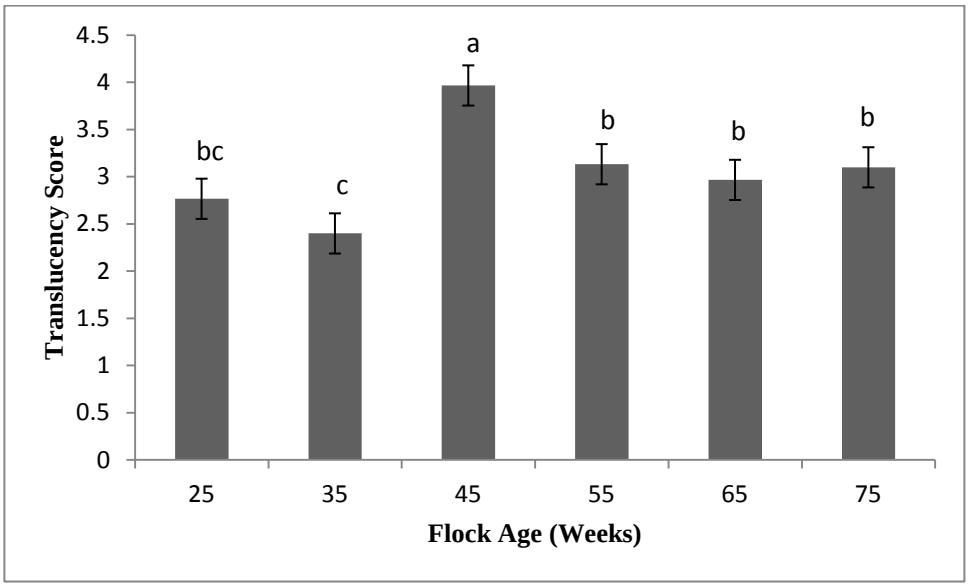

Figure 4.3.1.1 Translucency score at different ages (Mean±SE)

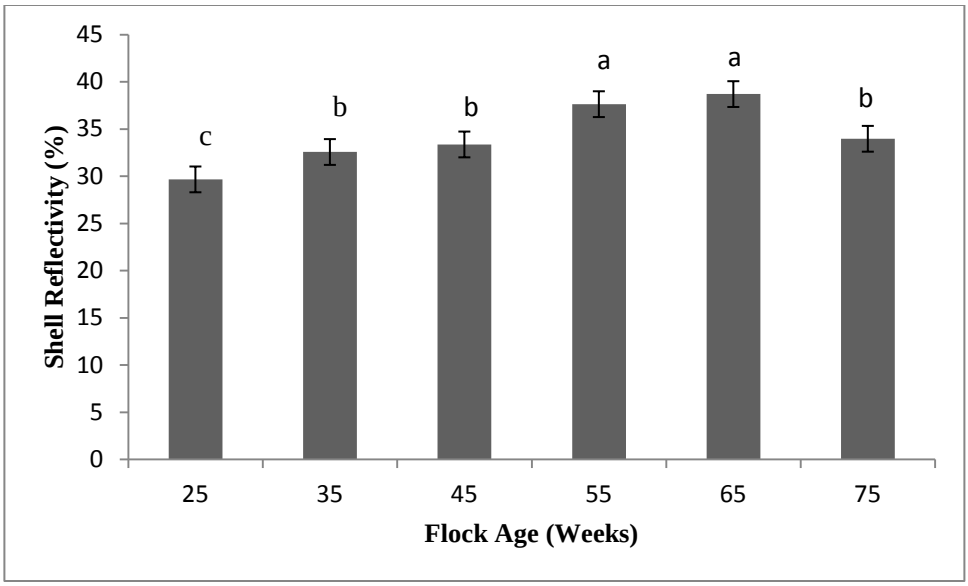

Figure 4.3.1.2 Shell reflectivity (%) at different ages (Mean±SE)

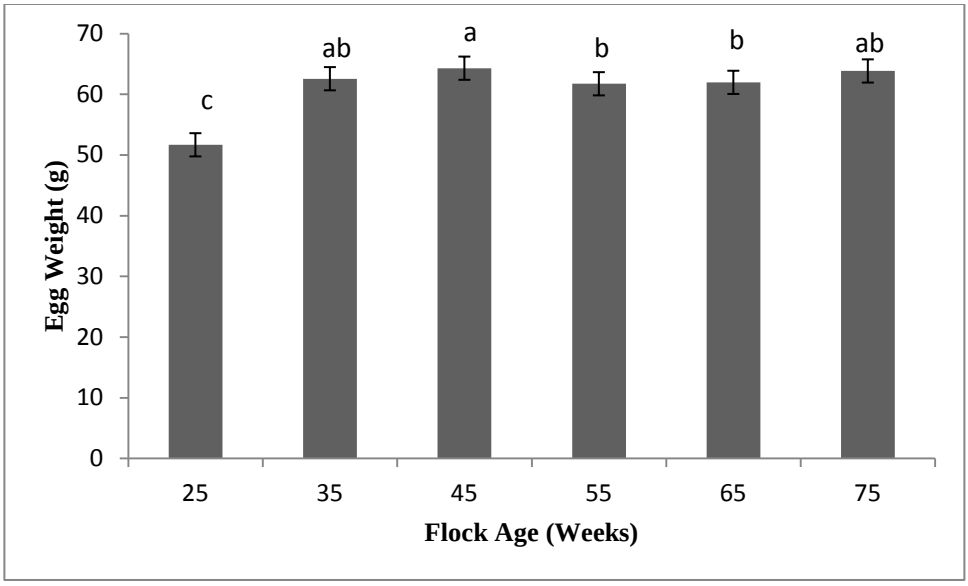

Figure 4.3.1.3 Egg weight (g) at different ages (Mean±SE)

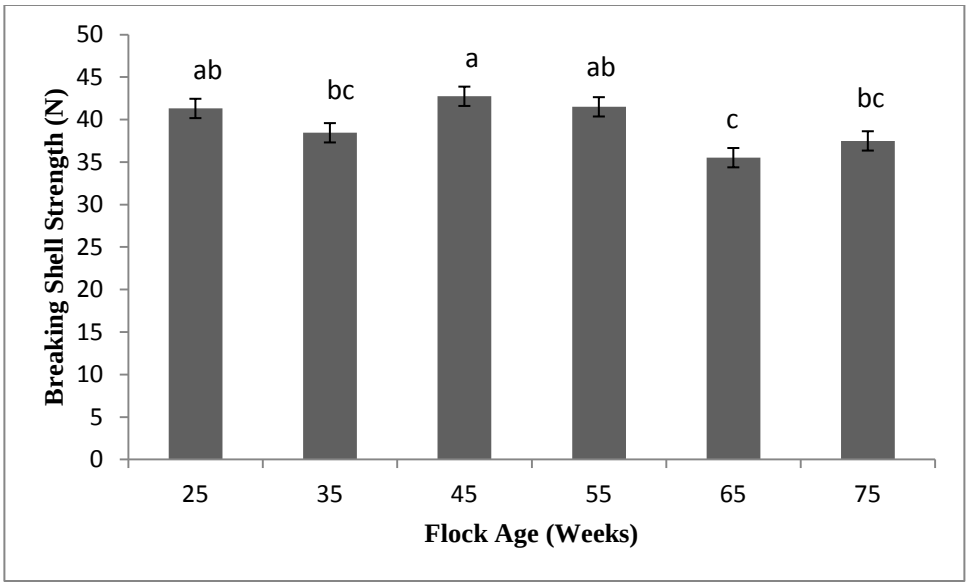

Figure 4.3.1.4 Breaking shell strength (N) at different ages (Mean±SE)

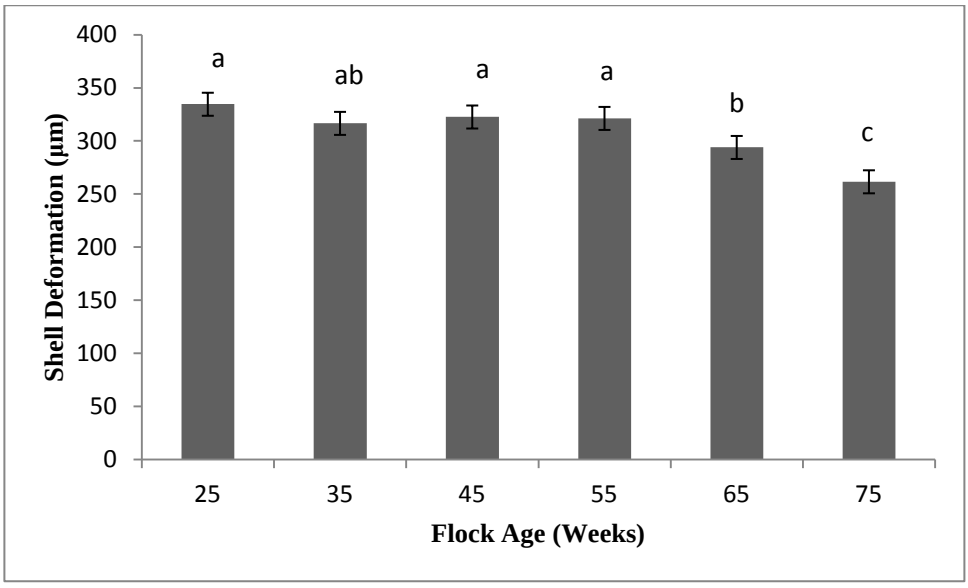

Figure 4.3.1.5 Shell deformation (µm) at different ages (Mean±SE)

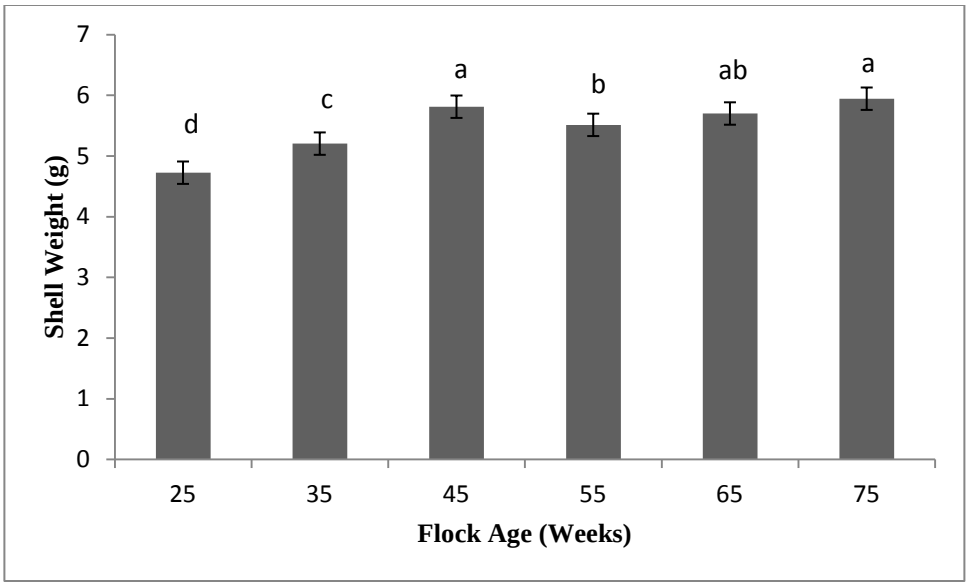

Figure 4.3.1.6 Shell weight (g) at different ages (Mean±SE)

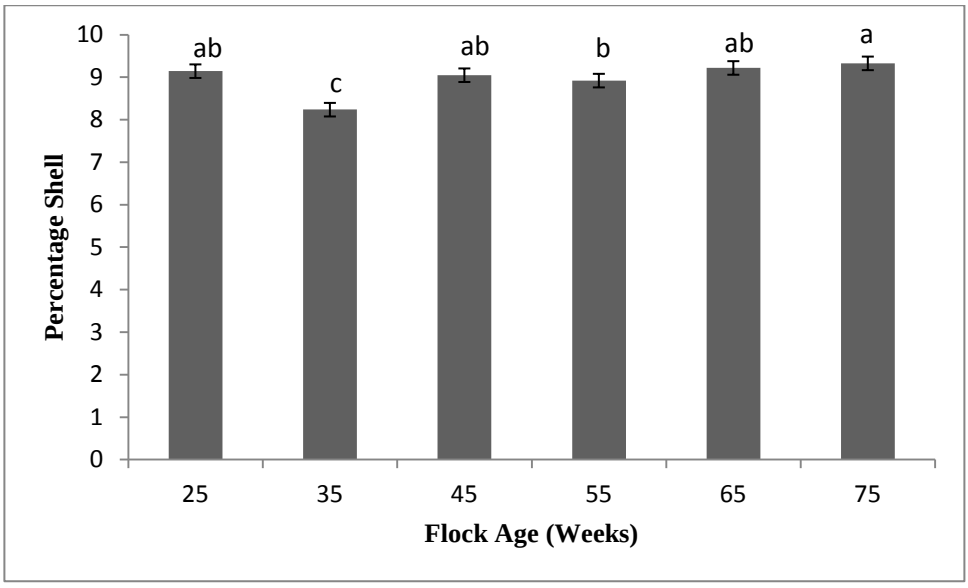

Figure 4.3.1.7 Percentage shell at different ages (Mean±SE)

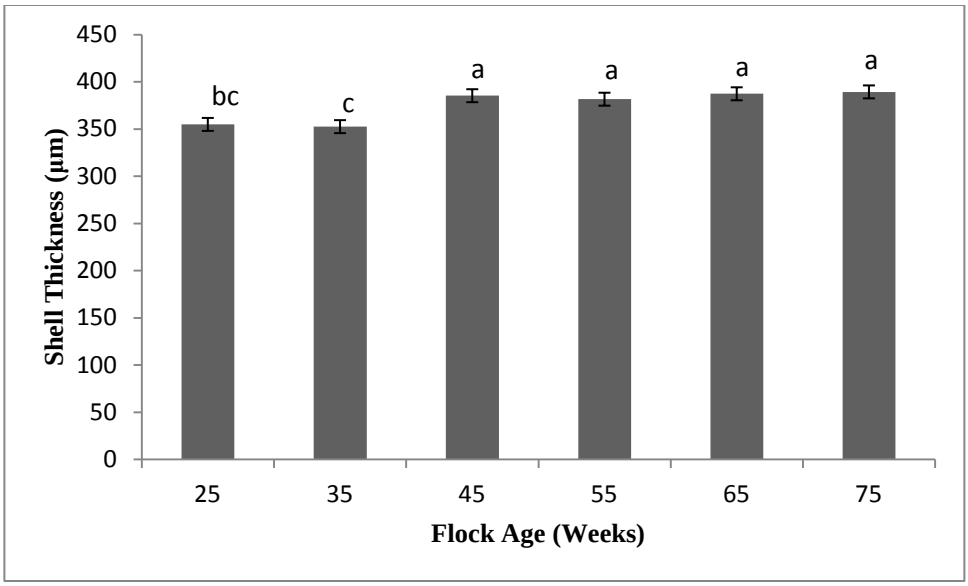

Figure 4.3.1.8 Shell thickness (µm) at different ages (Mean±SE)

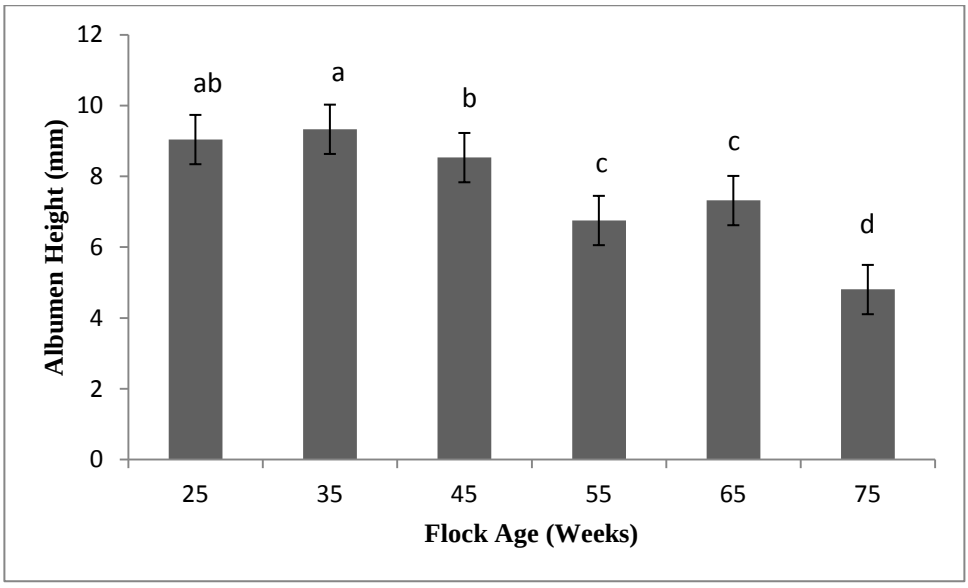

Figure 4.3.1.9 Albumen height (mm) at different ages (Mean±SE)

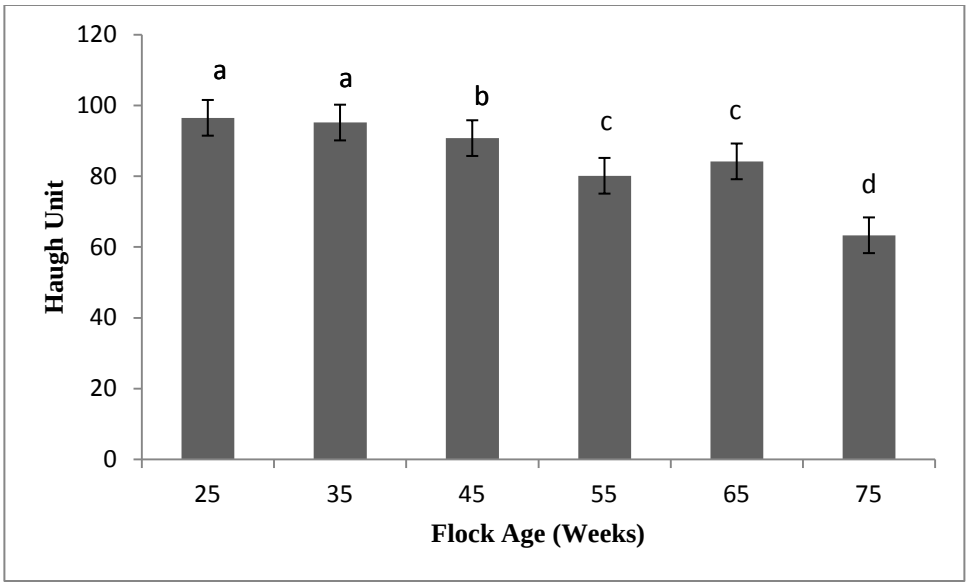

Figure 4.3.1.10 Haugh unit at different ages (Mean±SE)

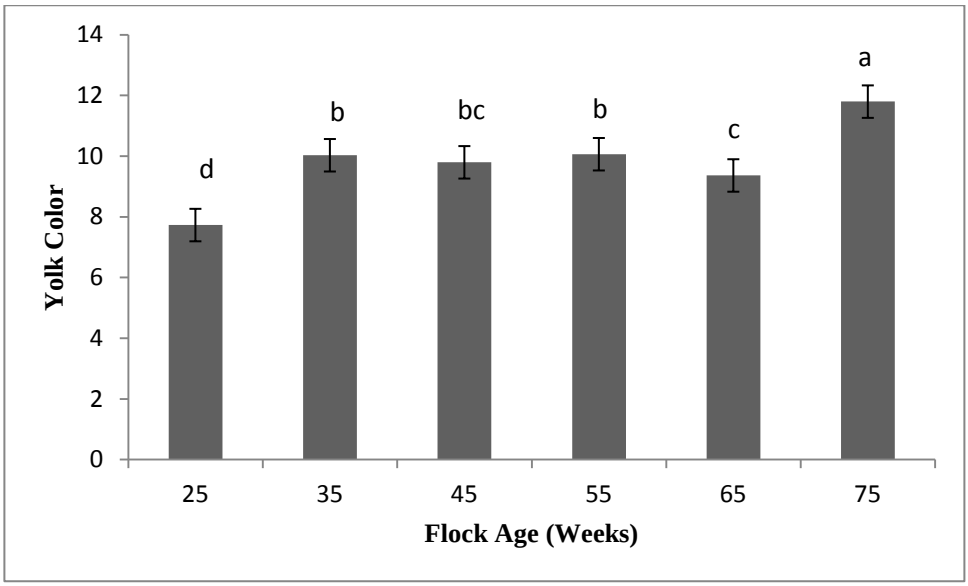

Figure 4.3.1.11 Yolk colour score at different ages (Mean±SE)

4.3.2 Estimation of the amount of cuticle

4.3.2.1 Shell reflectivity (%) and Spectrophotometry (L*a*b) measurements

All components of the L*a*b space system and shell reflectivity (%) of the stained eggs were statistically significantly different ($P \leq 0.05$) among age groups of the same flock as shown in Table 4.3.2.1.1. Shell reflectivity (%) of the stained eggs increased significantly with flock age being the highest at 65 wk. The SCI L* component of the L*a*b* space system was parallel in values to shell reflectivity and increased with flock age. The SCI a* component, where a more negative value indicates a greater amount of cuticle, generally decreased with flock age. SCI b* values showed slight increase and decrease followed by an increase in the last collection. SCE L*a*b* components were similar in values for SCI L*a*b* components, as shown in Table 4.3.2.1.1.

Table 4.3.2.1.1 Shell reflectivity (%) and L*a*b Values of stained eggshells

Variables	Flock age (weeks)						P value
	25	35	45	55	65	75	
Shell Reflectivity	26.30±0.84^{d}	25.77±0.59^{cd}	29.30±0.69^{b}	29.30±1.03^{b}	31.87±1.15^{a}	29.57±0.79^{ab}	<0.0001
SCI L	56.12±0.75^{d}	57.09±0.53^{cd}	59.79±0.65^{b}	60.44±0.93^{ab}	62.08±0.96^{a}	60.96±0.73^{ab}	<0.0001
SCI a	3.15±0.98^{a}	2.52±0.83^{a}	-0.85±0.92^{b}	-2.15±1.02^{b}	-1.35±0.97^{b}	-2.58±1.24^{b}	<0.0001
SCI b	34.47±0.42^{a}	34.67±0.50^{a}	32.42±0.46^{bc}	33.65±0.88^{ab}	30.76±0.58^{d}	31.29±0.54^{cd}	<0.0001
SCE L	55.90±0.76^{e}	56.92±0.54^{de}	59.68±0.66^{c}	60.27±0.94^{bc}	62.06±0.96^{ab}	60.88±0.74^{ac}	<0.0001
SCE a	3.18±0.98^{a}	2.55±0.83^{a}	-0.83±0.92^{b}	-2.14±1.02^{b}	-1.33±0.97b	-2.56±1.24^{b}	<0.0001
SCE b	34.72±0.42^{a}	34.89±0.51^{a}	32.49±0.45^{bc}	33.92±0.90^{ab}	30.79±0.59^{d}	31.37±0.56^{cd}	<0.0001

Values are Mean ± SE; SCI- Specular Component Included; SCE- Specular Component Excluded

$^{a, b, c, d}$ Values with different superscripts are significantly different from each other

4.3.2.2 Scanning Electron Microscopy of the cuticle surface

The total amount of cuticle cover was scored on the scale described in Table 2.2.4.1, Chapter 2 where 1 is a good intact cuticle and 4 is little or no cuticle. There was a significant effect of flock age on the amount of cuticle, as shown in Table 4.3.3.2.1. Scanning Electron Microscopy (SEM) of the cuticle showed that the amount of cuticle was lowest at the beginning and end of lay and highest at 55 wk. SEM mean values at different ages were in comparison to the values of SCI a* component of the L*a*b space by spectrophotometry, showing a general decrease in cuticle cover with flock age but with an increase at 75 wk.

Table 4.3.2.2.1 Scanning Electron Microscopy (SEM) values of cuticle cover

Variable	Flock age (weeks)						P value
	25	35	45	55	65	75	
Cuticle cover	2.57 ± 0.12^{b}	2.60 ± 0.09^{ab}	2.07 ± 0.11^{cd}	1.80 ± 0.15^{d}	2.03 ± 0.15^{d}	2.40 ± 0.15^{bc}	<0.0001

Values are Mean ± SE

$^{a, b, c, d}$ Values with different superscripts are significantly different from each other

4.3.3 Ultrastructural scoring of the shell mammillary layer

The incidence of all the eggshell mammillary layer features scored was significantly affected ($P\leq0.05$) by bird age except for mammillary cap size, cubic, depression and hole. The variables which were significantly affected by age are shown in graphs whereas those where there were no significant effects are summarized in Table 4.3.3.1. The incidence of confluence, cuffing and changed membrane decreased and cap quality was lower with increasing flock age (Figs. 4.3.3.1, 4.3.3.10, 4.3.3.11, 4.3.3.2). The incidence of alignment, Type A bodies, Type B bodies, the cubic cone formation and erosion increased as the flock grew older (Figs 4.3.3.5, 4.3.3.6, 4.3.3.7, 4.3.3.9,

4.3.3.12). The incidence of early fusion generally decreased and that of late fusion increased in early lay before remaining relatively constant (Figs. 4.3.3.3, 4.3.3.4). The incidence of aragonite was highest at 55 wk (Fig. 4.3.3.8). There was no significant effect of flock age on mammillary cap size variability, and the incidence of cubics, depression and holes (Table 4.3.3.1). Different superscripts ($^{a, b, c, d,}$) on the graphs indicate significant differences between different age groups for respective variables.

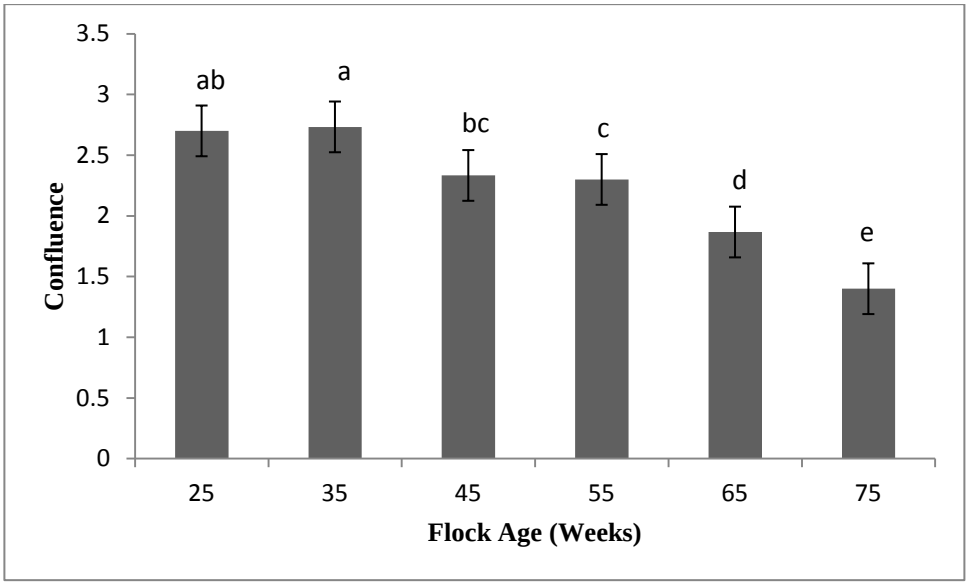

Figure 4.3.3.1 Incidence of confluence at different ages (Mean±SE)

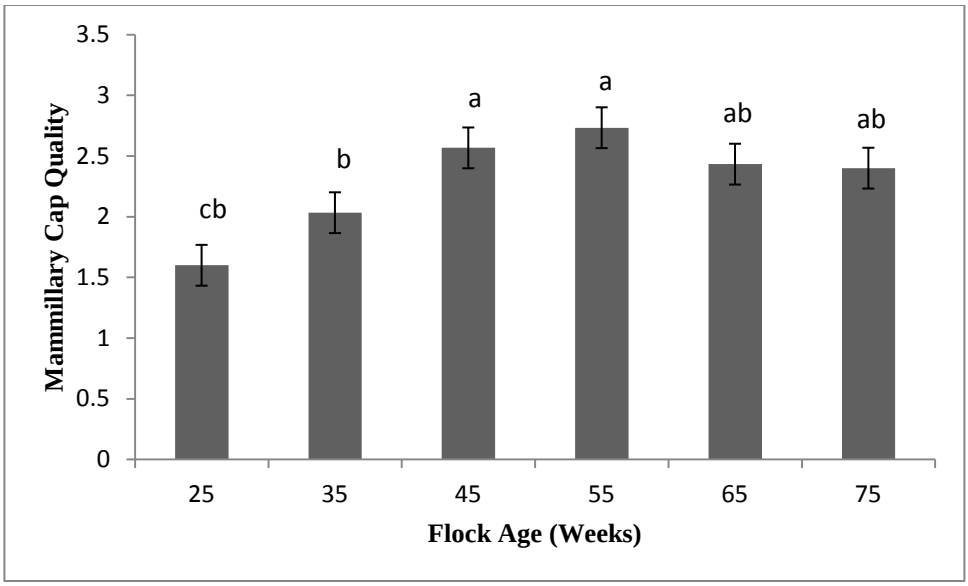

Figure 4.3.3.2 Incidence of mammillary cap quality at different ages (Mean±SE)

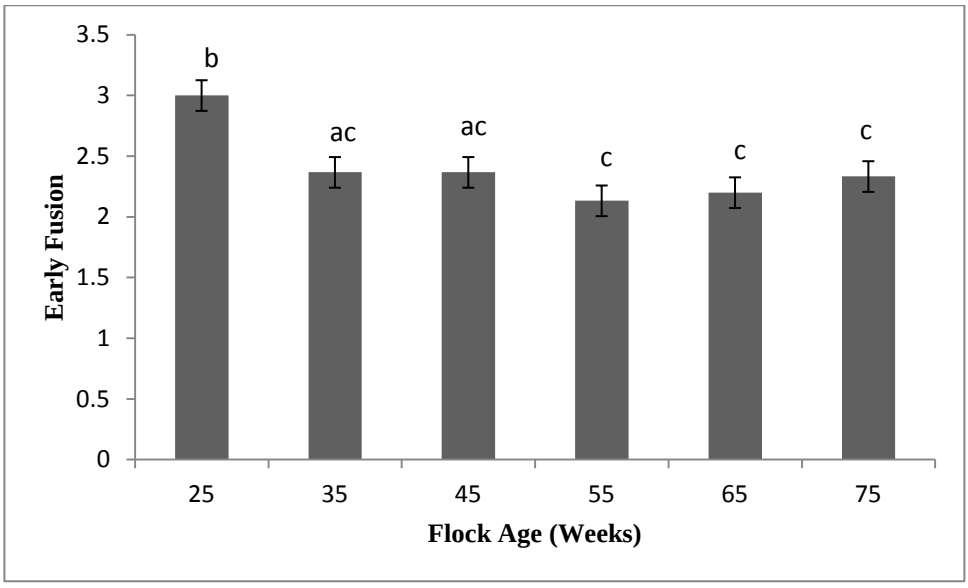

Figure 4.3.3.3 Incidence of early fusion at different ages (Mean±SE)

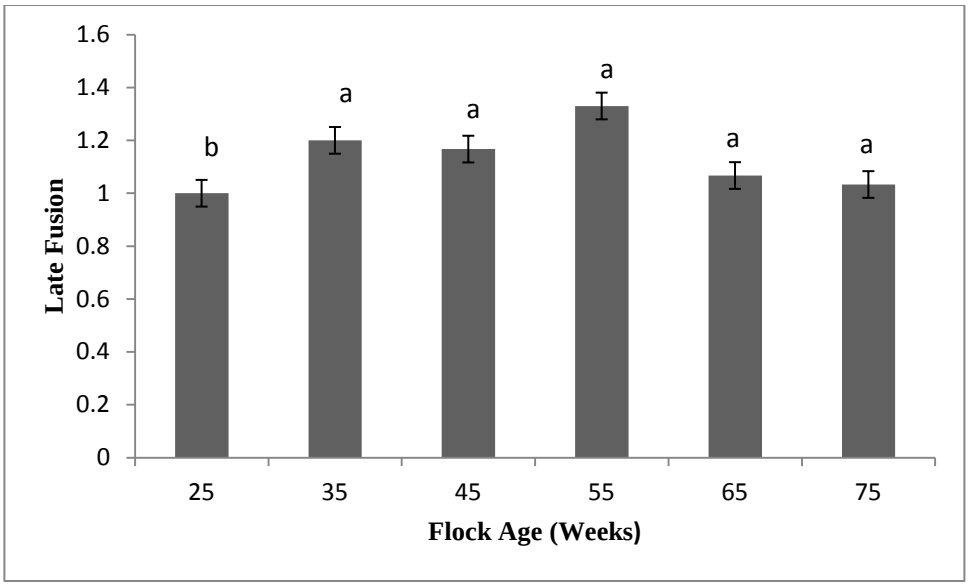

Figure 4.3.3.4 Incidence of late fusion at different ages (Mean±SE)

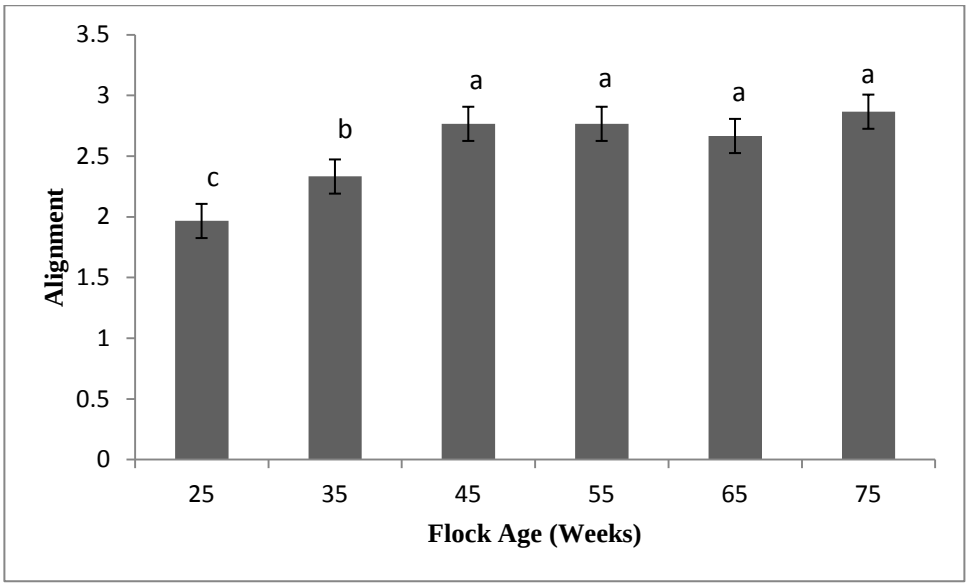

Figure 4.3.3.5 Incidence of alignment at different ages (Mean±SE)

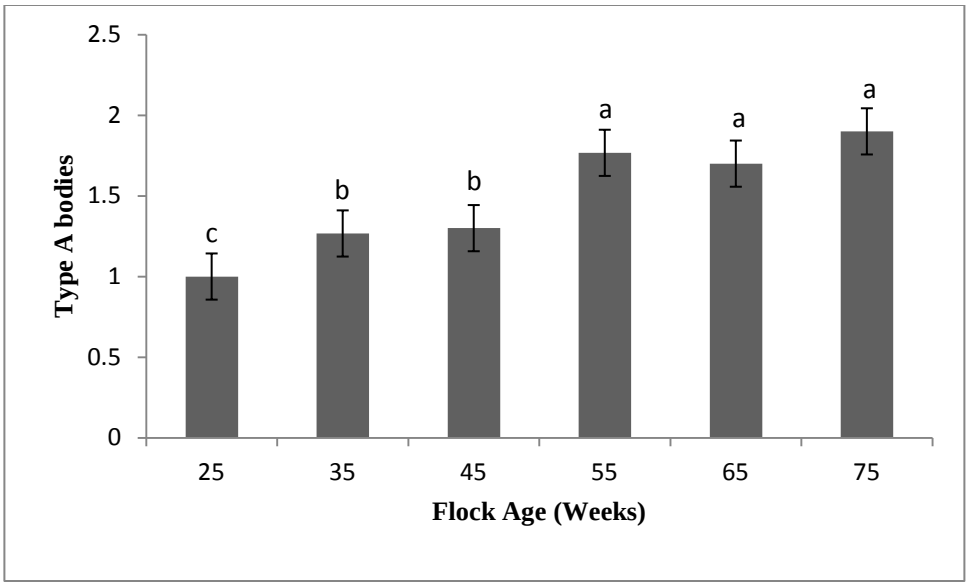

Figure 4.3.3.6 Incidence of Type A bodies at different ages (Mean±SE)

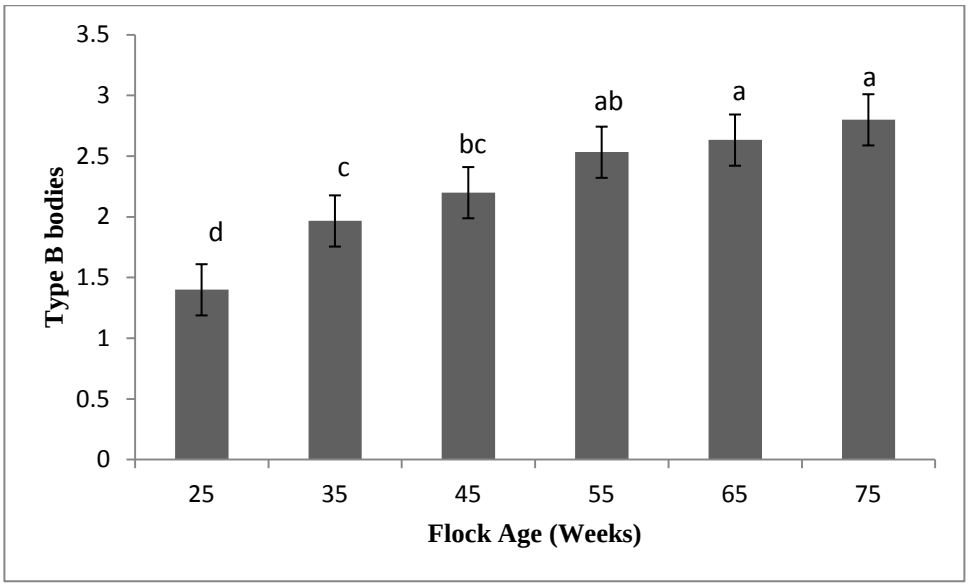

Figure 4.3.3.7 Incidence of Type B bodies at different ages (Mean±SE)

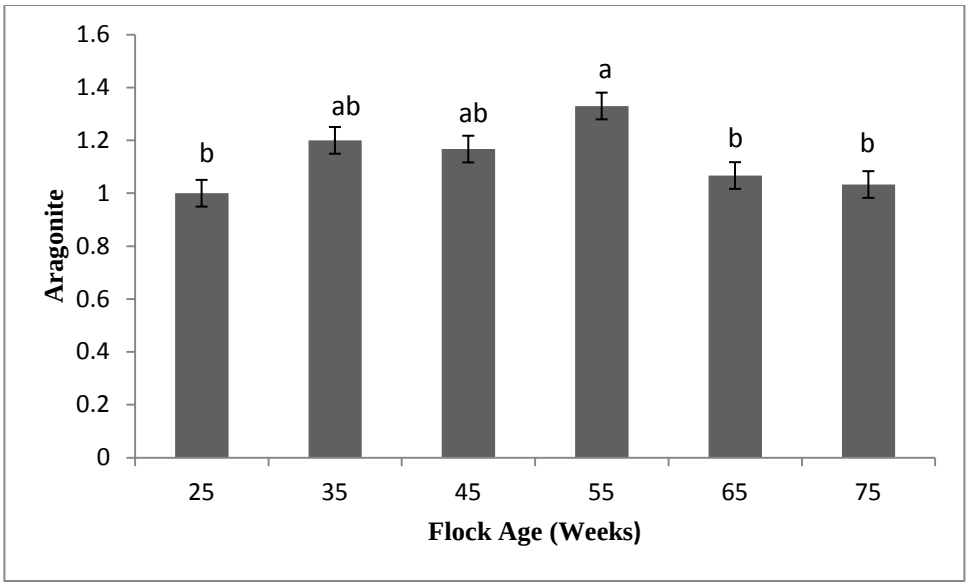

Figure 4.3.3.8 Incidence of aragonite at different ages (Mean±SE)

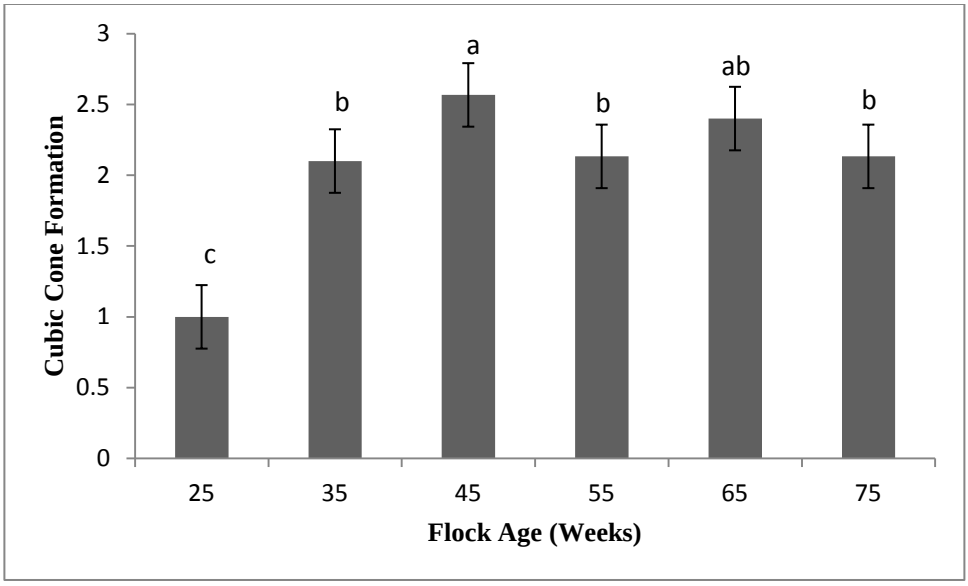

Figure 4.3.3.9 Incidence of cubic cone formation at different ages (Mean±SE)

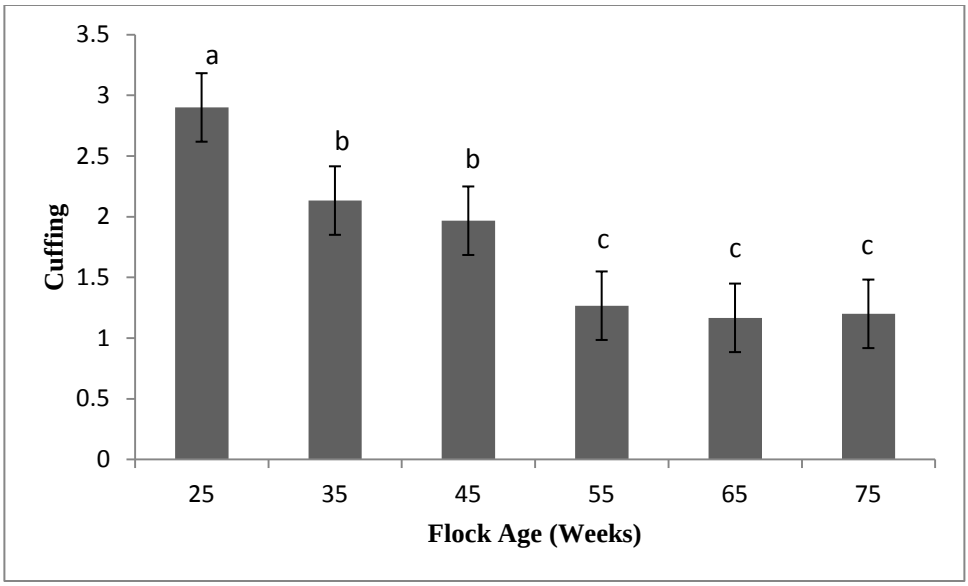

Figure 4.3.3.10 Incidence of cuffing at different ages (Mean±SE)

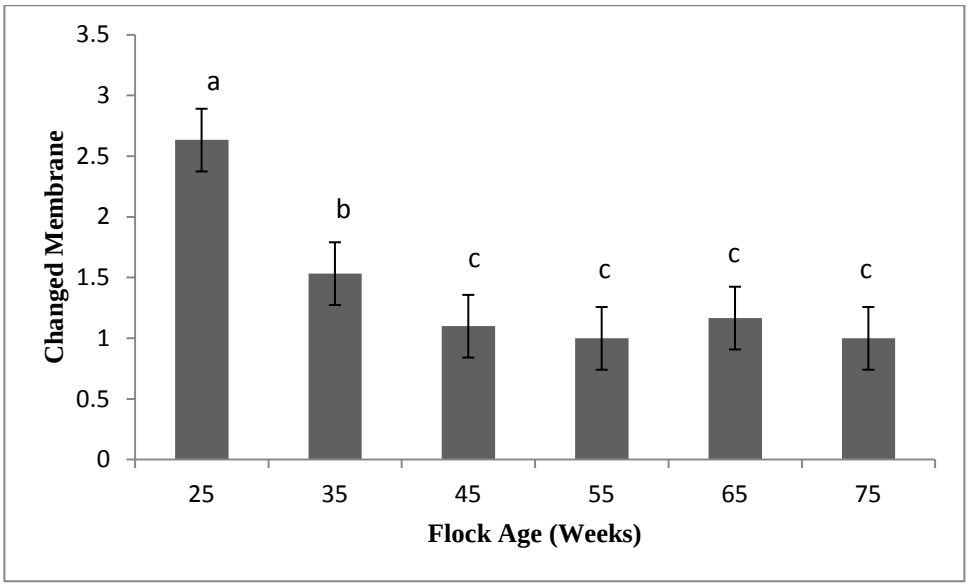

Figure 4.3.3.11 Incidence of changed membrane at different ages (Mean±SE)

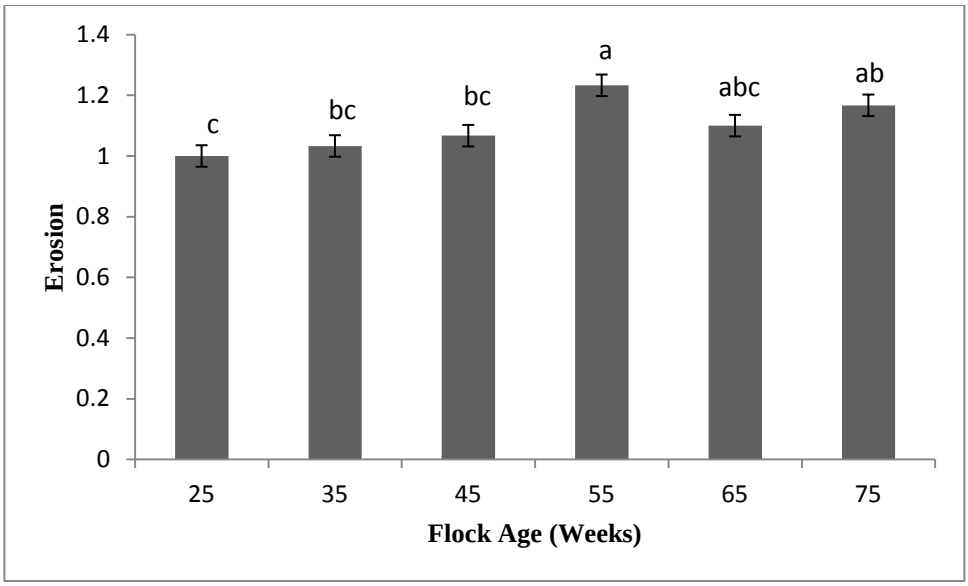

Figure 4.3.3.12 Incidence of erosion at different ages (Mean±SE)

Table 4.3.3.1 Shell ultrastructural variables not significantly affected by hen age

Variables	Flock age (weeks)						P value
	25	35	45	55	65	75	
Mammillary cap size	1.83±0.09	2.10±0.12	2.03±0.14	2.13±0.12	2.27±0.11	2.20±0.09	0.1135
Cubics	1.03±0.03	1.03±0.03	1.20±0.07	1.07±0.05	1.03±0.03	1.10±0.07	0.1704
Depression	1.00±0.00	1.20±0.09	1.10±0.06	1.07±0.05	1.10±0.06	1.20±0.10	0.2315
Holes	1.00±0.00	1.00±0.00	1.00±0.00	1.03±0.03	1.00±0.00	1.00±0.00	0.4193

Values are Mean ± SE

4.3.4 Egg Microbiology

There was a significant main effect of flock age on the total bacterial count (TBC) on the shell surface and total Enterobacteriaceae count (TEC) on the shell and in the shell crush as shown in Table 4.3.4.1. TBC in the shell crush did not significantly vary with flock age. TBC on the shell surface significantly increased towards the end of lay as shown in Table 4.3.4.1. TBC in shell crush tended to increase with the flock age and the highest cfu was recorded in 75 wk eggs. TEC on the shell surface at 25 wk was significantly different from the other collections while TEC in shell crush at 35 wk was significantly different from the rest of the collections. None of the egg internal contents were positive for bacteria. Two manure belt swabs were positive for *Salmonella* spp. One was *Salmonella* Infantis and another was *Salmonella* serovar 4, 12d.

Table 4.3.4.1 TBC and TEC (10 log cfu) on eggshell and in shell crush

Variables	Flock Age (weeks)						P Value
	25	35	45	55	65	75	
TBC on eggshell	4.20±0.16^{b}	3.472±0.09^{c}	2.71±0.18^{d}	3.46±0.11^{c}	4.29±0.05^{b}	4.78±0.04^{a}	<0.0001
TBC in shell crush	0.39±0.29^{b}	1.37±0.56^{ab}	1.09±0.36^{ab}	1.33±0.37^{ab}	1.82±0.42^{a}	2.09±0.60^{a}	0.1207
TEC on eggshell	2.59±0.60^{a}	1.11±0.37^{b}	1.06±0.36^{b}	1.05±0.44^{b}	0.96±0.39^{b}	0.80±0.33^{b}	0.0480
TEC in shell crush	0.11±0.07^{b}	1.99±0.66^{a}	0.76±0.32^{ab}	0.20±0.20^{b}	1.09±0.37^{ab}	1.40±0.48^{a}	0.0116

TBC- total bacterial count; TEC- total Enterobacteriaceae count; Values are Mean ± SE

$^{a, b, c, d}$ Values with different superscripts are significantly different from each other

4.4 Discussion

4.4.1 Eggshell and egg internal quality measurements

Translucency score was higher in the middle of the lay (45 wk) as these eggs were scored 5 days following collection of the eggs as compared to the rest of collections which were usually scored after 3 days. From the current research, it appears that translucency score is affected by both egg storage time and flock age. A slightly lower incidence of translucency at 25 and 35 wk is consistent with the findings of Solomon (1986) who reported minimum translucency in young bird eggs and an increase in its incidence with increasing flock age. As expected, shell reflectivity (%) increased with flock age. It has been suggested that, with increases in egg weight, shell reflectivity increases as the same amount of colour is distributed over a larger surface of the eggshell (Odabasi *et al.,* 2007). A relatively low shell reflectivity (%) in the 75 wk egg flock might also reflect an effect of diet as the flock was put onto a different diet at this age. Zita *et al.* (2009) have shown an increase in shell reflectivity (%) with increased flock age until 60 wk which is comparable with the current findings. Normally, egg weight (g) increases with age and similar results were obtained in the current study except for 55 and 65 week eggs which were slightly lower than the 45 week eggs. The slight decrease in egg weight at 55 weeks age might reflect the importance of diet as the flock was put on the owner formulated diet “mash from crumbles”. A spotty liver disease outbreak was also recorded at this age and this may have affected egg weight to some extent. These results are comparable to the findings of several authors (Guesdon *et al.,* 2006; Guesdon and Faure, 2004; Mallet *et al.,* 2006; Van Den Brand *et al.,* 2004; Novo *et al.,* 1997; Silversides, 1994; Rayan *et al.,* 2010; Tumova and Ledvinka, 2009; Berrang *et al.,* 1998 and Ferrante *et al.,* 2009) who reported an increase in egg weight with flock age. Shell breaking strength (BSN) was generally higher in the middle of the lay and decreased as the flock aged. Authors including Rodriguez-Navarro *et al.* (2002) and Rayan *et al.* (2010) have reported a decrease in BSN with increased flock age. Higher BSN at the flock peak production stage suggests that the shell is stronger in the middle of the lay. Shell deformation (μm) was relatively constant until 55 weeks and decreased towards the end of production. Decreased shell deformation indicates a less elastic shell and therefore poorer shell strength. Based on these findings, it is concluded that shell strength was at a maximum up to 55 wk of age and then decreased. The current results are contradictory to the findings of Zita *et al.* (2009) who reported an increase in the shell deformation values with flock age. Shell weight (g) generally increases with egg weight and in the current study

there was a positive correlation (r^2=0.467) between the two. Similarly, Zita *et al.* (2009), Nys (1986), Silversides and Budgell (2004) have recorded an increase in shell weight with flock age. Percentage shell remained relatively constant throughout the trial but was lower in the 35 week old flock. Most of the previous research has been conducted using conventional cages and there are few data on the percentage shell in free range production systems. Silversides and Scott (2001) recorded a decrease in percentage shell with increased flock age in ISA brown laying hens. However, the current findings are contradictory to the findings of Ronald *et al.* (1975) and Izat *et al.* (1985) who reported a decrease in percentage shell with increasing flock age. A slight increase in shell thickness (μm) with flock age in the current study can be compared to the findings of Tumova and Ledvinka (2009). Unlike the current findings, a significant decrease in shell thickness with increased flock age was recorded by Bell *et al.* (1978), Ronald *et al.* (1975). A slight increase followed by a consistent decrease in shell thickness by hen age was recorded by Garlich *et al.* (1984).

For the egg internal quality, albumen height (mm) decreased with flock age except for 65 weeks which was slightly higher than 55 weeks. This increase might be due to changes in flock diet. Silversides and Scott (2001), Silversides (1994) and Williams (1992) have recorded a decrease in albumen height with flock age. The relatively low albumen height in the 75 wk collection was contributed to, in part, by a time delay between egg collection and processing. Owing to a technical problem with the shell breaking strength machine, eggs collected (22/03/2012) at the age of 75 wk were processed on 27/04/2012 following storage at 4°C. A decrease in egg albumen height with increased storage time and temperature was reported by Karoui *et al.* (2008) and Silversides (1994). Similar to the albumen height values, Haugh unit showed slight increase in the 65 wk eggs. Haugh unit was positively correlated (r^2=0.945) with albumen height and the results can be compared to the findings of Zita *et al.* (2009) and Curtis *et al.* (1985). Yolk colour is dependent on the levels of pigment (xanthophylls) in the poultry diet and will also be influenced by feed intake of a particular diet. The yolk colour in the current study was low at 25 wk and remained constant in 35, 45, 55 and 55 wk and was higher 75 wk. These results most likely reflect the levels of yolk pigment in the different diets fed to the birds.

4.4.2 Estimation of the amount of cuticle

4.4.2.1 Shell reflectivity (%) and Spectrophotometry (L*a*b) measurements

Shell reflectivity (%) of the eggs stained with cuticle blue dye increased with flock age. This reflectivity is due to a combination of the underlying shell colour and the green colour imparted by the cuticle blue dye. The purpose of the stained eggshell reflectivity measurements was to compare its values with the SCI L* component of spectrophotometer. A positive correlation was recorded between shell reflectivity and SCI L* values.

The SCI L* component of the L*a*b system measures the grading between white and black and is similar to shell reflectivity, which simply indicates the colour lightness of the shell. In the current study, the increased L value with the flock age indicated that the amount of colour deposited decreased with flock age and this was observed despite the staining with cuticle blue dye. SCE L* was parallel to SCI L*. SCI a* measures the grading between red and green with green towards the negative end of the scale. The more negative value indicates the acquiring of more cuticle blue stain by the egg cuticle. With increasing flock age, the amount of cuticle deposited generally increased. Very few studies have used the L*a*b colour space system for quantifying the amount of cuticle in eggs from the same flock throughout its production cycle. Ruiz and Lunam (2000) recorded a thick cuticle in the peak production period compared to the beginning and end of lay. However, the SCI a* value for 75 wk suggests that the amount of cuticle was greater than for the other ages. However, a complication of the cuticle blue staining method is that the results for stained eggs will be influenced by the underlying shell colour which may or may not be directly related to the amount of cuticle present on the eggshell. The SCI b* component measures the colour grading between yellow and blue. In the current study SCI b* slightly decreased with the flock age.

4.4.2.2 Scanning Electron Microscopy (SEM) of the cuticle surface

Similar to the values of SCI a*, the SEM cuticle cover scoring system indicated maximum cuticle cover in the mid lay period. The values of SCI a* and SEM mean values cannot be compared directly as the piece cut from the eggshell for electron microscopy was smaller than the area covered by the spectrophotometric measurements. As viewed under the SEM, the amount of cuticle was lower at the

start and end of lay whereas SCI a* values show maximum cuticle towards the end of lay. In the investigations of Messens *et al.* (2005), the mean cuticle deposition declined as the flock aged.

4.4.3 Ultrastructural scoring of the shell mammillary layer

The incidence of the confluence was higher in the first 2 collections and then decreased as the flock advanced in age. Mammillary caps having more confluence provide strength to the shell, as it makes an even blanket on the cap surface, thus contributing to shell thickness and inhibiting microbial entrance (Solomon, 1992). A higher incidence of confluence can alter the pore distribution and influence the formation of the palisade layer (Solomon and Bain, online; Nascimento *et al.*, 1992). The low incidence of confluence in late lay suggests that shell ultrastructural quality deteriorates with flock age. Brackpool (1995) recorded a decrease in the incidence of confluence with increased flock age (33 vs 57wk).

A higher score for mammillary cap quality at peak production suggests that cap quality is affected by factors other than age as cap quality improved in the late lay period in the current study. A good cap quality gives extra strength to the mammillary layer and provides the maximum area for shell membrane attachment. A decrease in cap quality with flock age has been recorded by Nascimento *et al.* (1992) which is in contradiction to the current findings. In the studies of Brackpool (1995), cap quality was lower for 57 wk compared to 33 wk flock age.

Early fusion, which gives extra strength to the shell and enhances the effective thickness of the palisade columns (Solomon and Bain, online), decreased slightly with increased flock age. The incidence of early fusion did not change significantly with flock age in the studies of Nascimento *et al.* (1992). Decreased incidence of early fusion indicated the poor quality of mammillary layer towards the late lay period.

Late fusion generally increases with flock age and higher incidence of late fusion imparts a poorer quality to the shell. A slight increase was recorded in the incidence of late fusion with increased flock age. Solomon (1991) also recorded an increase in the incidence of late fusion with flock age.

The incidence of alignment was from moderate to high as the flock age increased. Areas of alignment may act as the points of initiation of micro and macro cracks which, in turn, put the shell at higher microbial risk. In the studies of Nascimento *et al.* (1992), there was no significant difference

(P>0.05) for alignment between different age groups but there was a high correlation between alignment and bacterial penetration.

A higher incidence of Type A bodies has the potential to decrease the strength of the mammillary layer strength by creating a gap between the mammillary layer surface and the shell membranes. Type A bodies are always deep in the mammillary layer and do not have any caps for attachment to the shell membranes. In the current study, the incidence of Type A bodies increased with increased flock age. A slight decrease was recorded in the 65 wk old flock eggs which might reflect a positive effect of the diet change on the ultra structure of the mammillary layer. A higher incidence of Type A bodies was recorded in older flock eggs by Rayan *et al*, (2010).

Type B bodies vary in size and may be numerous in the mammillary cone surface. Some of them are attached to the shell membranes. A high incidence of Type B bodies decreases the strength of the shell as mentioned by Solomon (1991). A high incidence of Type B bodies in older flock eggs was recorded by Rayan *et al.* (2010). However, Solomon and Bain (online) indicated a high incidence of Type B bodies in the young bird eggshells. A significant increase in the Type B bodies with the flock age was recorded by Nascimento *et al.* (1992).

Aragonite is amorphous calcium which is normally found in the eggshells of reptiles (Solomon, 1992). The incidence of aragonite was higher at peak production age (55 wk) while towards the end of the lay most of the eggshells were free from aragonite. Nascimento *et al.* (1992) reported a high incidence of aragonite in late lay and found a higher correlation of aragonite with the penetration of bacteria. No significant difference was recorded for aragonite incidence with the flock age in the studies of Rayan *et al.* (2010).

Cubic cone formation is an ultrastructural variation that imparts poor quality to shell. In the current study, a high incidence of cubic cone formation was recorded at peak production. Similarly, Brackpool (1995) has recorded a higher incidence of cubic cone formation in 57 wk flock eggs versus 33wk.

Cuffing is the deposition of extra calcium between the bases of the mammillary cores. A higher incidence of cuffing gives strength to the shell ultrastructure by reinforcing the mammillary cones. With increased flock age, the incidence of cuffing decreased. Extra crystalline cuffs at the junction of

the cone and palisade layers assist in early fusion of the palisade columns and thereby decrease the lateral distribution of stress within the shell (Solomon and Bain, online). Brackpool (1995) recorded a higher incidence of cuffing in 57 wk compared to 33 wk of flock age eggs.

A higher incidence of changed membrane in the 25 wk flock followed by decrease with flock age was recorded in the current study. The collections at 55, 65 and 75 wk showed no incidence of changed membranes. A higher incidence of changed membrane has also been recorded by Brackpool (1995) in 33wk flock eggs versus 57wk. Changed membrane is thought to decrease shell strength (Solomon, 1992).

The incidence of erosion increased slightly with flock age and was highest at 55 wk. A higher incidence of erosion can decrease shell strength and make it more prone to microbial attack (Solomon, 1992). In the studies of Brackpool (1995) the incidence of erosion was increased with flock age.

The incidence of mammillary cap size variability, cubic, depression and the hole did not change significantly ($P>0.05$) with flock age. Highly variable mammillary caps, and high incidence of cubic, depression and hole all negatively affect shell ultrastructure. Overall, the incidence of ultrastructural features which have a positive effect on shell quality decreased with increasing flock age and the incidence of negative features increased.

4.4.4 Egg microbiology

The egg and eggshell microbiology experiments showed that the total bacterial count (TBC) was higher in the early and late lay periods. It can be concluded that the TBC on eggshells remained low in the mid lay period. Similarly, TBC in shell crush was not significantly affected by flock age. In the studies of De Reu *et al.* (2005b, 2006c) and Protias *et al.* (2003a) there was no significant effect on hen age of bacterial eggshell contamination. Unlike the TBC prevalence, the total Enterobacteriaceae count (TEC) on the shell was significantly higher at 25 wk (2.6 log cfu) flock and reduced to less than 1 log cfu/mL rinsate in the last collection. The higher TEC in shell crush in the early and late lay periods might be more due to some other factors rather than age. The current results indicated that the total bacterial load on the eggshell is lower in the peak production period compared to early and late lay period. Out of the total manure swabs processed, the two samples positive for indicate that

Salmonella Infantis was present on the farm but probably in low numbers. Good hygienic practices on the farm could be a contributing factor to the low total bacterial load as well as *Salmonella* negative eggs.

Chapter 5

Comparison of Conventional Cage System to Free Range

5.1 Introduction

In Australia, conventional cage and free range are the main egg production systems. The estimated retail share of the Australian egg production market in 2010 from conventional and free range production systems was 58.6% and 28.4%, respectively (AECL Annual Report, 2010/11). A trend towards free range production is increasing as its market share value in the year 2010 (28.4%) was higher compared to 2009 which was 26.6% (AECL Annual Report, 2010). Eggs obtained from a conventional cage system have been reported as being cleaner and having better overall egg quality compared to free range eggs in the studies of many authors including Dukic-Stojcic *et al.* (2009). Consumers always prefer an egg in which the albumen is firm, the yolk has a dense colour, the egg is of an appropriate size with intact shell and free from pathogens.

In this chapter, the results presented in Chapters 3 and 4 are compared to investigate the effect of production system, either conventional cage (CC) or free range (FR), on egg quality parameters and egg microbial load.

5.2 Materials and methods

The details of the materials and methods are presented in Chapters 2, 3 and 4. In brief, eggs were collected at 25, 35, 45, 55, 65, and 75 wk of age from one cage flock and one free range flock. The two flocks were 4 weeks apart in age. The cage flock was molted at the age of 62 wk. During the study period, a total of 900 eggs was processed from each production system. Out of 900 eggs from each flock, 540 eggs were processed for the determination of traditional egg external and internal quality parameters, amount of cuticle estimation and the scoring of eggshell ultrastructural features. The remaining 360 eggs were processed for egg microbiology in which the total bacterial load on eggshells, shell crush and internal contents was recovered. Eggs were processed as explained in Chapters 2, 3 and 4. Data were analyzed using a two way analysis of variance (ANOVA) with production system and flock age as independent variables (2 x 6 factorial) as described in Chapter 2 section 2.5.

5.3 Results

5.3.1 Eggshell and egg internal quality measurements

For the eggshell quality parameters, there was a significant (P≤0.05) main effect of production system and interaction between production system and flock age for translucency score, shell reflectivity (%), egg weight (g), shell weight (g) and shell thickness (µm). Breaking shell strength (BSN), shell deformation (µm) and percentage shell were not significantly affected by production system although there was a significant interaction between production system and flock age as shown in Table B in the Appendix. For the egg internal quality, there was a significant main effect of production system and significant interaction between production system and flock age for albumen height (mm), Haugh unit and yolk colour. Translucency score (Fig. 5.3.1.1), shell reflectivity (Fig. 5.3.1.2), breaking shell strength (Fig. 5.3.1.7) and shell deformation (Fig. 5.3.1.8) were higher in the free range (FR) flock whereas egg weight (Fig. 5.3.1.3), shell weight (Fig. 5.3.1.4), percentage shell (Fig. 5.3.1.5) and shell thickness (Fig. 5.3.1.6) were higher in the cage production system (CC). The egg internal quality parameters albumen height (Fig. 5.3.1.9), Haugh unit (Fig. 5.3.1.10) and yolk colour (Fig. 5.3.1.10) were higher in the CC production system versus FR. All the eggshell quality and egg internal quality parameters are presented in the following graphs and Table B in the appendix.

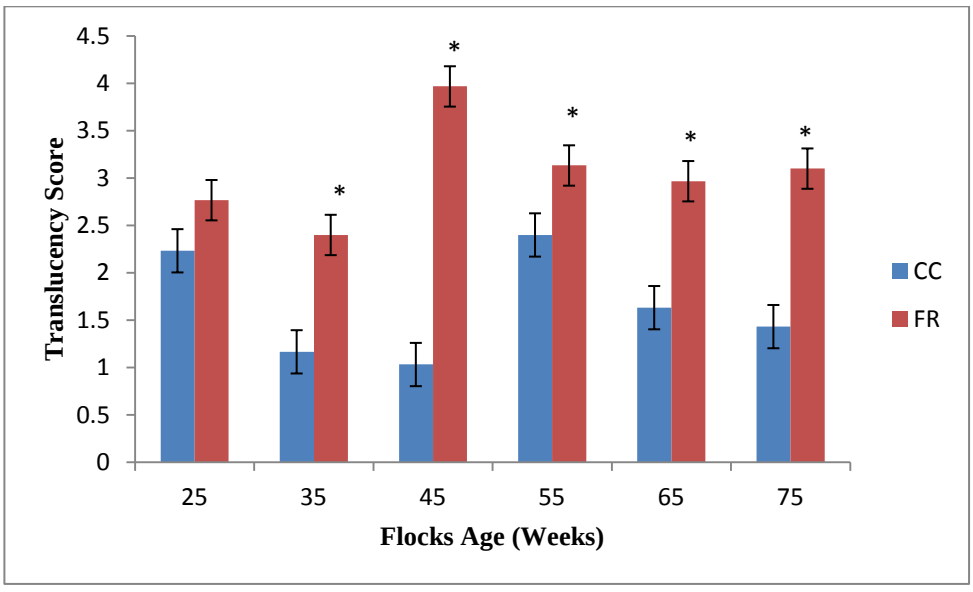

Figure 5.3.1.1 Mean ± SE. * Significantly different from cage production system

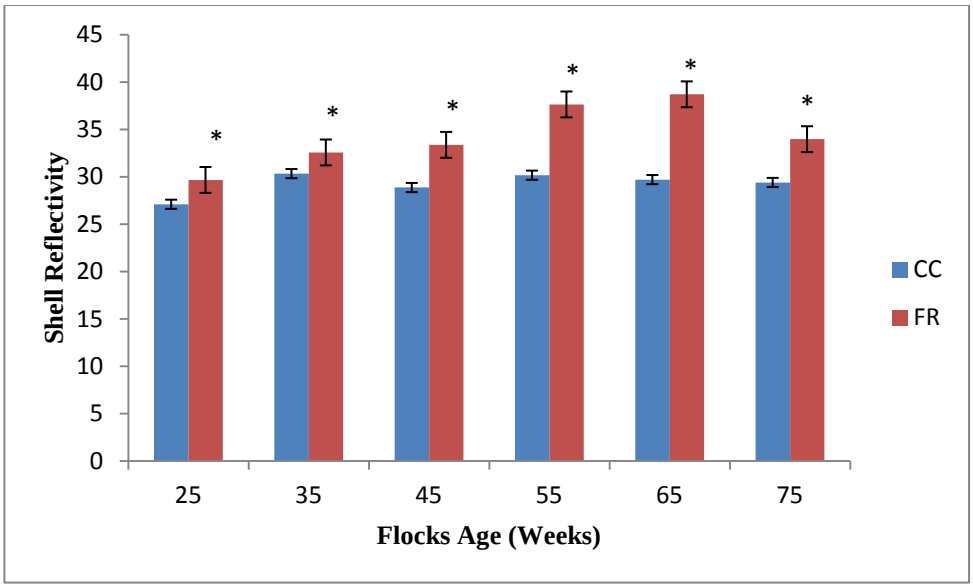

Figure 5.3.1.2 Mean ± SE. * Significantly different from cage production system

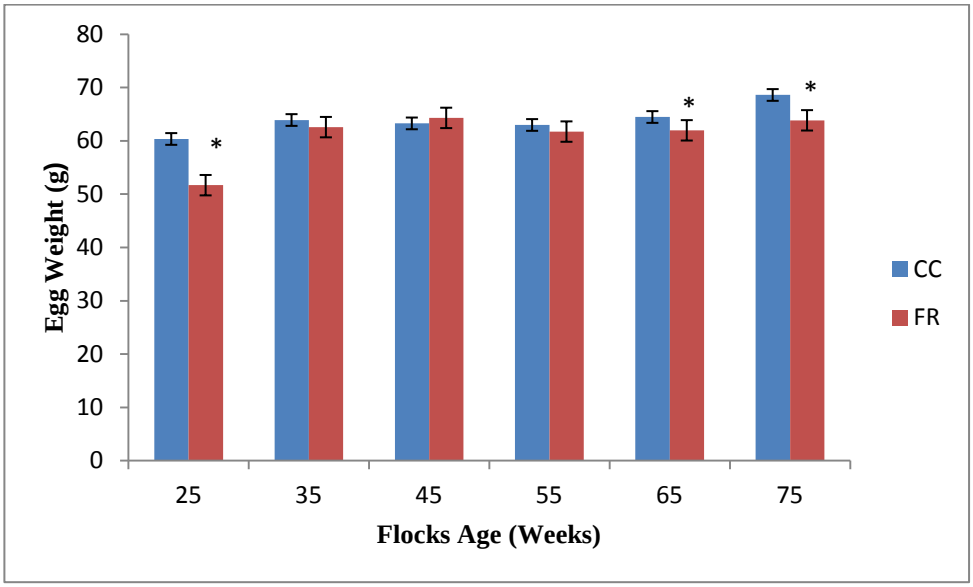

Figure 5.3.1.3 Mean ± SE. * Significantly different from cage production system

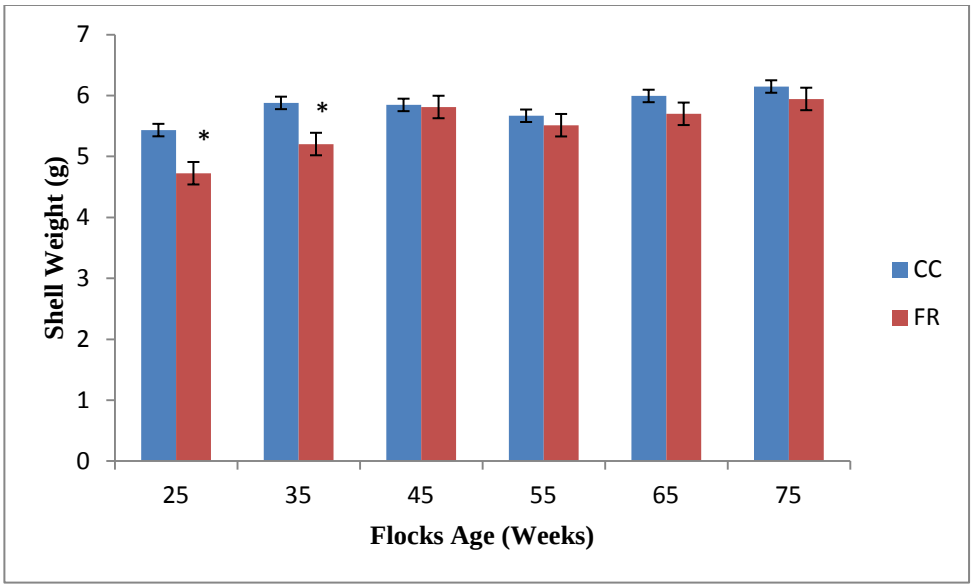

Figure 5.3.1.4 Mean ± SE. * Significantly different from cage production system

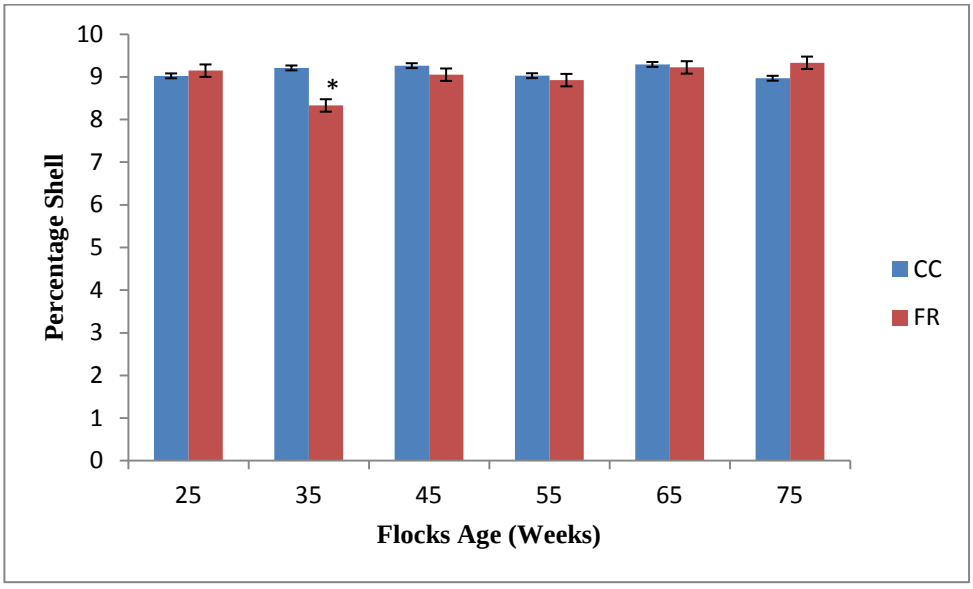

Figure 5.3.1.5 Mean ± SE. * Significantly different from cage production system

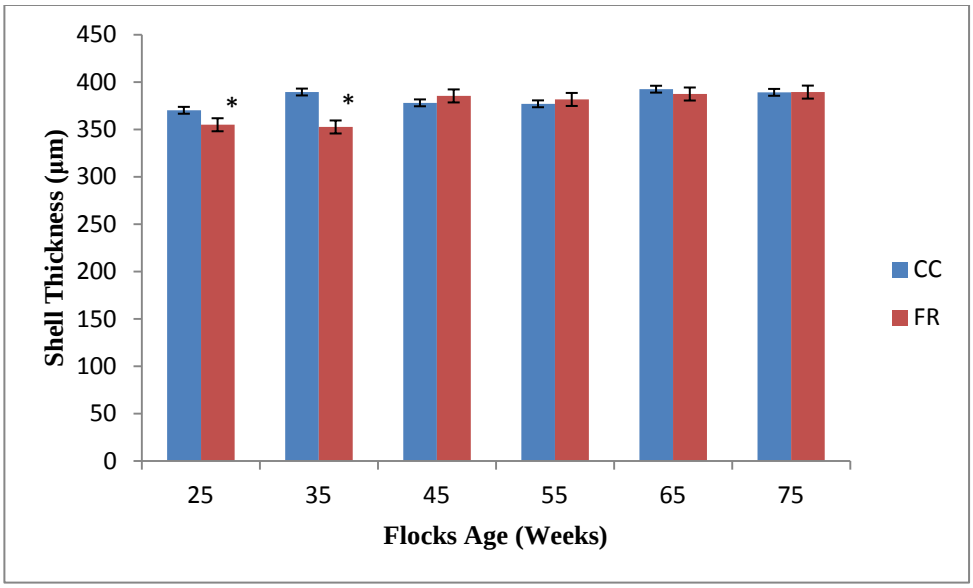

Figure 5.3.1.6 Mean ± SE. * Significantly different from cage production system

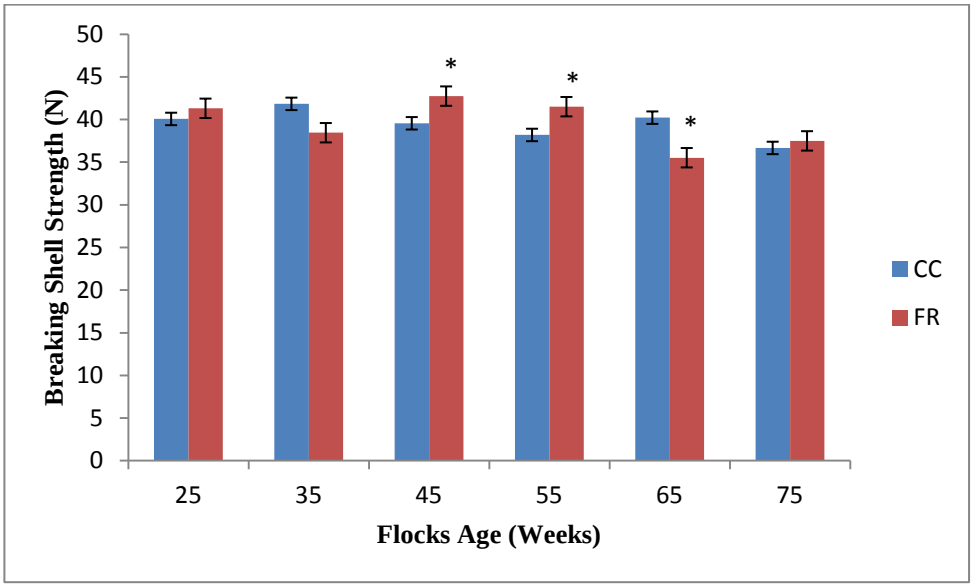

Figure 5.3.1.7 Mean ± SE. * Significantly different from cage production system

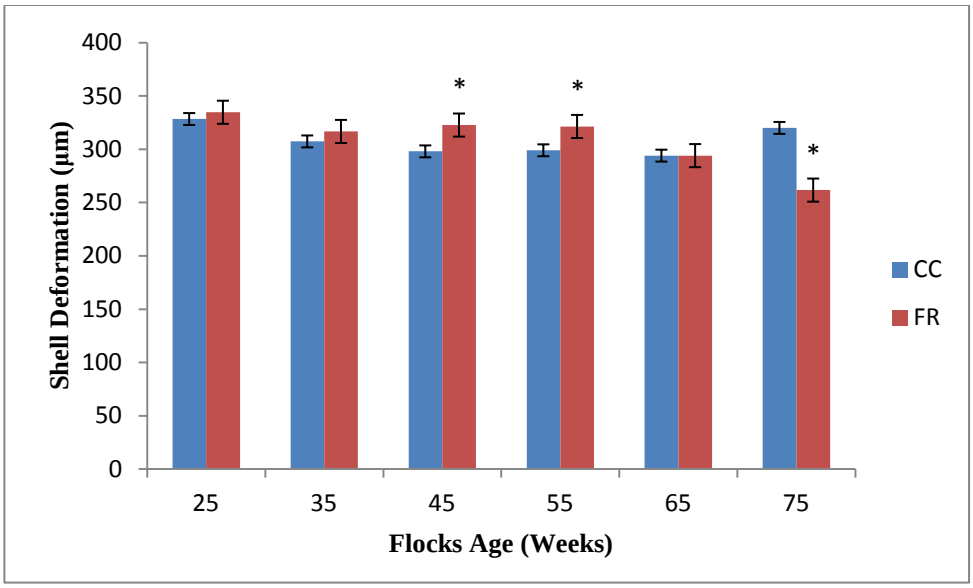

Figure 5.3.1.8 Mean ± SE. * Significantly different from cage production system

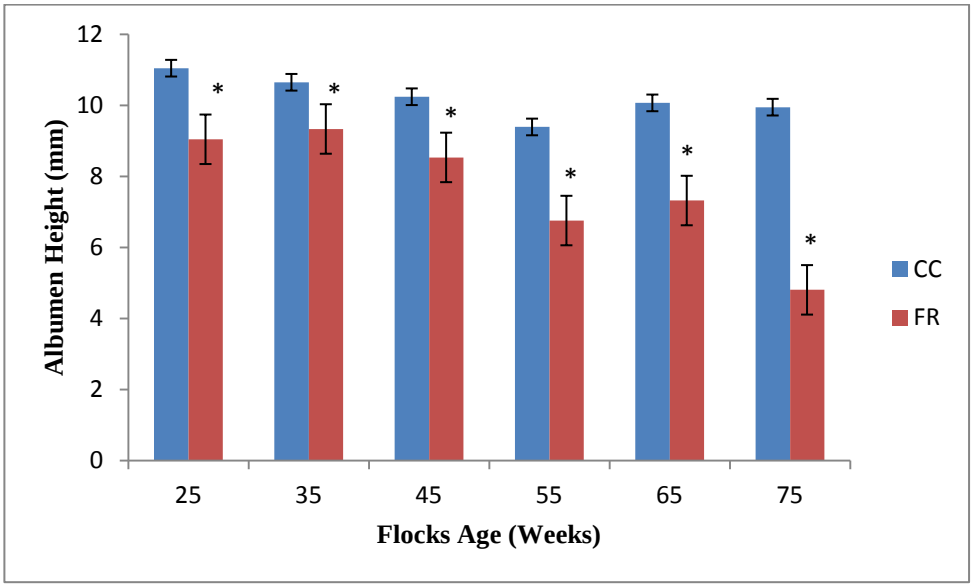

Figure 5.3.1.9 Mean ± SE. * significantly different from cage production system

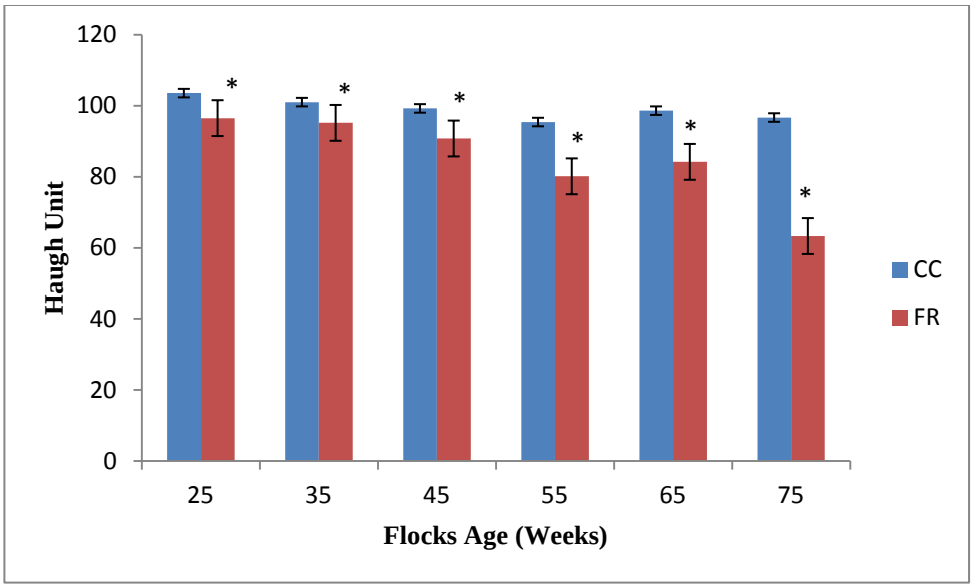

Figure 5.3.1.10 Mean ± SE. * Significantly different from cage production system

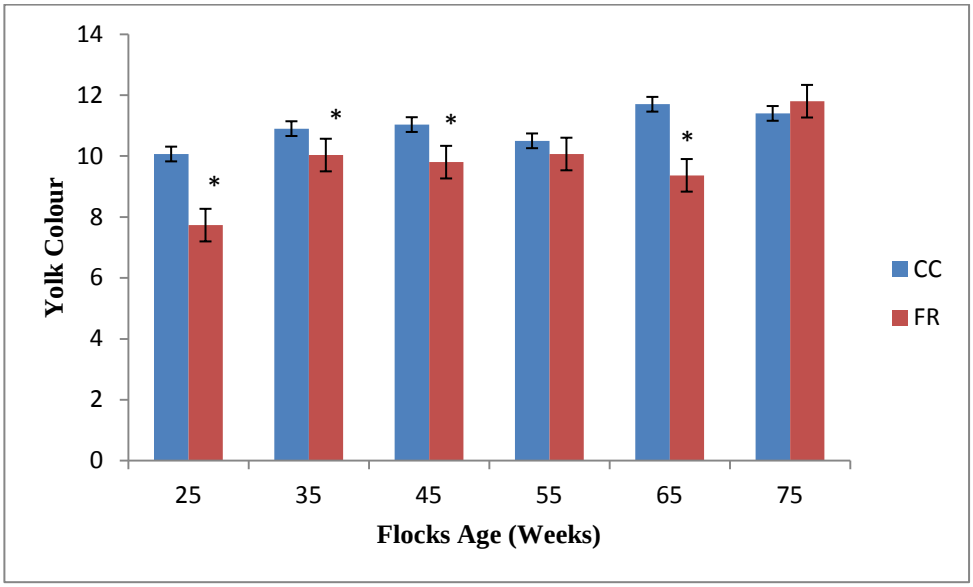

Figure 5.3.1.11 Mean ± SE. * Significantly different from cage production system

5.3.2 Estimation of the amount of cuticle

5.3.2.1 Shell reflectivity (%) and Spectrophotometry (L*a*b) measurements

There was a significant main effect of production system, flock age and a significant interaction between production system and flock age for shell reflectivity of stained eggs, SCI L*, SCI b*, SCE L* and SCE b* components of the L*a*b space system as shown in Table 5.3.2.1.1. There was a significant main effect of a production system and flock age for SCI a* and SCE a* components of the L*a*b space system but no significant interaction between the two. Shell reflectivity, SCI L*, SCE L*, SCI a*, SCE a*, SCI b* and SCE b* values were higher in the free range (FR) compared to the cage production system.

Table 5.3.2.1.1 Shell reflectivity (%) and spectrophotometry (L*a*b) values of stained eggshells

Variables	Flocks age (weeks)												P value		
	Conventional Cages						Free Range								
	25	35	45	55	65	75	25	35	45	55	65	75	P	A	P*A
Shell Reflectivity	19.27 ±0.45	24.53 ±0.65	25.83 ±1.35	23.57 ±0.83	24.40 ±0.55	21.97 ±0.80	*26.30 ±0.84	*25.77 ±0.59	*29.30 ±0.69	*29.30 ±1.03	*31.87 ±1.15	*29.57 ±0.79	<0.0001	<0.0001	0.0004
SCI L	51.06 ±0.49	54.76 ±0.61	56.47 ±1.23	54.28 ±0.86	55.09 ±0.53	53.09 ±0.86	*56.12 ±0.75	*57.09 ±0.53	*59.79 ±0.65	*60.44 ±0.93	*62.08 ±0.96	*60.96 ±0.73	<0.0001	<0.0001	0.0029
SCI a	-2.67 ±0.75	0.26 ±0.78	-3.84 ±1.20	-4.83 ±1.14	-1.05 ±0.66	-4.61 ±1.01	*3.15 ±0.98	2.52 ±0.83	-0.85 ±0.92	-2.15 ±1.02	-1.35 ±0.97	-2.58 ±1.24	<0.0001	<0.0001	0.0699
SCI b	32.31 ±0.30	32.4 ±0.41	28.46 ±0.64	29.44 ±0.29	31.92 ±0.43	28.97 ±0.42	*34.47 ±0.42	*34.67 ±0.50	*32.42 ±0.45	*33.65 ±0.88	30.76 ±0.58	*31.29 ±0.54	<0.0001	<0.0001	<0.0001
SCE L	50.79 ±0.50	54.57 ±0.62	56.37 ±1.22	54.07 ±0.89	54.95 ±0.54	52.93 ±0.87	*55.90 ±0.76	*56.92 ±0.54	*59.68 ±0.66	*60.27 ±0.94	*62.06 ±0.96	*60.88 ±0.74	<0.0001	<0.0001	0.0026
SCE a	-2.71 ±0.76	0.27 ±0.79	-3.84 ±1.21	-4.85 ±1.14	-1.04 ±0.67	-4.63 ±1.01	*3.18 ±0.98	2.55 ±0.83	-0.83 ±0.92	-2.14 ±1.02	-1.33 ±0.97	-2.56 ±1.24	<0.0001	<0.0001	0.0684
SCE b	32.65 ±0.30	32.63 ±0.42	28.57 ±0.65	29.63 ±0.29	32.11 ±0.44	29.19 ±0.42	*34.72 ±0.42	*34.89 ±0.51	*32.49 ±0.45	*33.92 ±0.90	30.79 ±0.59	*31.37 ±0.56	<0.0001	<0.0001	<0.0001

Mean ± SE. SCI- Specular component included; SCE- Specular component excluded

P- Production system; A- Age; P*A- Production system & Flock age interaction

* Values indicate significantly different from cage system at the same age

5.3.2.2 Scanning Electron Microscopy (SEM) of the cuticle surface

There was a significant main effect of flock age but no significant main effect of production system or interaction between flock age and production system on the total amount of cuticle on the eggshell as shown in Table 5.3.2.2.1. However, cuticle cover at 25 and 75 wk was significantly lower in the free range as compared with the conventional cage system when direct comparisons were made.

Table 5.3.2.2.1 Scanning Electron Microscopy (SEM) values of cuticle cover

Variable	Flocks age (weeks)												P value		
	Conventional Cages						Free Range								
	25	35	45	55	65	75	25	35	45	55	65	75	P	A	P*A
Cuticle cover	2.07 ±0.14	2.45 ±0.16	2.17 ±0.15	1.80 ±0.19	2.20 ±0.15	1.87 ±0.16	*2.57 ±0.12	2.60 ±0.09	2.07 ±0.11	1.80 ±0.15	2.03 ±0.15	*2.40 ±0.15	0.0743	<0.0001	0.0581

Values are Mean ± SE. * Values indicate significantly different from cage system at the same age

P- Production system; A- Age; P*A- Production system & Flock age interaction

5.3.3 Ultrastructural scoring of the shell mammillary layer

For the ultrastructural variables of the mammillary layer, a significant main effect of production system was recorded for: mammillary cap size (Fig. 5.3.3.1), mammillary cap quality (Fig. 5.3.3.3), early fusion (Fig. 5.3.3.4), Type A bodies (Fig. 5.3.3.7), Type B bodies (Fig. 5.3.3.8), aragonite (Fig. 5.3.3.9), cuffing (Fig. 5.3.3.12) and erosion (Fig. 5.3.3.14). A significant interaction between production system and flock age ($P<0.05$) was found for mammillary cap size, late fusion (Fig. 5.3.3.5), alignment (Fig. 5.3.3.6), Type A bodies (Fig. 5.3.3.7), Type B bodies (Fig. 5.3.3.8), cubic cone formation (Fig. 5.3.3.11), cuffing (Fig. 5.3.3.), and changed membrane (Fig. 5.3.3.13). There was no significant effect of production system nor interaction between production system and flock age for the incidence of confluence (Fig. 5.3.3.2), cubics (Fig. 5.3.3.10), depression (Fig. 5.3.3.14) or hole (Fig. 5.3.3.16). Variability of mammillary cap size, the incidence of poor mammillary cap quality, incidence of late fusion, alignment, Type A bodies, Type B bodies and cubic cone formation were greater in free range (FR) versus cage system (CC) and increased with flock age in both production systems. The incidence of confluence and early fusion were greater in CC and decreased with age in both production systems. All the mammillary layer ultrastructural variables are shown in the graphs below.

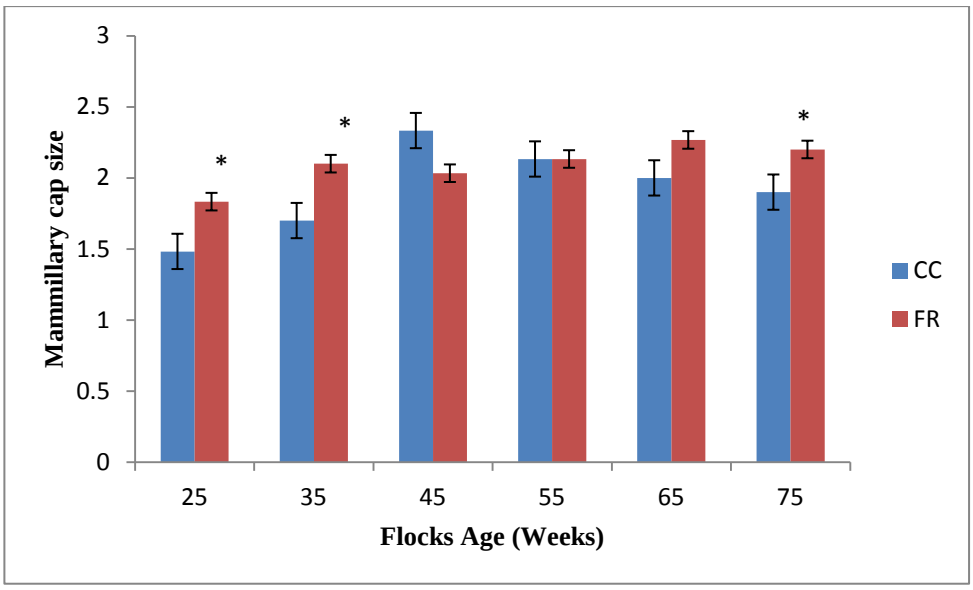

Figure 5.3.3.1 Mean ± SE. * Significantly different from cage production system

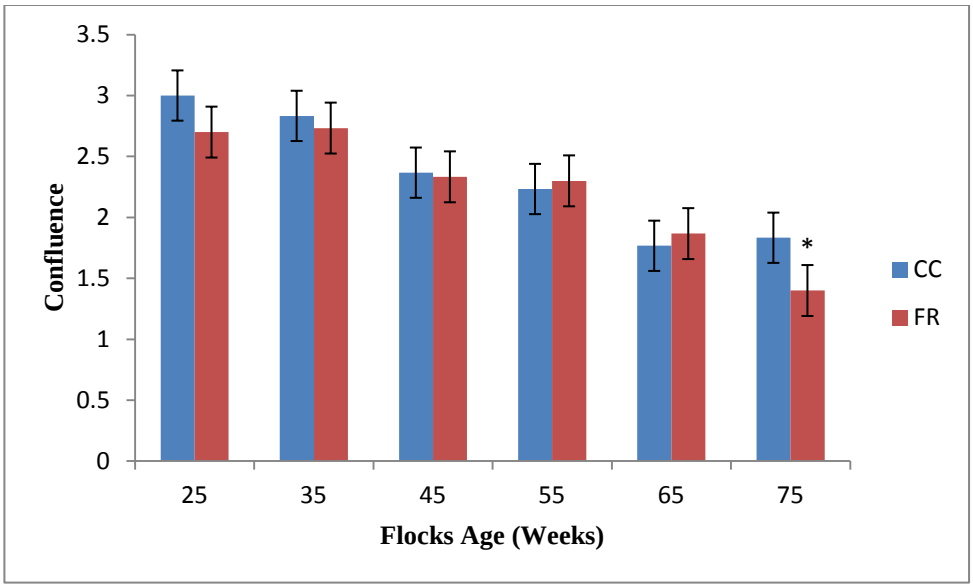

Figure 5.3.3.2 Mean ± SE. * Significantly different from cage production system

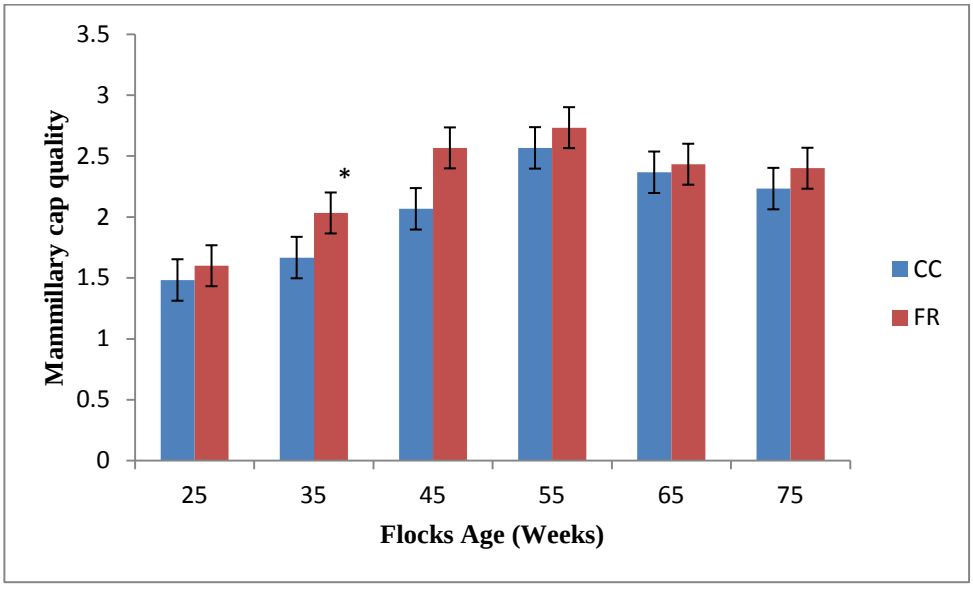

Figure 5.3.3.3 Mean ± SE. * Significantly different from cage production system

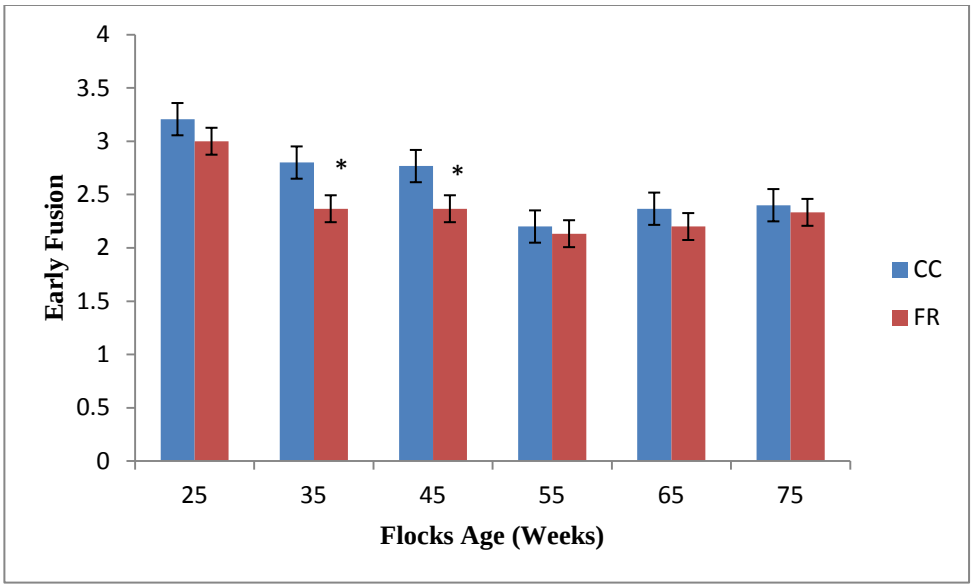

Figure 5.3.3.4 Mean ± SE. * Significantly different from cage production system

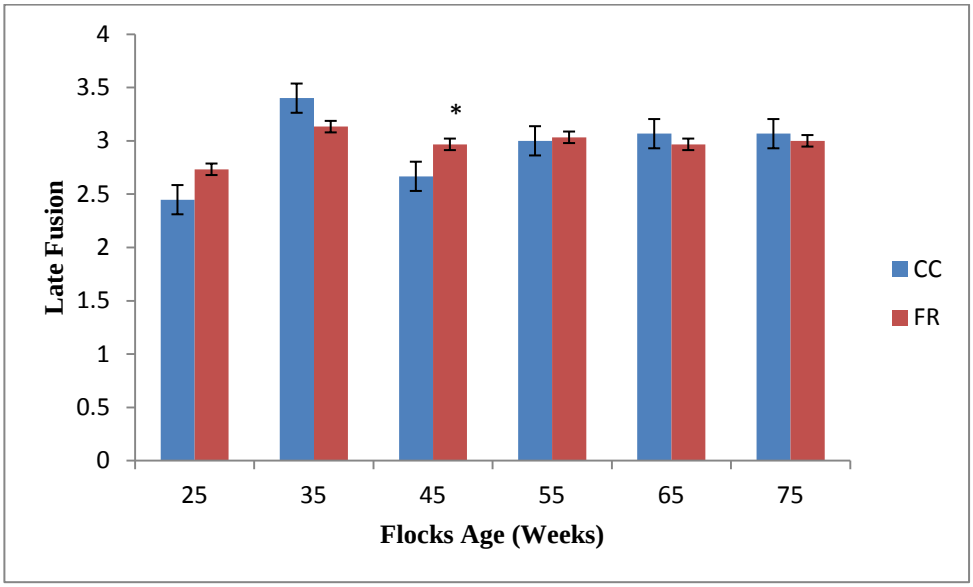

Figure 5.3.3.5 Mean ± SE. * Significantly different from cage production system

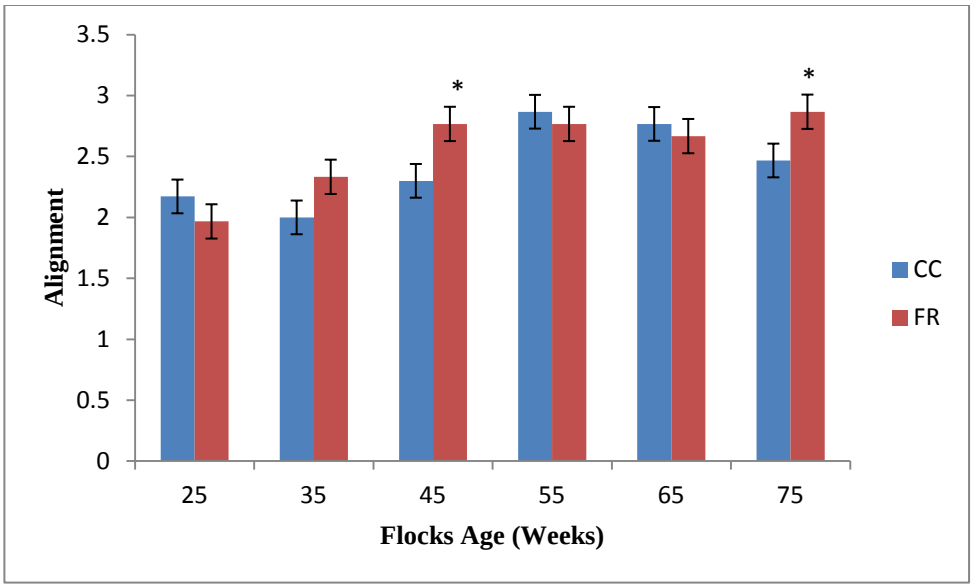

Figure 5.3.3.6 Mean ± SE. * Significantly different from cage production system

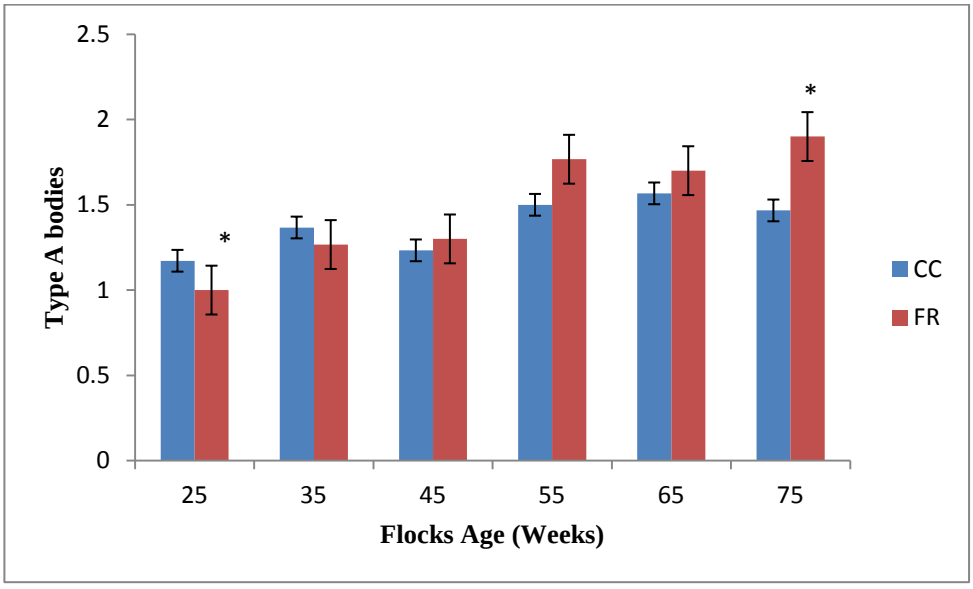

Figure 5.3.3.7 Mean ± SE. * Significantly different from cage production system

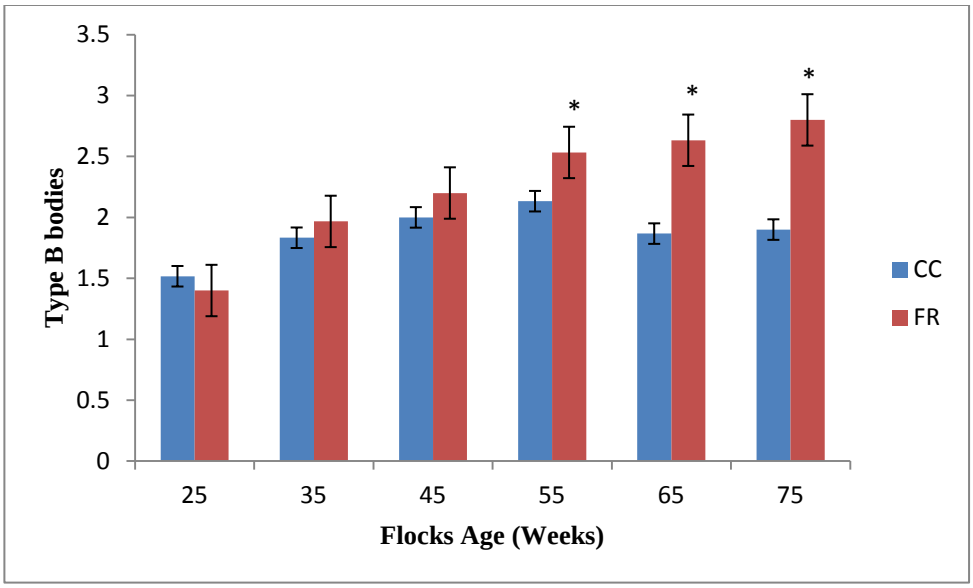

Figure 5.3.3.8 Mean ± SE. * Significantly different from cage production system

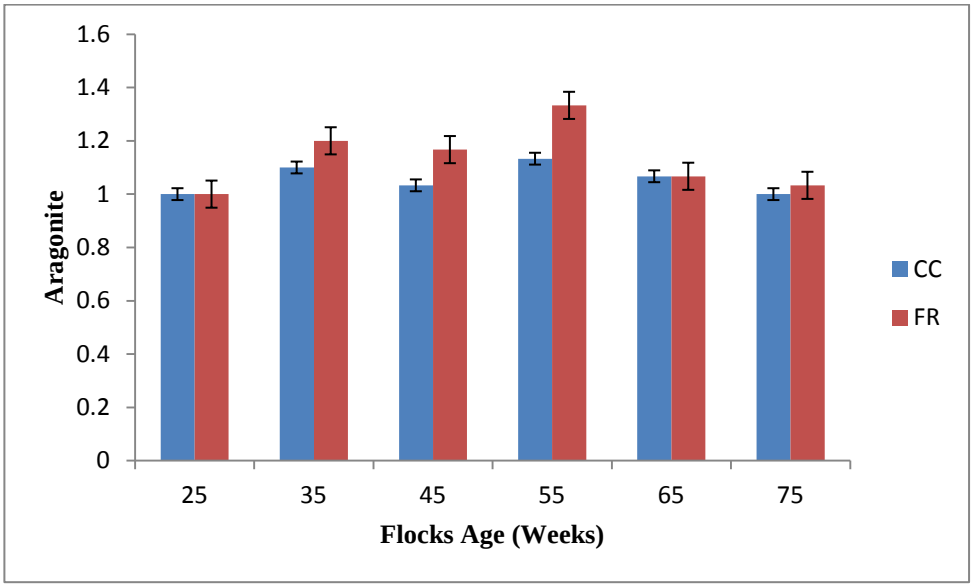

Figure 5.3.3.9 Mean ± SE. Not significantly different between production systems

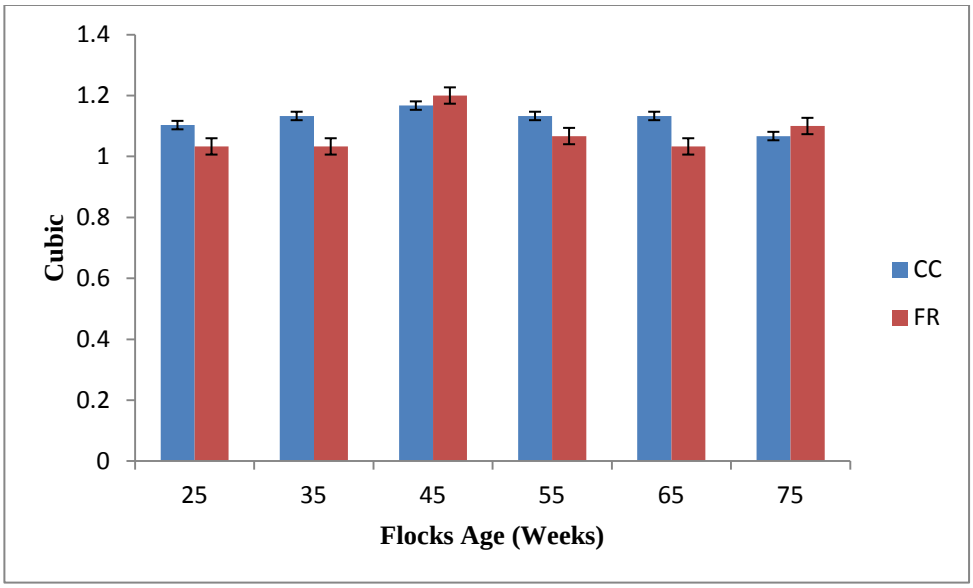

Figure 5.3.3.10 Mean ± SE. Not significantly different between production systems

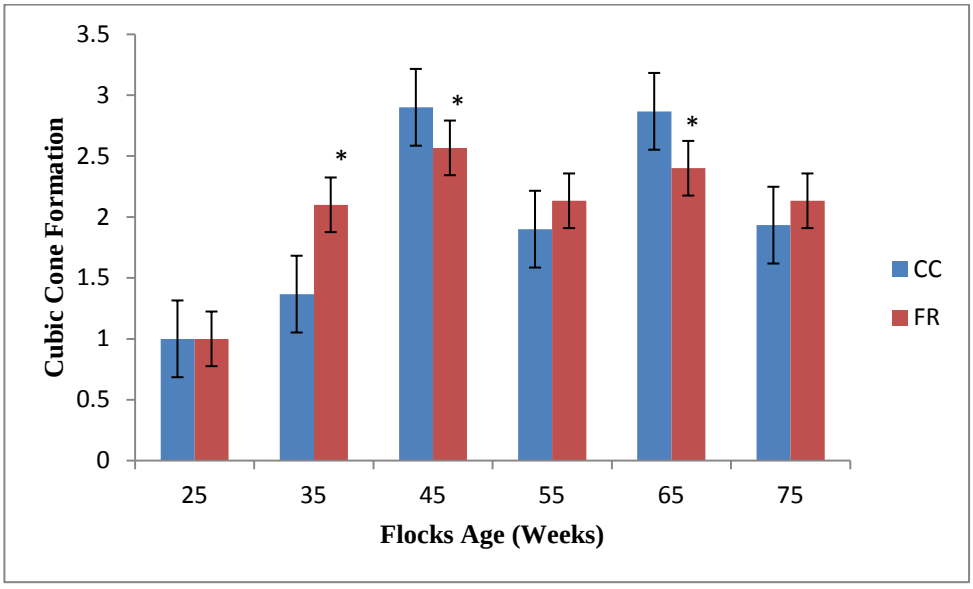

Figure 5.3.3.11 Mean ± SE. * Significantly different from cage production system

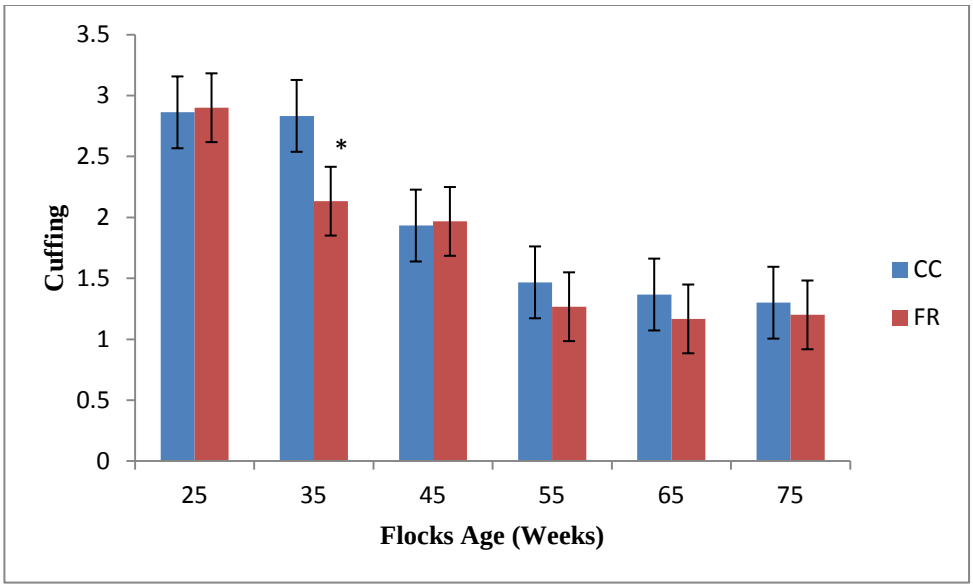

Figure 5.3.3.12 Mean ± SE. * Significantly different from cage production system

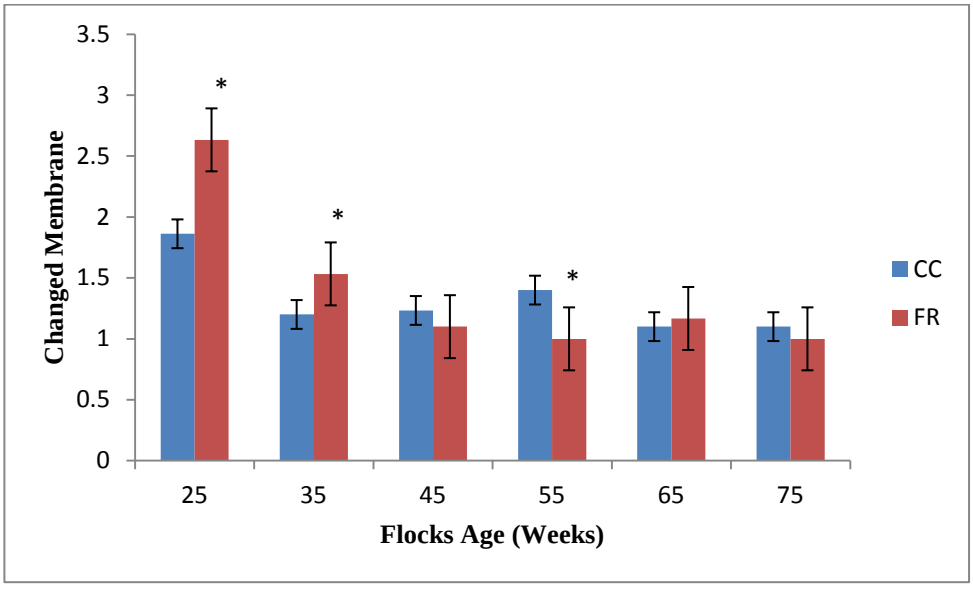

Figure 5.3.3.13 Mean ± SE. * Significantly different from cage production system

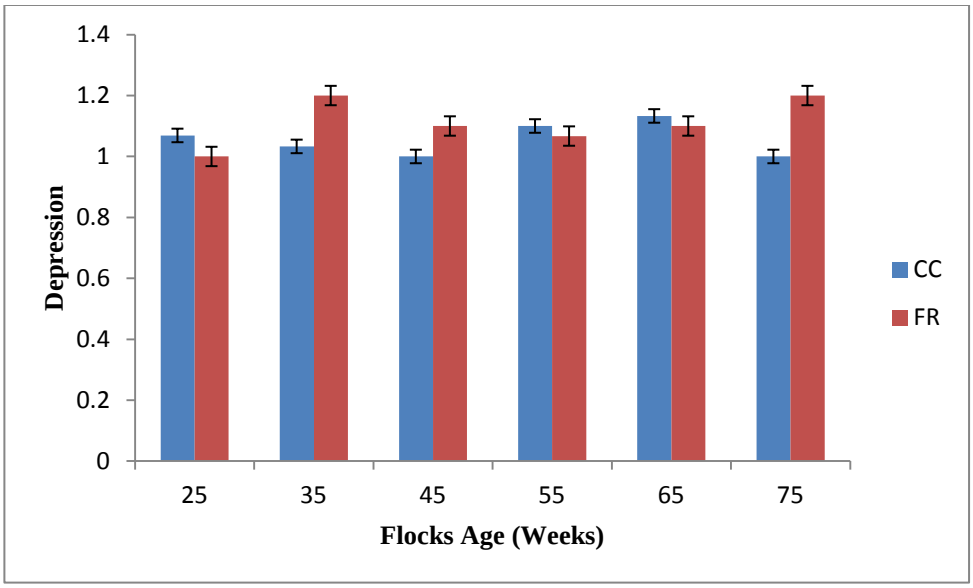

Figure 5.3.3.14 Mean ± SE. Not significantly different between production systems

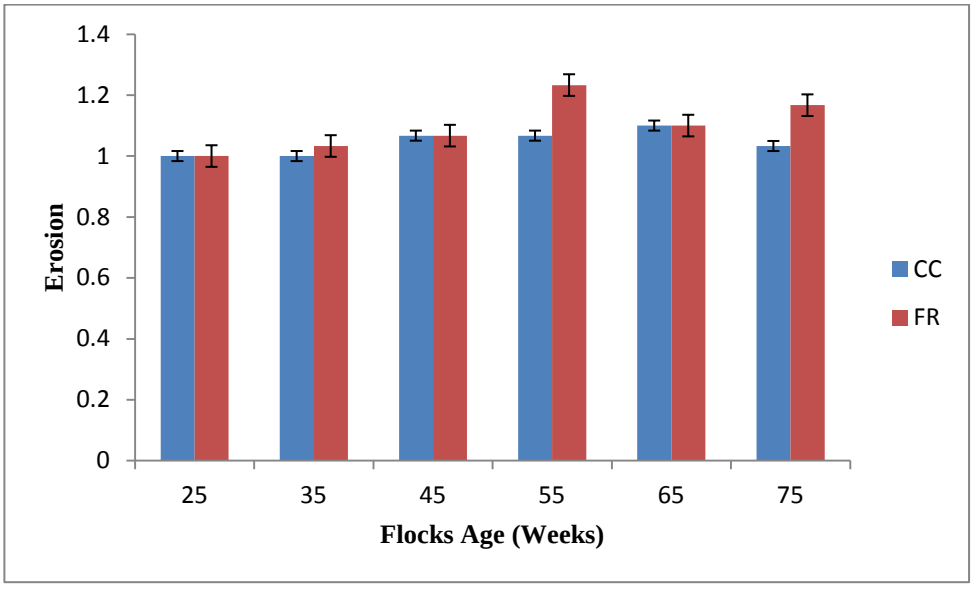

Figure 5.3.3.15 Mean ± SE. Not significantly different between production systems

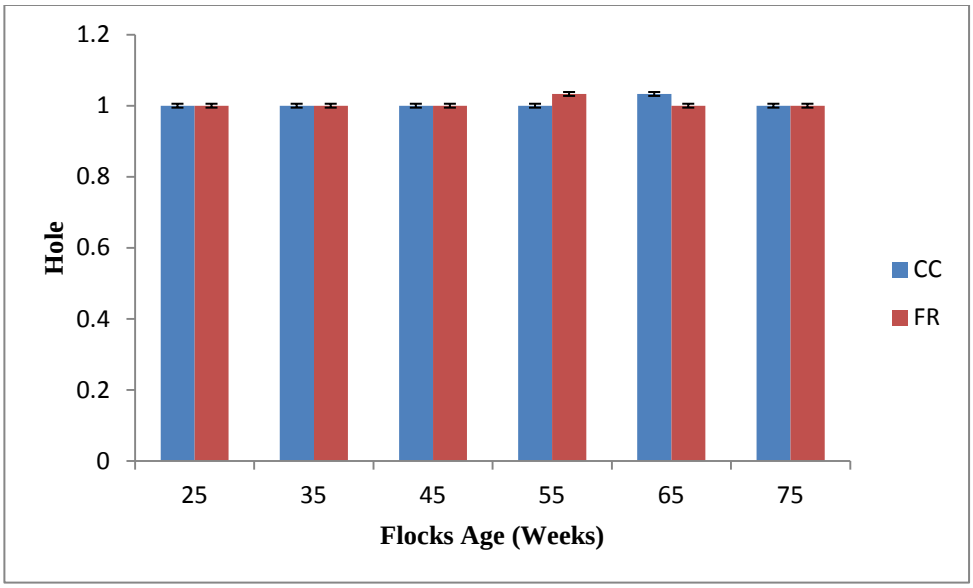

Figure 5.3.3.16 Mean ± SE. Not significantly different between production systems

5.3.4 Egg Microbiology

A significant main effect of production system and interaction between flock age and production system was recorded only for total bacterial count (TBC) on the eggshell surface. There was no significant main effect of production system or interaction between production system and flock age for TBC and total Enterobacteriaceae count (TEC) in shell crush. None of the egg internal contents or swab samples from the cage production system tested positive for *Salmonella*. However, two manure swabs from the free range production system tested positive for *Salmonella* Infantis.

Table 5.3.4.1 TBC and TEC (10 log cfu) on eggshell and in shell crush

Variables	Flocks Age (weeks)												P Value		
	Conventional Cages						Free Range								
	25	35	45	55	65	75	25	35	45	55	65	75	P	A	P*A
TBC on eggshell	3.42 ±0.08	1.55 ±0.52	3.09 ±0.35	3.48 ±0.59	3.29 ±0.22	4.07 ±0.11	*3.81 ±0.12	*2.50 ±0.33	2.89 ±0.20	3.47 ±0.29	*3.79 ±0.15	*4.39 ±0.09	<0.0001	<0.0001	0.0012
TBC in shell crush	0.35 ±0.35	1.56 ±0.53	1.31 ±0.45	1.19 ±0.41	1.96 ±0.55	0.86 ±0.48	0.34 ±0.22	1.46 ±0.37	1.20 ±0.28	1.26 ±0.26	1.88 ±0.33	1.47 ±0.40	0.6112	0.0332	0.5984
TEC on eggshell	1.63 ±0.45	0.58 ±0.39	1.79 ±0.52	3.08 ±0.36	1.57 ±0.44	1.68 ±0.47	2.10 ±0.38	0.84 ±0.27	1.42 ±0.31	*2.06 ±0.35	*1.26 ±0.29	1.32 ±0.29	0.0671	0.0265	0.0133
TEC in shell crush	0.00 ±0.00	1.51 ±0.41	1.71 ±0.38	0.20 ±0.20	0.20 ±0.20	0.43 ±0.29	0.05 ±0.03	1.74 ±0.38	1.23 ±0.26	0.20 ±0.13	0.64 ±0.22	0.91 ±0.29	0.2111	<0.0001	0.0709

TBC- total bacterial count; TEC- total Enterobacteriaceae count; Values are Mean ± SE

* Values indicate significantly different from cage system at the same age

P- Production system; A- Age; P*A- Production system & Flock age interaction

5.4 Discussion

5.4.1 Eggshell and egg internal quality measurements

Overall, translucency score was higher in the free range than cage flock eggs. One possible reason for the higher translucency score in free range eggs might be the time delay between collection and scoring time as these eggs were scored 3 to 4 days after collection on arriving to the egg laboratory while the cage eggs were scored on the day of collection. The cage eggs were darker brown in colour than the free range. Shell reflectivity (%) was higher in cage eggs versus eggs from birds on litter and increased with the flock age in both systems in the findings of Tumova *et al.* (2011). Cage eggs were darker brown in colour in the findings of Sekeroglu *et al.* (2010) compared to eggs from free range and litter systems which is in comparison to the current findings. In the current study, shell reflectivity generally increased to a greater extent in the free range flock, with increased flock age.

Egg weight in the cage system flock was significantly higher than for the free range birds at all flock ages. In the free range flock, egg weight (g) increased between 25 and 35 weeks and then remained relatively constant. Unlike the free range, in the cage production system there was a consistent slight increase in egg weight with increased flock age. These observations are in comparison to the findings of Wang *et al.* (2009) and Van Den Brand *et al.* (2004) in which a greater increase in egg weight was observed in free range eggs. A slightly higher egg weight in a cage flock compared to a free range flock was reported by Varguez-Montero *et al.* (2012) and Pavlovski *et al.* (2004) which is similar to the current findings. Similarly, a higher egg weight in a cage flock compared to free range and litter systems has been recorded by Petek et al (2009); Mostert *et al.* (1995); Sekeroglu *et al.* (2010) and Yakubu *et al.* (2007). However, Englmaierova and Tumova (online); Hidalgo *et al.*, (2008); Hughes *et al.* (1985); Pistekova *et al.* (2006); Sencic *et al.* (2006); Tumova and Ebeid (2005) and Zemkova *et al.* (2007) reported a higher egg weight in free range and litter systems versus cage system. In the findings of Tumova *et al.* (2011), egg weight was not significantly different between the cage and free range production system of brown layers. Therefore, it appears that there is no consistent effect of the production system on egg weight which can be affected by a range of other factors.

Shell weight and shell thickness were higher for cage eggs versus free range in the present study. No significant difference was recorded for shell weight between cage and floor pen and litter system eggs in the studies of Banga-Mboko *et al.* (2010); Petek *et al.* (2009) and Tumova *et al.* (2011). In the

studies of Abrahamsson *et al.* (1998), higher shell weight was recorded for cage eggs compared to an aviary system. Shell thickness was not significantly different between free range and cage systems in the studies of Wang *et al.* (2009); Tumova *et al.* (2011) and Van De Brand *et al.* (2004). In the studies of Mostert *et al.* (1995), housing system accounted for only 1 % of the difference in shell thickness. Higher shell thickness was recorded in free range eggs compared to a cage system in the findings of Mostert *et al.* (1995); Petek *et al.* (2009); Sencic *et al.* (2006); Hughes *et al.* (1985) and Banga-Mboko *et al.* (2010). The current results for higher shell thickness in cage eggs cannot directly be compared to previous research findings as most of the authors compared cage eggs with different production systems from the free range production system of the present study.

Housing system had little effect on shell breaking strength in the present study. Breaking strength was higher for free range than for cage at 45 and 55 weeks but the reverse was the case at 65 wk. Hidalgo *et al.* (2008) reported higher shell strength in conventional cage eggs compared to all other systems and a higher value for shell breaking strength was recorded in cage eggs than for a litter system in the studies of Tumova *et al.* (2011). Wang *et al.* (2009) and Tumova and Ledvinka (2009) reported an increase in shell strength towards peak production followed by a subsequent decrease with flock age in cage and outdoor flocks. The mean shell strength was also higher in the outdoor flock in the studies of Wang *et al.* (2009). Mertens *et al.* (2006) have reported weak shell strength in free range eggs compared to cage eggs. No significant difference was recorded in the studies of Sekeroglu *et al.* (2010); Petek *et al.* (2009) and Clerici *et al.* (Online) for shell breaking strength in cage and free range flocks.

Shell deformation (μm) showed a similar pattern to breaking shell strength indicating that housing system had little effect on shell elasticity or fragility. There was a significant interaction ($P<0.0001$) between the production system and flock age for shell deformation being higher for cage than free range at 45 and 55 wk. Shell deformation was greater in conventional cage eggs versus all other systems in the findings of Hidalgo *et al.* (2008). In the studies of Abrahamsson *et al.* (1998), higher shell deformation was recorded for cage eggs compared to aviary system. No significant effect of the production system on shell deformation was recorded by Tanaka and Hurnik (1992).

In the current study, percentage shell was not significantly affected by production system which can be compared to the findings of Sekeroglu *et al.* (2010) who recorded no significant effect of

production systems on percentage shell. A higher percentage shell has been recorded in cage system eggs versus other systems by Hidalgo *et al.* (2008). A slightly higher percentage shell was recorded for free range egg versus cage system by Wang *et al.* (2009) and Van De Brand *et al.* (2004) which is in contradiction to the current findings.

Albumen height (mm) was statistically significantly different ($P \leq 0.05$) between the two production systems at different ages. However, as indicated previously, there was a longer interval of time between egg collection and egg analysis of the free range eggs which would explain much of this difference. Wang *et al.* (2009) reported that albumen height was variable in cage eggs with increasing hen age but linearly decreased in outdoor hen eggs. A relatively higher albumen height was recorded in free range eggs than cages with an overall decrease with flock age in the studies of Van Den Brand *et al.* (2004). Wang *et al.* (2009) and Varguez-Montero *et al.* (2012) recorded no significant effect for albumen height among different production system.

A statistically significant effect ($P \leq 0.05$) of production system and interaction between production systems and flock age was found for Haugh unit and this followed a similar pattern to albumen height. Haugh unit decreased with flock age more in the free range whereas in cage system it followed a consistent decline. Tumova *et al.* (2011), Tumova and Ebeid (2005), Lichovnikova and Zeman (2008) and Ronald *et al.* (1979) reported higher Haugh unit in cage eggs versus other systems. Wang *et al.* (2009) reported a slight decrease and then increase in Haugh unit in outdoor at the same time as a linear decrease occurred in a cage production system (26-50 wk flock age). Free range eggs had lowest Haugh unit scores in the studies of Hidalgo *et al.* (2008) as compared to other systems. In the studies of Mostert *et al.* (1995); Petek *et al.* (2009) and Sekeroglu *et al.* (2010) there was no significant effect of the production system on Haugh unit. Pavlovski *et al.* (2004) reported a higher Haugh unit in free range compared to a cage and deep litter system.

Yolk colour was generally more consistent for the cage production system. Petek *et al.* (2009); Varguez-Montero *et al.* (2012) and Sencic *et al.* (2006) recorded a significantly higher yolk colour in free range compared to cage eggs. In the studies of Sekeroglu *et al.* (2010), there was no significant difference in yolk colour between free range and cage eggs. Singh *et al.* (2009) recorded a higher yolk colour for floor flock versus cage system eggs. It can be concluded that yolk colour varies more with the flock nutrition than age or production system.

5.4.2 Estimation of the amount of cuticle

5.4.2.1 Shell reflectivity (%) and Spectrophotometry (L*a*b) measurements

A higher shell reflectivity (%) in free range versus cage eggs indicated that free range eggs were lighter in colour. The purpose of recording shell reflectivity of stained eggs was to correlate the shell reflectivity values with the values of the SCI L* component of spectrophotometer measurements.

The L* component of the L*a*b space system was in positive correlation to shell reflectivity and increased in both production systems. The higher values of L* for free range compared to cage eggs indicated less pigment in free range compared to cage system eggs. From the current spectrophotometric measurements of stained eggs, it can be concluded that shell reflectivity and SCI L* values are less significantly affected by MST blue stain. The a* is the most important component in the L*a*b space system that shows the amount of the MST stain acquired by the cuticle. The more negative values in the cage system versus free range indicated more cuticle present. From the spectrophotometric measurements of the stained eggs, it can be concluded that free range eggs had less cuticle as compared with the cage system. The lack of significant interaction between flock age and production system indicated that flock age had similar effects on the amount of cuticle deposition in both production systems. The reliability of the MST cuticle blue stain has been tested in the current experiments and showed a high correlation between the MST staining and the presence of cuticle as verified by SEM.

The b* component of the L*a*b colour space system is the grading between blue and yellow where yellow is towards the positive end of the scale and blue is towards the negative end. There were significant main differences between production system with free range flocks being higher. This component is of less importance compared to SCI L* and SCI a*.

5.4.2.2 Scanning Electron Microscopy (SEM) of the cuticle surface

There was no significant difference between production systems and no significant interaction between production systems and flock age for the amount of cuticle present quantified by SEM. The amount of cuticle was only significantly different between the two production systems at the ages of 25 and 75 wk. Overall, the amount of cuticle cover tended to be higher for cage eggs as compared to free range. Comparing the amount of cuticle of free range with cage system eggs by SEM

quantification, it can be concluded that production system had little or no effect on the amount of cuticle cover laid upon eggshell. The difference between the values of a* and SEM quantification could be due to a small specimen size in SEM compared to the whole eggshell surface in spectrophotometry.

5.4.3 Ultrastructural scoring of the shell mammillary layer

The overall variability of mammillary cap size was higher in free range eggs compared to cage eggs. More variable mammillary cap size leads to poor membrane attachment and poor cap quality which can affect the overall quality of the shell. Overall, cap quality was poorer in free range compared to cage eggs. Good caps quality cones show higher affinity for membrane attachment, thus making the shell stronger. A higher incidence of early fusion has a positive impact on shell strength and vice versa (Solomon, 1991). The overall incidence of early fusion was slightly higher in cage eggs versus free range. A higher incidence of early fusion increases the effective thickness of palisade columns (Solomon, 1991) and is a positive feature of the mammillary layer. Increased incidence of cuffing has a positive effect on the mammillary layer, and its higher incidence in cage eggs suggests better ultrastructural quality.

Late fusion, alignment, cubics, cubic cone formation, changed membrane, depression and hole negatively affect mammillary layer quality. Their incidence was not significantly different between the two production systems. The higher incidence of mammillary layer variables like Type A bodies, Type B bodies and aragonite in free range eggs compared to cage eggs indicates better quality of cage eggs.

5.4.4 Egg microbiology

Understanding of external contamination of shells is important in order to evaluate the shelf life and food safety of commercial eggs. In most of cases, egg internal contamination results from penetration of bacteria deposited on the shell surface after it is being laid (Schoeni *et al.,* 1995). *Salmonella* poisoning related to egg products has attracted attention of food safety authorities in Australia and all over the world. The shell wash method used in the current study has been used successfully by other workers for microbial enumeration from the eggshell surface (De Reu *et al.*, 2005b). In the current study, the total bacterial count (TBC) on shell surface was significantly higher in free range eggs

compared to cage eggs. On average, 20-30 times more bacteria have been isolated from the surface of litter floor eggs compared to a wire floor (Quarles *et al.*, 1970). There is a greater chance of eggshell contamination in litter and free range systems compared to cages as freshly laid eggs can be contaminated when coming in contact with contaminated surfaces (Gentry and Quarles, 1972; Harry, 1963). Harry (1963) found 15 times more bacterial load on the eggshell surfaces of deep litter eggs compared to cage eggs. Total aerobic flora were higher (more than 1.0 log) on eggs from an aviary housing system compared to conventional and furnished cage systems (De Reu *et al.,* 2005b). In the studies of Wall *et al.* (2008), the total bacterial load and Enterobacteriaceae on the eggshell was significantly higher for furnished cage eggs compared to conventional cage eggs. In the present study, the TBC in shell crush was not significantly different between production systems and remained below 2 log cfu/mL of rinsate throughout production in both systems. The total Enterobacteriaceae count (TEC) on the shell and shell crush was not affected by the production system and compared to TBC, was quite low. Enterobacteriaceae are the main bacteria that cause food poisoning. Bacterial contamination of the eggshell is greatly affected by factors such as, diet (Smith *et al.,* 2000) and poultry house environment (De Reu *et al.,* 2005b). Wall *et al.* (2008) found a significantly higher Enterobacteriaceae count on eggshells from furnished cage production (12.3%) compared to conventional cages (5.8%) but the egg internal contents from both production systems were free from bacteria. In the present study, egg internal contents from both production systems were free from bacteria. Daughtry *et al.* (2005) could not isolate *Salmonella* from egg internal contents collected from different production systems in Australia. Comparing different housing systems, De Reu *et al.* (Online) obtained lower (1.9 %) contamination of internal contents of furnished cage eggs compared to non cage system eggs (2.3%). Previous studies (De Reu *et al.,* 2005b; Mallet *et al.,* 2006; Smeltzer *et al.,* 1979; Wall *et al.,* 2008; Quarles *et al.,* 1970) have shown that eggs produced by cage systems have generally lower bacterial count compared to other production systems but the differences found between the cage and non cage system eggs in terms of contamination are less pronounced under commercial conditions (De Reu *et al.,* 2008). The lack of differences in total microbial load between the cage and free range production system could be attributed to proper hygienic measurements followed on the farms during the study period.

Chapter 6

Horizontal Study - Conventional Cage Production System

6.1 Introduction

The aim of this short study was to investigate differences between flocks of the same breed (Hy Line brown layer), on the same farm but of different ages, on egg quality parameters and egg microbial load. A horizontal study was conducted by collecting eggs from these flocks housed in a conventional cage production system.

6.2 Materials and methods

Eggs were collected from flocks at 22, 39, 55 and 79 wk of age. The 79 wk flock had been molted at the age of 62 wk. All flocks were housed on the same farm in two adjacent environmentally controlled identical sheds. The conventional cage farm was located in the region of Tamworth NSW. All flocks received locally formulated feed containing wheat (or wheat plus sorghum), soybean meal, meat meal, vegetable oil, limestone and yolk colour pigment as major components. From each flock, 150 eggs were collected with a total number of 600 eggs from all four flocks. Ninety eggs from each flock were processed for egg quality experiments while the remaining 60 eggs were used for egg microbiology. From each flock, 30 eggs were processed for each of the following procedures, (a total of 120 eggs from all the four flocks).

- Traditional egg internal and external quality measurements
- Estimation of the amount of cuticle
- Shell ultrastructural scoring

The remaining 60 eggs from each flock were processed for egg microbiology. Eggs processed for the shell wash procedure were further processed for shell crush and internal contents. Thus a total of 240 eggs were processed from all the four flocks for egg microbiology.

Eggs were processed as described in Chapter 2, general materials and methods. Data were analyzed using Statview Software (SAS Institute Inc., Version 5.0.1.0) as explained in Chapter 2 section 2.5.

6.3 Results

6.3.1 Eggshell and egg internal quality measurements

All traditional eggshell quality parameters including translucency score were significantly different ($P<0.05$) among the age groups. Translucency score was lowest in the 39 week flock and highest in the 79 week flock with the other two flocks intermediate. Shell reflectivity (%) increased linearly with increased age of the flock (Fig. 6.3.1.2). Egg weight was lower for the 22 wk flock than for all other flocks (Fig. 6.3.1.3). Shell breaking shell strength (N) was lower in the 55 week flock than for all the other flocks which were not significantly different from one another (Fig. 6.3.1.4). The pattern was similar but slightly different for shell deformation (μm) which was highest in the 22 week flock and generally lower for the older flocks with the 55 wk being the lowest (Fig. 6.3.1.5). However, there was considerable overlap between the three older flocks. Shell weight (g) was significantly lower in the 22 week flock. The 39 and 55 week flocks were not significantly different from each other and the same applied to the 39 and 79 week flocks (Fig. 6.3.1.6). Percentage shell was significantly higher for the 22 week flock as compared with the other three flocks which were not significantly different from one another (Fig. 6.3.1.7). For shell thickness (μm), the 22 and 39 week flocks were significantly higher than the 55 week flock whereas the 79 week flock was not different from any of the other flocks (Fig. 6.3.1.8).

There was a statistically main significant effect ($P<0.05$) of flock age on all the egg internal quality parameters. Albumen height (mm) and Haugh unit declined linearly with flock age with each flock being significantly different from all others (Fig. 6.3.1.9 and 6.3.1.10). Yolk colour was higher in the 22 and 39 wk flocks which were not significantly different from each other and was lower in the 55 and 79 wk flocks which were not different from each other (Fig. 6.3.1.11).

All the traditional eggshell and egg internal quality variables are shown in the graphs.

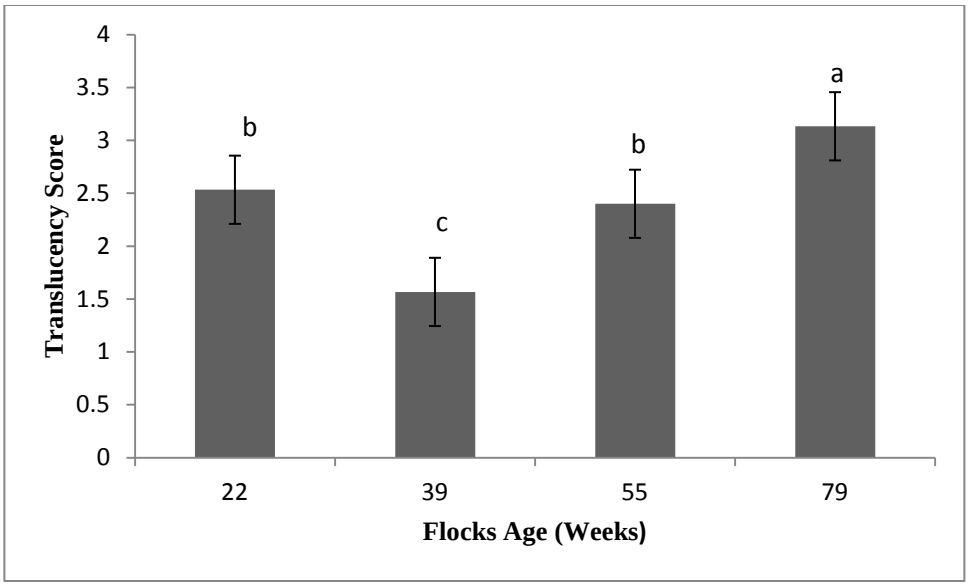

Figure 6.3.1.1 Translucency score of different aged flocks (Mean ± SE)

[a, b, c] Different superscripts indicate significant differences

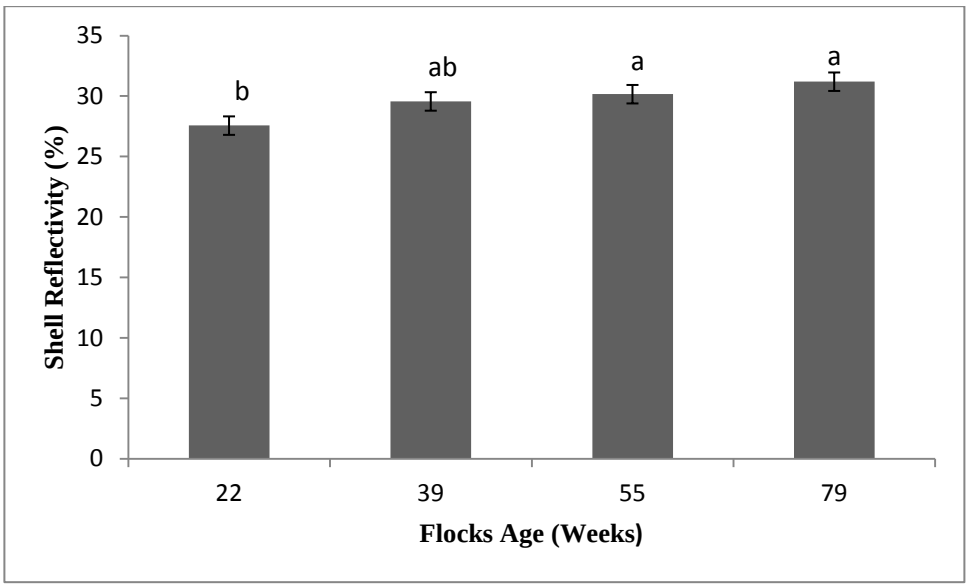

Figure 6.3.1.2 Shell reflectivity (%) of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

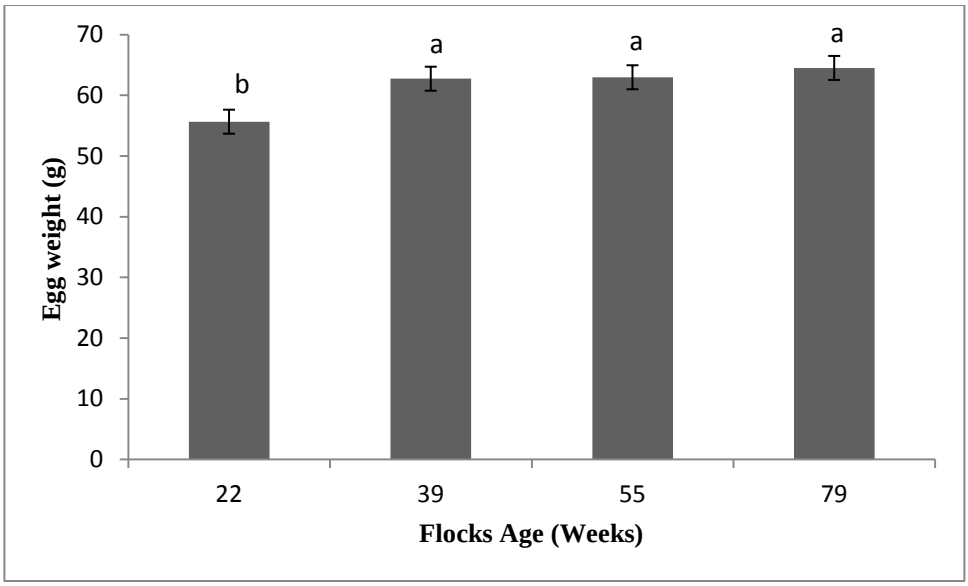

Figure 6.3.1.3 Egg weight (g) of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

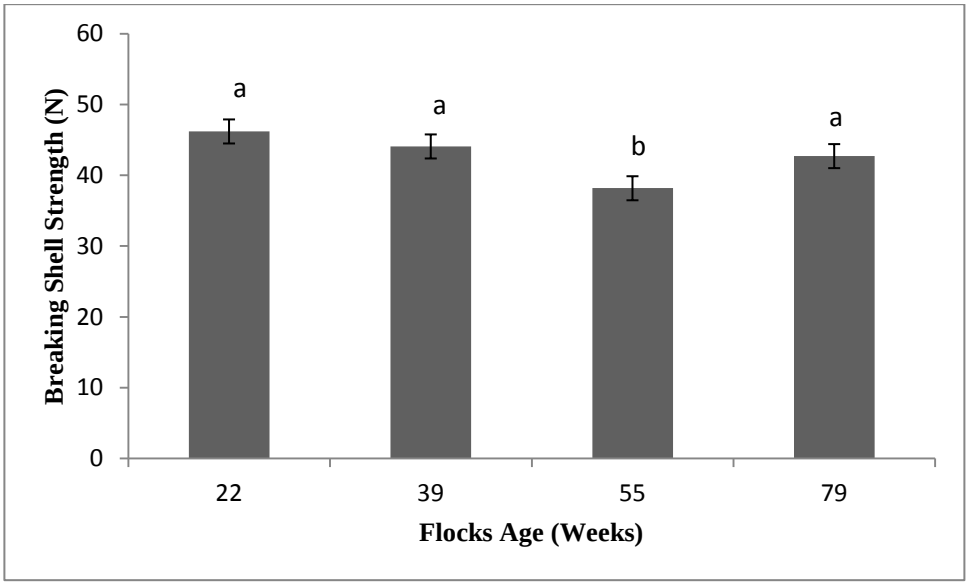

Figure 6.3.1.4 Breaking shell strength (N) of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

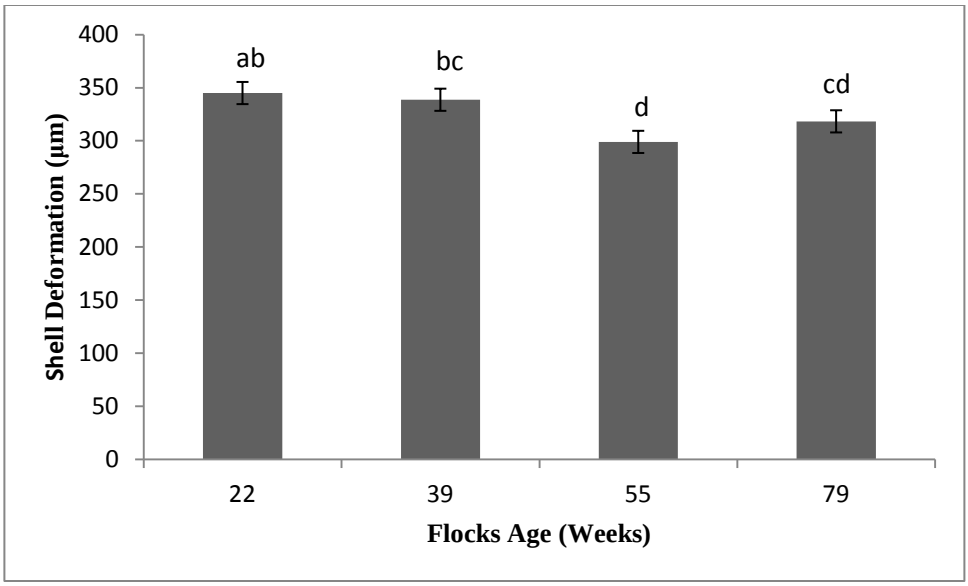

Figure 6.3.1.5 Shell deformation (µm) of different aged flocks (Mean ± SE)

[a, b, c, d] Different superscripts indicate significant differences

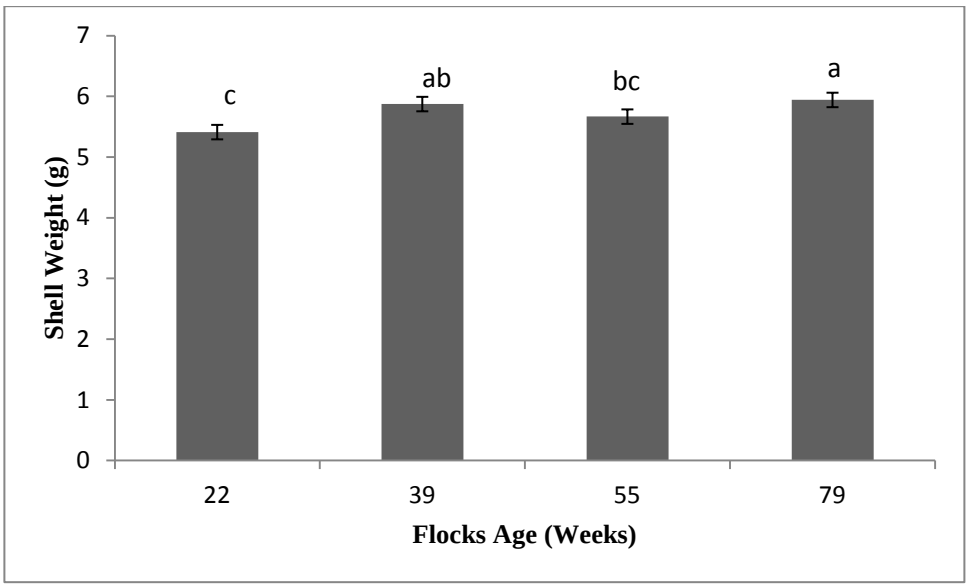

Figure 6.3.1.6 Shell weight (g) of different aged flocks (Mean ± SE)

[a, b, c] Different superscripts indicate significant differences

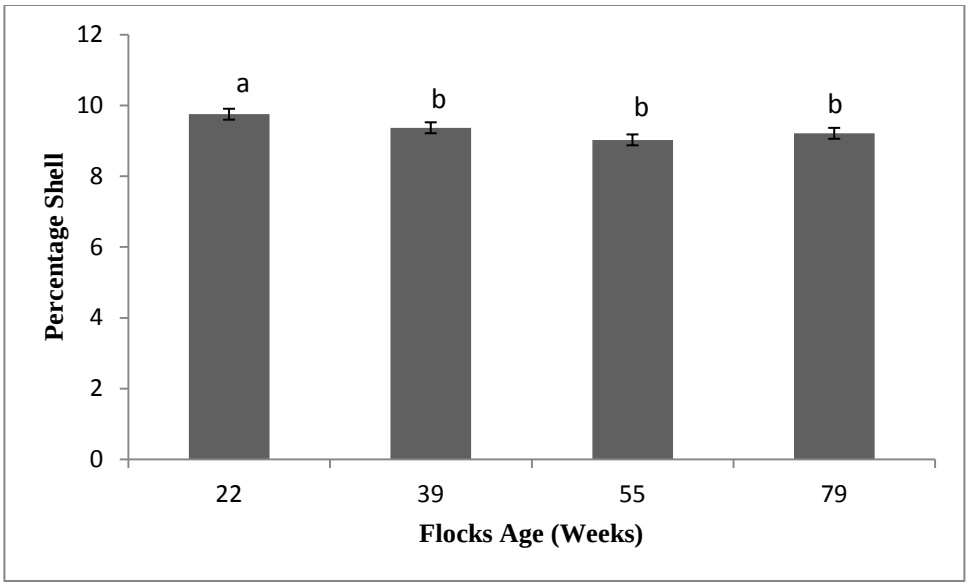

Figure 6.3.1.7 Percentage shell of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

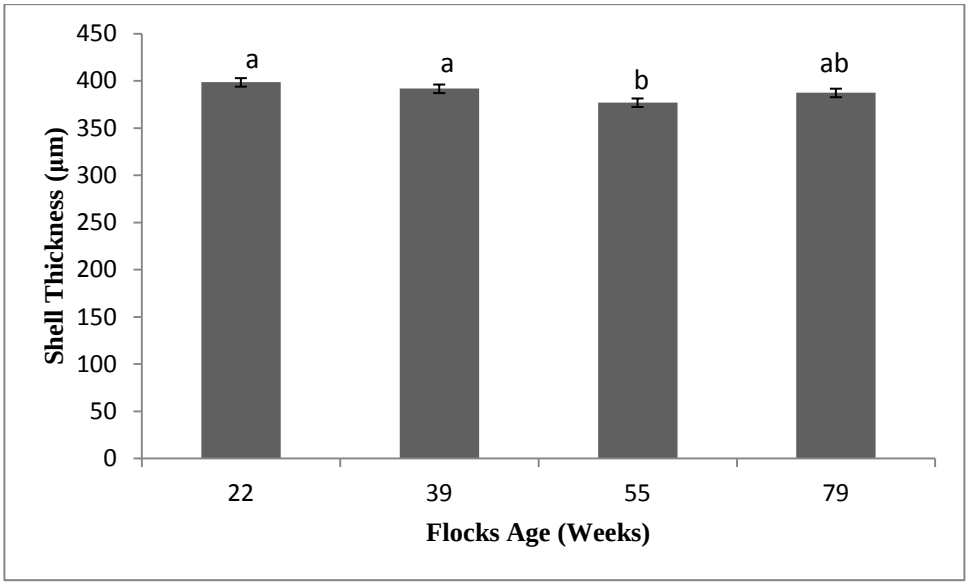

Figure 6.3.1.8 Shell thickness (µm) of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

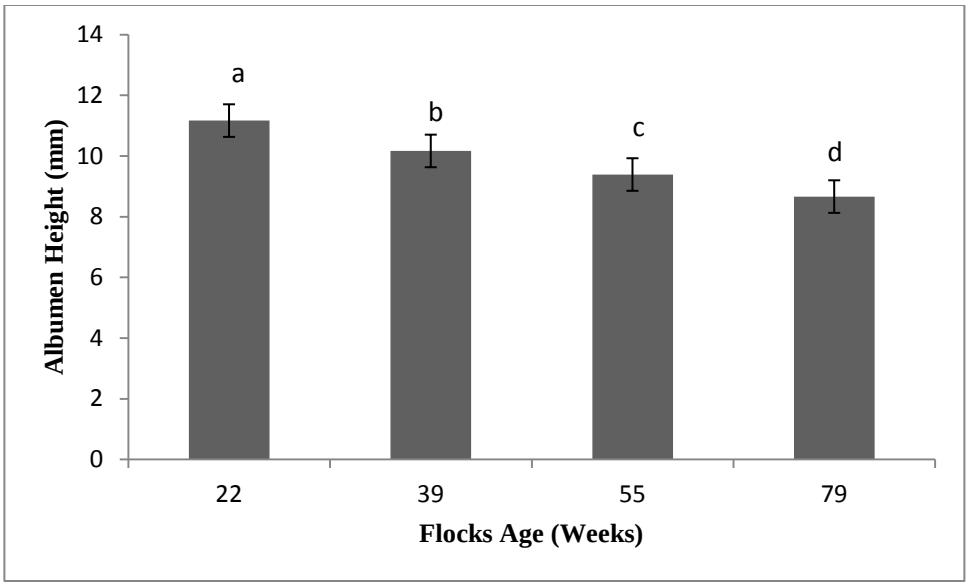

Figure 6.3.1.9 Albumen height (mm) of different aged flocks (Mean ± SE)

[a, b, c, d] Different superscripts indicate significant differences

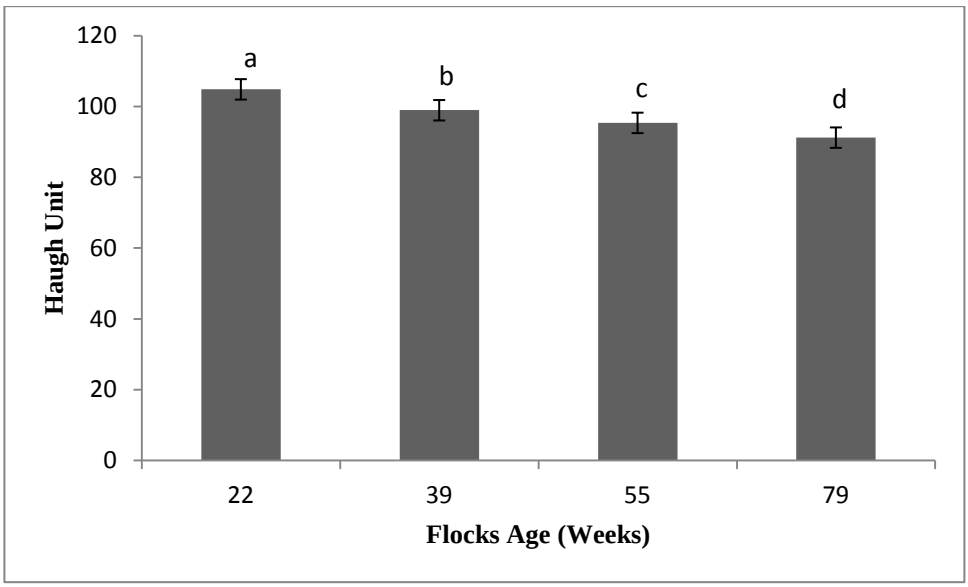

Figure 6.3.1.10 Haugh unit of different aged flocks (Mean ± SE)

[a, b, c, d] Different superscripts indicate significant differences

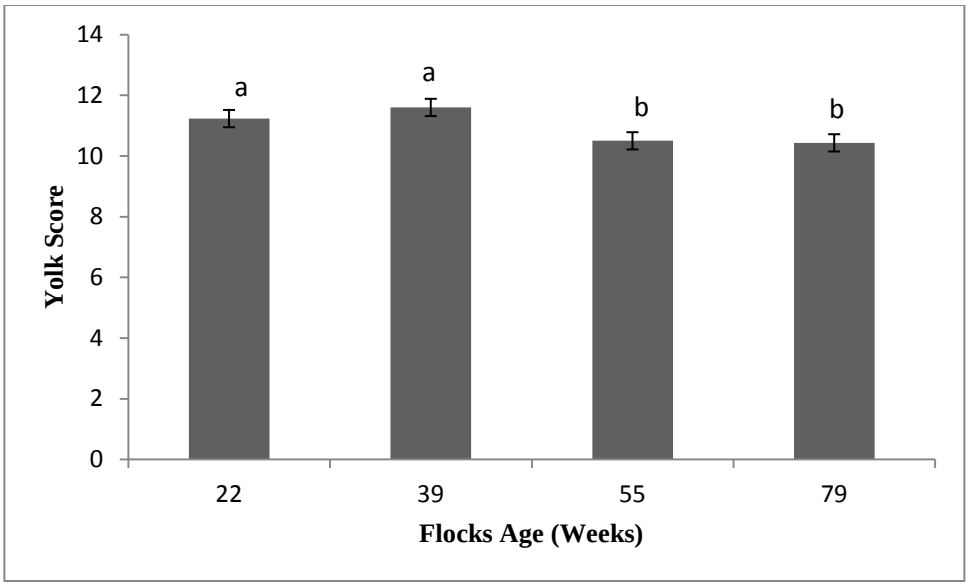

Figure 6.3.1.11 Yolk score of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

6.3.2 Estimation of the amount of cuticle

6.3.2.1 Shell reflectivity (%) and Spectrophotometry (L*a*b) measurements

The shell reflectivity (%), SCI L* and SCE L* values of eggshell stained with cuticle blue dye increased with increased flock age as shown in Table 6.3.2.1.1. SCI a* and SCE a* values were higher in the 55 wk flock and lowest in the 22 and 79 wk flocks, with the 39 wk flock being intermediate and not significantly from the other three flocks. SCI b* and SCE b* values were only significantly different between 55 and 79 wk flocks.

Table 6.3.2.1.1 Shell reflectivity and L*a*b Values of stained eggshells

Variables	Flocks age (weeks)				P value
	22	39	55	79	
Shell Reflectivity	21.87±0.62[b]	22.67±0.61[b]	23.57±0.81[b]	26.00±0.85[a]	0.0009
SCI L	53.08±0.72[b]	53.57±0.61[b]	54.28±0.86[b]	56.88±0.81[a]	0.0039
SCI a	-0.71±1.08[a]	-2.41±0.82[ab]	-4.83±1.14[b]	-1.30±1.32[a]	0.0478
SCI b	29.91±0.28	29.89±0.21	29.44±0.29	30.55±0.36	0.0670
SCE L	52.96±0.73[b]	53.63±0.63[b]	54.07±0.89[b]	56.78±0.82[a]	0.0040
SCE a	-0.70±1.08[a]	-2.41±0.83[ab]	-4.85±1.14[b]	-1.29±1.32[a]	0.0466
SCE b	30.05±0.29	30.04±0.22	29.63±0.30	30.65±0.37	0.1194

SCI- Specular Component Included; SCE- Specular Component Excluded; Values are Mean ± SE

a, b Values with different superscripts are significantly different from each other

6.3.2.2 Scanning Electron Microscopy of the cuticle surface

The score allocated to cuticular cover under the scanning electron microscope (SEM) was significantly lower at 55 weeks which indicates that more cuticle was present in this flock. A relatively high value for the 79 wk flock egg indicates that eggs from this flock had the least amount of cuticle as shown in Table 6.3.2.2.1.

Table 6.3.2.2.1 Scanning Electron Microscopy (SEM) values of cuticle cover

Variable	Flocks age (weeks)				P value
	22	39	55	79	
Cuticle cover	2.30±0.15[a]	2.23±0.14[ab]	1.80±0.19[b]	2.57±0.16[a]	0.0074

Values are Mean ± SE

[a, b] Values with different superscripts are significantly different from each other

6.3.3 Ultrastructural scoring of the shell mammillary layer

The variation in mammillary cap size was similar for all flocks as shown Fig. 6.3.3.1. The incidence of confluence was higher for the 22 wk flock than for the older flocks except that the 55 wk was not significantly different from all other flocks (Fig. 6.3.3.2). The quality of the mammillary caps was highest for the 22 wk flock and lowest for the 55 and 79 wk flocks with the 39 wk flock intermediate (Fig. 6.3.3.3). Early fusion decreased linearly as flock age increased whereas late fusion increased with increased flock age as shown in Fig. 6.3.3.4 and 6.3.3.5, respectively. The incidence of mammillary alignment was lower in the 22 wk flock, as compared with the other three flock ages (Fig. 6.3.3.6). The cubic cone formation was highest in the 39 week flock, lowest in the 79 week flock with 22 and 55 week flocks intermediate (Fig. 6.3.3.7). The incidence of cuffing was higher in the 39 wk flock than for all other flocks (Fig. 6.3.3.8). The incidence of changed membrane was lower in the 22 wk flock than for all other flock ages (Fig. 6.3.3.9). The incidence of depression was

highest in the 79 wk flock and lowest in the 22 and 39 wk flocks with the 55 wk flock intermediate (Fig. 6.3.3.10). Mammillary layer ultrastructural features for which the incidence was not significantly different among the flocks were Type A bodies, Type B bodies, aragonite, cubics and erosion as shown in Table 6.3.3.1. All the mammillary ultra structure variables which were statistically significantly different among the flocks are presented in graphs whereas variables not significantly different among flocks are shown in Table 6.3.3.1.

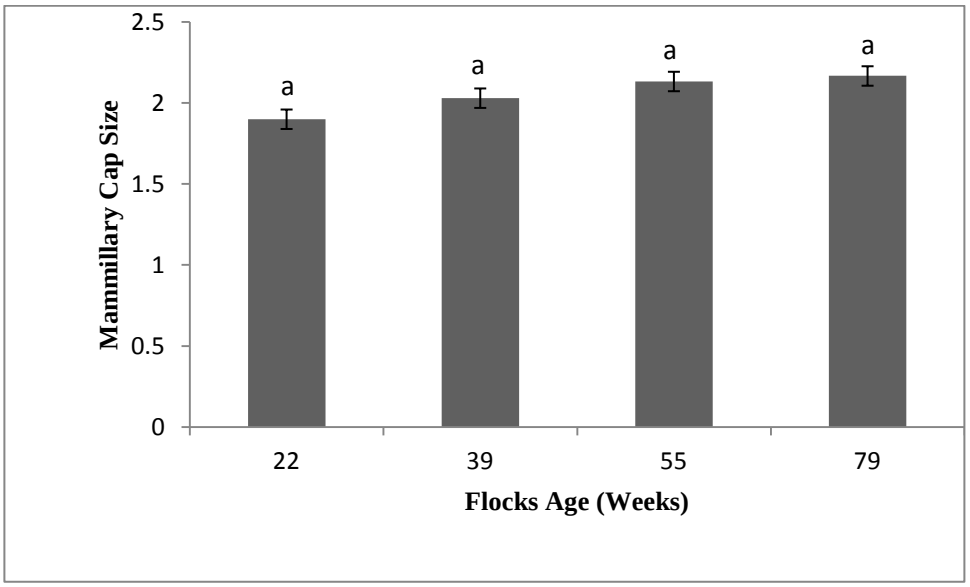

Figure 6.3.3.1 Incidence of mammillary cap size variability of different aged flocks (Mean ± SE)
[a, b, c] Different superscripts indicate significant differences

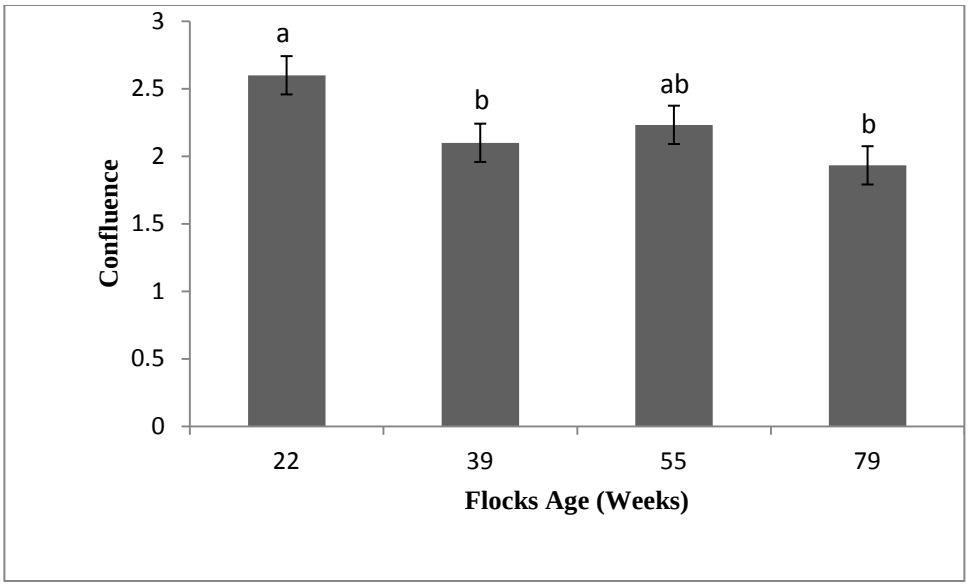

Figure 6.3.3.2 Incidence of confluence of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

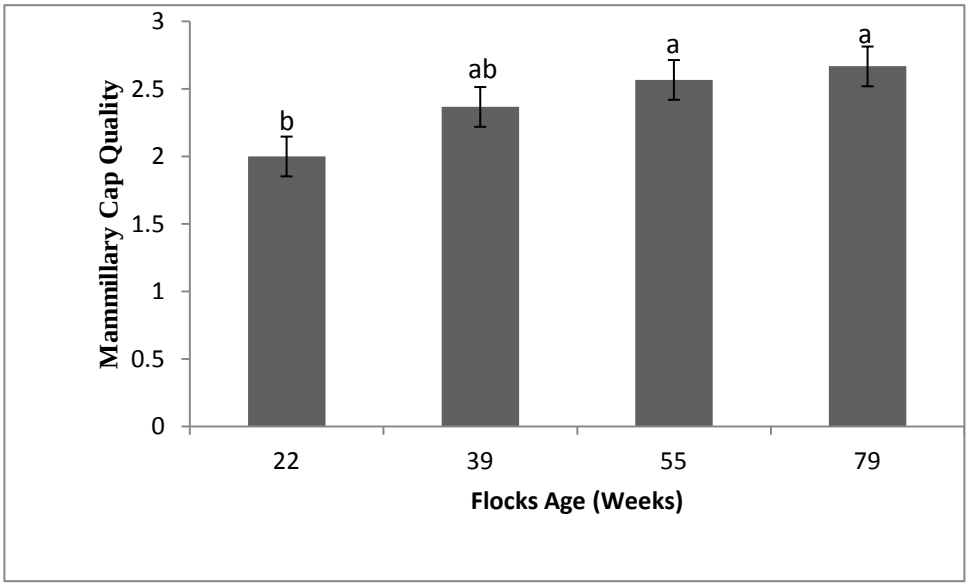

Figure 6.3.3.3 Incidence of mammillary cap quality of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

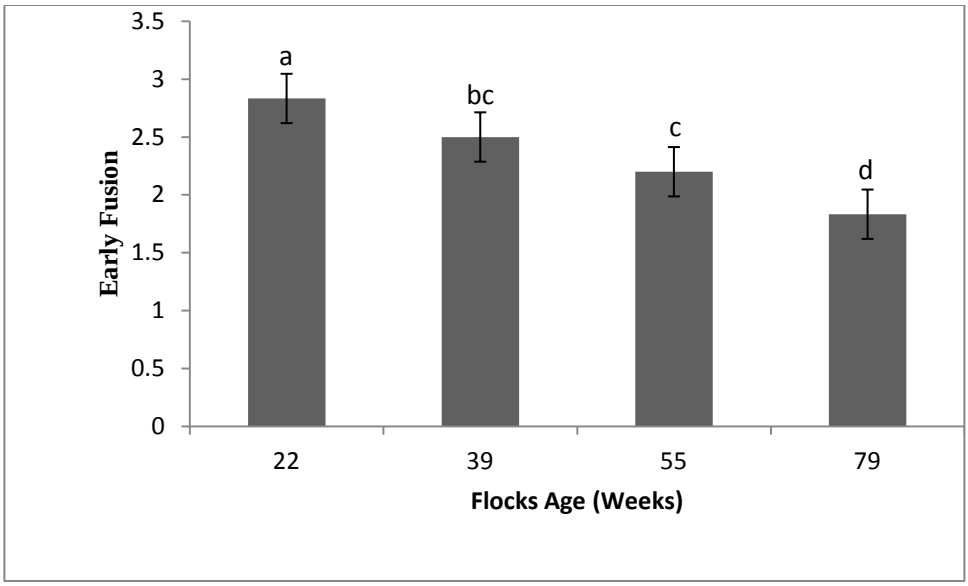

Figure 6.3.3.4 Incidence of early fusion of different aged flocks (Mean ± SE)

[a, b, c, d] Different superscripts indicate significant differences

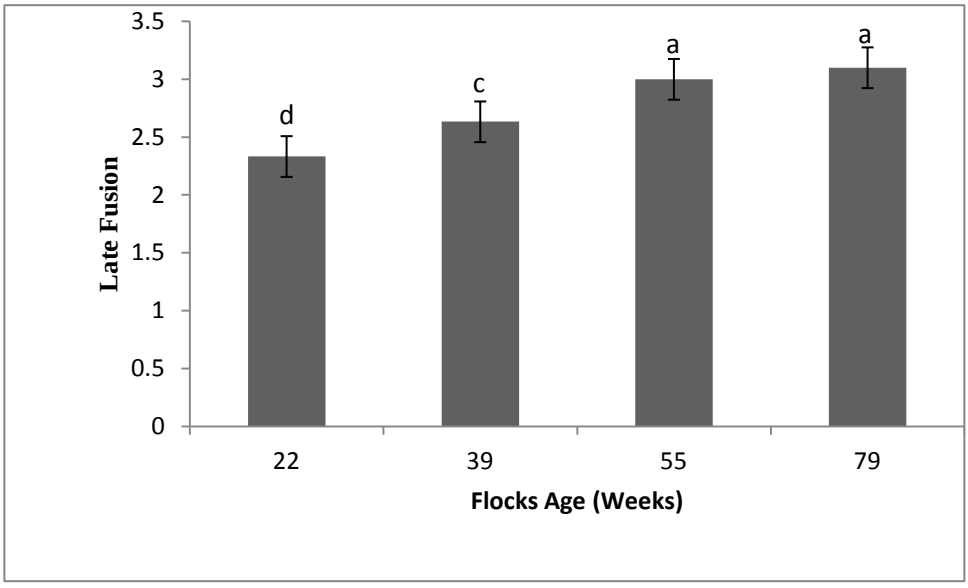

Figure 6.3.3.5 Incidence of late fusion of different aged flocks (Mean ± SE)

[a, b, c, d] Different superscripts indicate significant differences

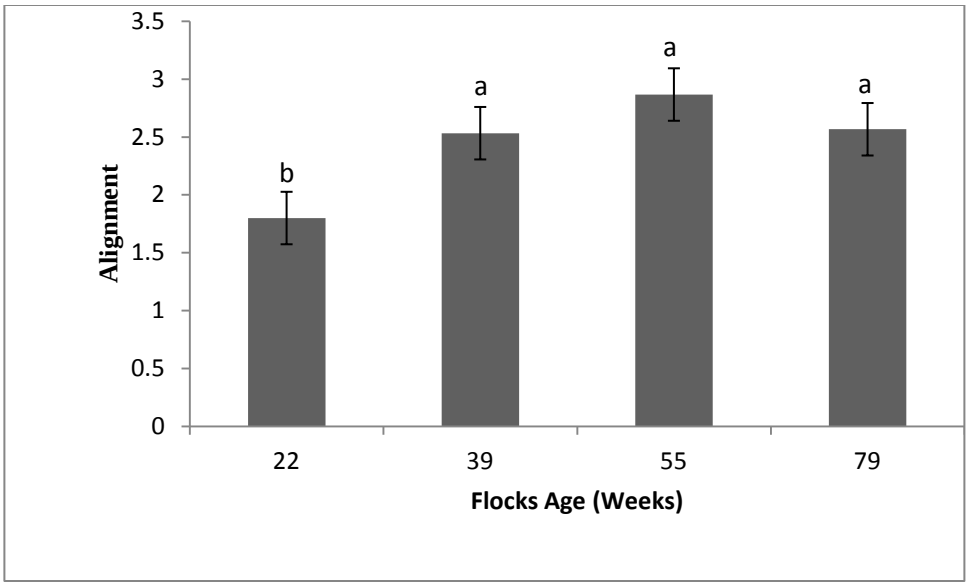

Figure 6.3.3.6 Incidence of alignment of different aged flocks (Mean ± SE)

[a, b, c] Different superscripts indicate significant differences

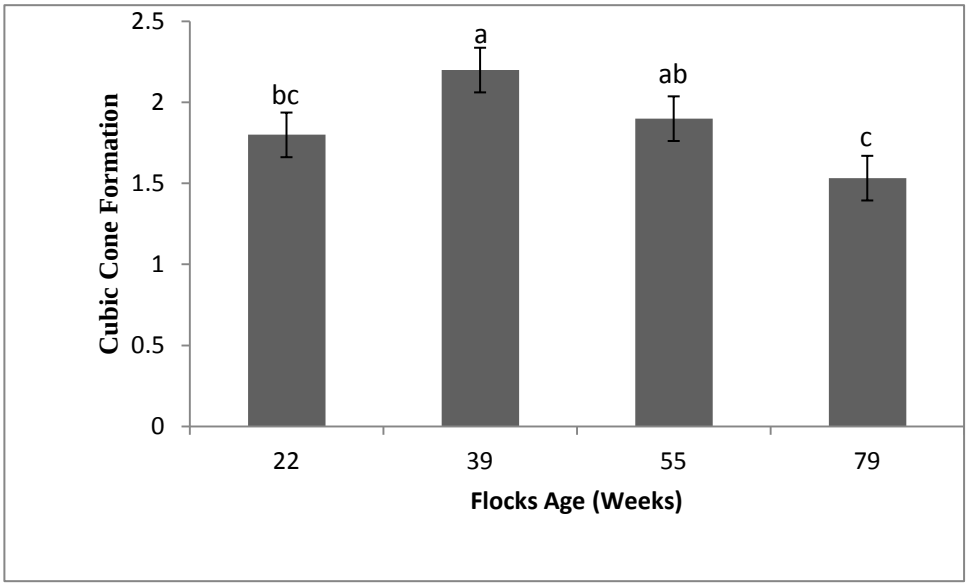

Figure 6.3.3.7 Incidence of cubic cone formation of different aged flocks (Mean ± SE)

[a, b, c] Different superscripts indicate significant differences

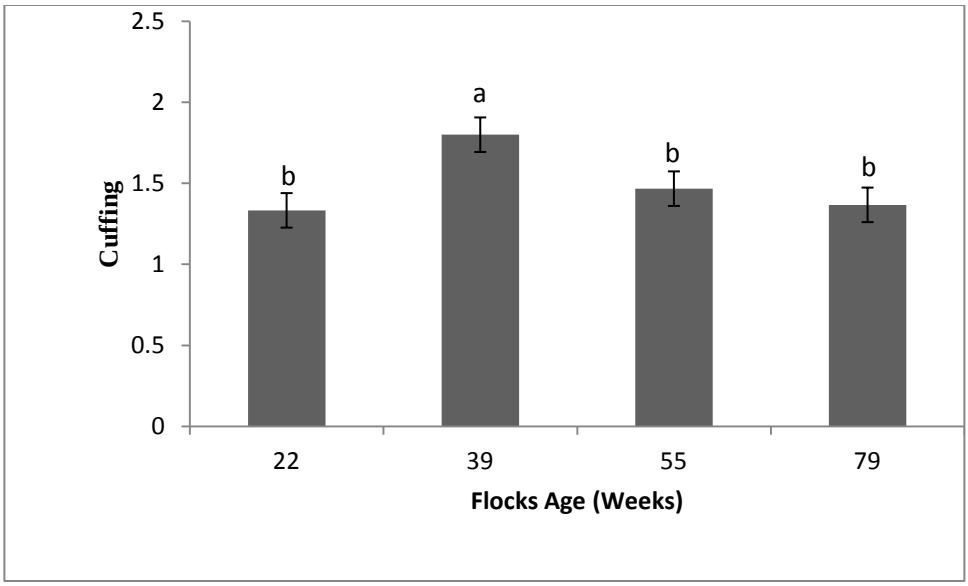

Figure 6.3.3.8 Incidence of cuffing of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

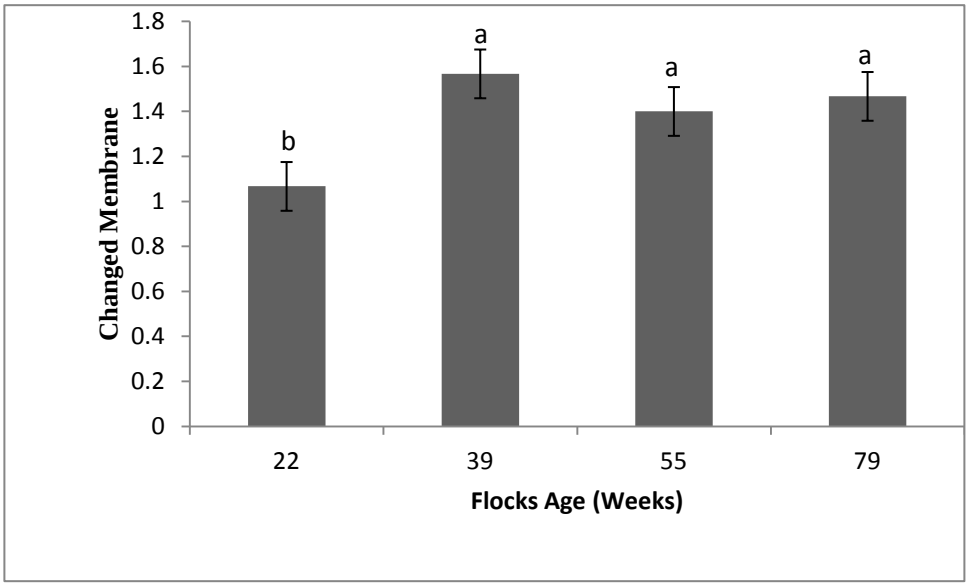

Figure 6.3.3.9 Incidence of changed membrane of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

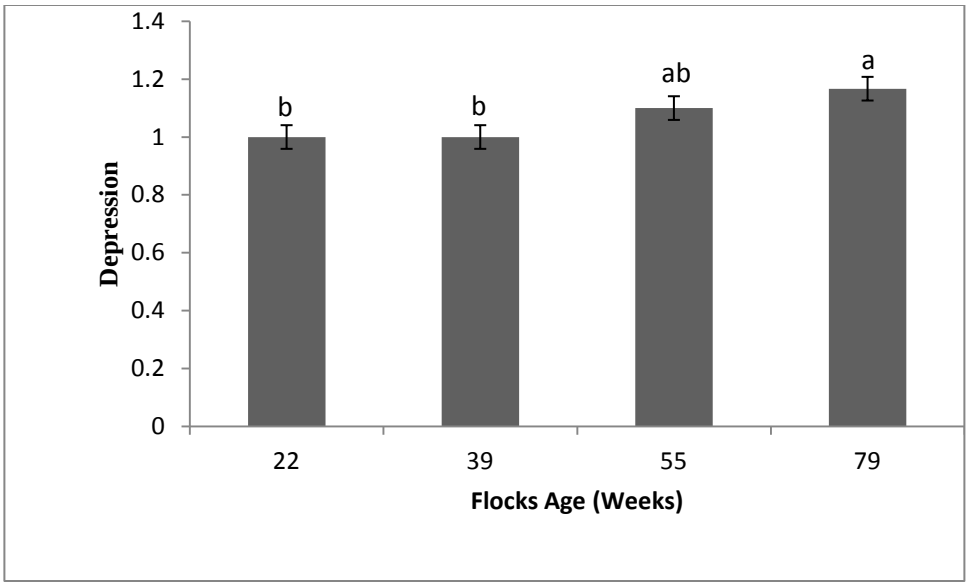

Figure 6.3.3.10 Incidence of depression of different aged flocks (Mean ± SE)

[a, b] Different superscripts indicate significant differences

Table 6.3.3.1 Shell ultrastructural variables not affected by flocks age

Variables	Flocks age (weeks)				P value
	22	39	55	79	
Type A bodies	1.40±0.09	1.37±0.09	1.50±0.09	1.63±0.11	0.2103
Type B bodies	2.07±0.15	2.07±0.11	2.13±0.12	2.47±0.16	0.1313
Aragonite	1.33±0.11	1.27±0.08	1.13±0.09	1.27±0.11	0.5402
Cubic	1.27±0.09	1.20±0.07	1.13±0.08	1.23±0.08	0.6969
Erosion	1.10±0.06	1.27±0.09	1.07±0.05	1.20±0.07	0.1706

Values are Mean ± SE

6.3.4 Egg microbiology

The total microbial count (TBC) on the eggshell and in shell crush was significantly lower in the 79 wk flock as shown in Table 6.3.4.1. The total Enterobacteriaceae count (TEC) on the eggshell surface in the 39 wk flock was zero while in the shell crush of eggs from 22 and 79 wk flocks Enterobacteriaceae were not isolated/detected. Egg internal contents were free from bacteria and all the egg belt swabs and manure swabs were negative for *Salmonella* recovery.

Table 6.3.4.1 TBC and TEC (10 log cfu) on eggshell and in shell crush

Variables	Flocks Age (weeks)				P Value
	22	39	55	79	
TBC on eggshell	3.48 ± 0.39^{a}	3.88 ± 0.14^{a}	3.48 ± 0.59^{a}	0.83 ± 0.56^{b}	<0.0001
TBC in shell crush	0.48 ± 0.32^{bc}	1.44 ± 0.40^{a}	1.19 ± 0.41^{ab}	0.00 ± 0.00^{c}	0.0143
TEC on eggshell	1.11 ± 0.47^{b}	0.00 ± 0.00^{c}	3.18 ± 0.36^{a}	2.71 ± 0.47^{a}	<0.0001
TEC in shell crush	0.00 ± 0.00^{b}	1.30 ± 0.44^{a}	0.20 ± 0.20^{b}	0.00 ± 0.00^{b}	0.0011

TBC- total bacterial count; TEC- total Enterobacteriaceae count; Values are Mean ± SE

$^{a, b, c}$ Values with different superscripts are significantly different from each other

6.4 Discussion

6.4.1 Eggshell and egg internal quality measurements

Flock age had a statistically significant effect ($P \leq 0.05$) all egg external and internal quality variables. Similar findings were recorded in the longitudinal study by following the same flock throughout its production cycle.

Translucency score was lower in the 39 week flock and highest in the 79 week old flock. Although there are overall differences among the different aged flocks for translucency score, it has also been noted that translucency varies within a flock. Freshly laid eggs show relatively low translucency and its incidence increases with storage time reaching a maximum at about 6-7 days. After a week of storage in the refrigerator, translucency remains relatively stable for most eggs. In the present study, all eggs were collected at the same time and the time interval between the egg being laid and scored for translucency was the same for all flocks at 2 days following lay. It has been suggested that translucency occurs due to the escape of moisture through the shell membranes into the mammillary layer. Dried shell pieces do not show any translucent spots but the translucent spots reappear when the shell is soaked in water for more than 24 hours. A correlation between the incidence of translucency and the ease of microbial penetration has been proposed by some authors (Chousalkar *et al.*, 2010; Solomon, 1992). Under the scanning electron microscope, translucent spots did not show any consistent variation in the mammillary layer.

Shell reflectivity (%) is an important parameter as pigment in the shell has various effects ranging from shell quality aspects to antimicrobial activity. Generally, shell reflectivity increases with flock age as reported by several authors including Odabasi *et al.* (2007), Zita *et al.* (2009) and Tumova and Ledvinka (2009). In the current study, shell reflectivity (%) significantly increased ($P \leq 0.05$) and similar findings were recorded in longitudinal studies outlined in Chapters 3 and 4.

Egg weight (g), one of the important economic variables for producers, increases with flock age. A positive correlation between egg weight and flock age was recorded in the current study which can be compared to the findings of many authors such as Wang *et al.* (2009); Rayan *et al.* (2010); Rossi and Pompei (1995); Johnston and Gous (2007); Van Den Brand *et al.* (2004); Novo *et al.* (1997); Rizzi and Chiericato (2005); Garlich *et al.* (1984); Izat *et al.* (1986); Ronald (1979); Ledvinka *et al.* (2011);

Ferrante *et al.* (2009); Berrang *et al.* (1998); Roland, Sr. *et al.* (1975) and Odabasi *et al.* (2007). So far, no one has recorded a decrease in egg weight with flock age in a properly managed commercial flock. From the findings of Fisher (1969) it is concluded that feeding protein deficient diets to a layer flock will reduce egg weight.

Shell breaking shell strength (BSN) is the force in Newtons that causes the breakage of an intact shell. High BSN is indicative of a good quality eggshell. A slightly lower BSN value in the 55 wk old flock suggests decreased eggshell quality in older flocks. The higher BSN in 79 wk flock, as compared to the 55 wk flock, may reflect a positive effect of molting on eggshell quality as the flock was molted at 62 wk of age.

The linear decrease in eggshell deformation (μm) values with the flock age (to 55 weeks) suggests that shell quality decreased as the flock entered the mid lay period. A slightly higher value in the 79 wk flock might be explained by improvement in shell quality due to molting.

Although shell weight increased with increasing flock age, the percentage shell was lower for the three older flocks. This indicates that the increase in shell weight was not keeping up with the increase in egg size. A decrease in percentage shell with flock age has been recorded by Silversides and Scott (2001) and Zita *et al.* (2009). Shell thickness was lowest in the 55 wk flock. As flock age increased, shell thickness decreased in the studies of Rayan *et al.* (2010) while Roland *et al.* (1975) reported an increase in shell thickness with increased flock age. However, the current study cannot be directly compared to the above mentioned authors as they followed the same flock at different ages.

The albumen height (mm) measurement indicates the quality of albumen and is maximum for thick viscous albumen. The viscosity and height of albumen were lower with increased flock age in the present study. It is not clear what types of changes occur in the albumen with hen age. In the studies of Zita *et al.* (2009), processing eggs from the same flock (20-60 week old), albumen height decreased with increased flock age.. From the horizontal study, it can be concluded that eggs from different flocks of the same breed vary significantly in egg quality parameters were comparable to the results from the longitudinal flock age. Age is considered the single most important factor affecting albumen quality (Williams, 1992).

Haugh unit is still used as a reliable indicator for determining the quality of albumen despite criticism from various researchers (Silversides, 1994 and Silversides and Scott, 2001). Haugh unit was parallel to the values for albumen height and, in the present study, Haugh unit decreased with increased flock age. Williams (1992) indicated a positive correlation of the Haugh unit with albumen height.

The lower yolk colour in the 55 and 79 wk flocks eggs is not easily explained. Yolk colour mostly depends on the amount of xanthophylls in the diet. The slight increase followed by a decrease in the intensity of yolk colour may have been due to different diets being fed to the different flocks, despite being on the same farm, or to different feed intakes. No clear indication from the previous research has shown the age effect on yolk colour.

6.4.2 Estimation of the amount of cuticle

6.4.2.1 Shell reflectivity (%) and Spectrophotometry (L*a*b) measurements

The shell reflectivity (%) increases with increased flock age, found in the present study, have also been reported by earlier studies (Odabasi *et al.*, 2007; Zita *et al.*, 2009 and Tumova and Ledvinka, 2009). The reflectivity of cuticle blue stained eggs increased with flock age, consistent with the lighter underlying colour of the eggs. The current findings confirm that shell reflectivity increases with increased flock age and similar findings have been recorded in the longitudinal studies, chapters 3 and 4. The explanation may be that, with increased flock age, egg weight increases and the same amount of pigment is deposited on a relatively larger surface of eggshell, as suggested previously by Odabasi *et al.* (2007).

The SCI L* component of the L*a*b colour space system measures the grading between white (100) and black (0) colour. In brown coloured eggs, the value is low whereas for white eggs it is high. SCI L* has a positive correlation with shell reflectivity (%). Both SCI L* and SCE L* values increased linearly with flock age, indicating that the eggs were lighter in colour with increased flock age.

SCI a* measures the grading between green (negative) and red (positive). The more negative the value the more green the egg surface which indicates more amount of cuticle present. SCI a* and SCE a* values were similar to each other. The current results showed that the amount of cuticle deposition increased towards the mid lay period (55 wk) followed by a decrease towards late lay (79

wk). Similar results were obtained in longitudinal studies (chapters 3 and 4) in which the amount of cuticle was higher in the mid lay period compared to early and late lay periods.

SCI b* and SCE b* values were not significantly different among the flocks. Using Konica Minolta spectrophotometer (CM-2600d) for measurements of the stained eggs with MST cuticle blue, the most important component (L*a*b) of interest is a* which shows the amount of stain acquired by the eggshell cuticle.

6.4.2.2 Scanning Electron Microscopy (SEM) of the cuticle surface

The eggs from the 55 week flock had more cuticle compared to the other flocks. These findings are in agreement with the works of Messens *et al.* (2005) and Ruiz and Lunam (2000) who reported less cuticle in the late lay period. Although the SEM cuticle scoring and SCI a* values showed different patterns among the flock ages, there was still an overall consistency. One of the reasons for this difference is the size of the specimen viewed under SEM and SEM accuracy as compared to the surface of egg measured by the spectrophotometer.

6.4.3 Ultrastructural scoring of the shell mammillary layer

The variability of the mammillary cap size increased with increased flock age. More variable cap size may affect the cap quality and adherence to shell membranes during its formation which in turn affects the overall shell quality. Confluence, a positive ultrastructure variable, decreased with increased age. Similar findings have been recorded by Brackpool (1995). Solomon (1991) has mentioned that extra confluence alters the position of pores and affects palisade layer formation. Mammillary cap quality always affects membrane attachment and, in the current study, cap quality declined with increasing flock age, as reported also by Nascimento *et al.* (1992) and Brackpool (1995). Early fusion has a positive effect on shell mammillary strength (Parsons, 1982) and, in the current study, a linear decrease in early fusion with increased flock age indicates that shell ultrastructural quality decreases with increased age. Unlike early fusion, a higher incidence of late fusion would decrease shell quality. Solomon (1991, 1992) considered late fusion as a negative factor on the mammillary layer quality. A high incidence of alignment increases the risk of crack propagation and the risk of microbial penetration. A relatively high incidence was recorded in the 55 week flock. A slightly, but not significantly, lower incidence of alignment in the 79 wk flock might

reflect the positive effect of molting on shell ultrastructure as the flock was molted at the age of 62 wk. The incidence of type A, Type B bodies, aragonite, cubic and erosion was not significantly different among flocks although higher incidence has a negative effect on ultrastructural shell quality. Cuffing is considered a positive variable and its higher incidence increases shell strength. Decreased incidence with older flock age (55 and 79 week) helps to explain the negative effect of flock age on shell ultrastructure. Brackpool (1995) recorded a higher incidence of cuffing in 57 vs 33 wk flocks which is contradictory to our current findings. A higher incidence of cubic cone formation and changed membrane might have a negative effect on the shell ultrastructure quality. Brackpool (1995) recorded a higher incidence of cubic cone formation and changed membranes in 57 wk flock eggs versus 33 wk. Increased incidence of depression and erosion make the shell more prone to microbial attack. Similar to the longitudinal studies (Chapters 3 and 4), the incidence of erosion and depression were not significantly different among flock ages. Overall, it can be concluded from the SEM of the mammillary layer that the incidence of all negatively affecting variables though significant among flocks age but still remains low.

6.4.4 Egg microbiology

Generally, bacteria load on the eggshell surface increases with the flock age (Wall *et al.,* 2008). The lower total bacterial count (TBC) on eggshells in 55 and 79 wk old flock might be due to a technical error in the procedures as these eggs were processed separately from 22 and 39 wk eggs. There was also a gap of 7 days in the processing time by storing the 55 and 79 wk eggs at 4°C. In the studies of Wall *et al.,* (2008) the number of aerobic counts on the shell was higher at 28 week versus 62 week while the Enterobacteriaceae count was higher for 62 week compared to 28 week flock age. In the studies of De Reu *et al.* (2005b, 2006c) and Protias *et al.* (2003a), following the same flock at different ages, there was no significant effect of hen age on bacterial eggshell contamination. In the current study, higher bacterial load on shell surface and in shell crush was recorded in peak production (39 and 55 wk). The total Enterobacteriaceae count (TEC) on the shell was highest at 55 wk and in the shell crush at 39 wk. Overall, the bacterial load on eggs collected during this experiment was low. The egg internal contents were free from bacteria and all the swabs processed were negative for *Salmonella* indicating that either *Salmonella* was not present on the farm or could not transfer to the flock due to good husbandry practices. However, a large number of samplings is needed before making any solid conclusions. As mentioned in Chapter 3, the overall total bacterial

load and egg internal contents negative for bacteria further elaborate the effectiveness of hygiene practices on the target farm.

Overall, it can be concluded that flocks age do affect significantly the eggshell quality and microbial load present on the eggshell.

Chapter 7

Quantification of the Amount of Protoporphyrin IX in Cuticle and True Shell of Australian Hy Line Brown Eggs

7.1 Introduction

Brown, white or tinted eggs are linked to the genotype of the hen. However, eggs of many colours are laid by different avian species and considerable attention has been paid to the eggshells of the domestic quail. The eggshell colour of brown eggs is a quality aspect for consumers (Mertens *et al.*, 2010) as brown eggs dominate commercial markets in many countries including Australia. Eggshell pigment has been shown to have antimicrobial properties, particularly against gram positive bacteria (Ishikawa *et al.*, 2010). Shell pigment is also used as an assessment tool for stress and disease condition in laying birds (Martinez-de la Puente, *et al.*, 2007; Moreno and Osorno, 2003; Mertens *et al.*, 2010). Shell colour has been linked to egg quality parameters in the brown eggs (Curtis *et al.*, 1985; Jones *et al.*, 2010) and may be related to shell ultrastructure (Richards and Deeming, 2001). Protoporphyrin has been shown to be the main eggshell pigment found in the eggs of laying hens, in addition to other pigments such as zinc porphyrin, biliverdin and zinc biliverdin (Miksik *et al.*, 1996; Kennedy and Vevers, 1973, 1976; Mertens *et al.*, 2010; With, 1973). Previous research has suggested that most of the protoporphyrin is located in the cuticle (Miksik *et al.*, 2007; Sparks, 1994). However, the study of Nys *et al.* (1991) which investigated the kinetics of protoporphyrin deposition found that approximately 75% of the protoporphyrin was laid down in association with the calcareous part of the shell. Therefore, the purpose of the present study was to quantify the amount of protoporphyrin IX from the cuticle and true shell layer in brown eggs.

7.2 Materials and methods

Eggs were collected from conventional cage flocks (Hy Line brown) which were 33, 50 and 67 wk of age. The 67wk flock was subjected to an induced molt at the age of 62 weeks. Eggs were divided into two groups and used for the following investigations.

Colour measurements of eggshells of unstained and stained eggs, with and without cuticle and Scanning Electron Microscopy (SEM) of the eggshell surface of these eggs

Protoporphyrin IX quantification from shells, with and without cuticle, by spectrophotometry

7.2.1 Colour measurements of unstained, stained, cuticle removed and re stained eggs and their SEM

This study was conducted to verify the reliability of MST cuticle blue dye as an indicator of the presence of cuticle. It also investigated the effectiveness of the use of EDTA to remove the cuticle without eroding into the true shell.

Thirty eggs from each age group (33, 50 and 67 week) were included in this study. Eggs selected were free from extraneous calcareous materials. Shell colour (L*a*b) was measured before staining using a Konica Minolta spectrophotometer (CM-2600d) in addition to measuring shell reflectivity (%) using a shell reflectivity meter (TSS U.K.). Eggs were soaked in MST Cuticle blue dye (MS Technologies, Europe Ltd) for 1 minute and rinsed in distilled water to remove excess stain. After drying, shell colour (L*a*b) and shell reflectivity were measured on the stained eggs. For the thorough and careful removal of the cuticle, the method described by Leleu *et al.* (2011) was followed with slight modification. Each egg, individually, was soaked in an EDTA solution (0.34 M, pH 7.5) for 5 minutes and the cuticle was carefully scrubbed off in the running tap water using a small soft brush. Eggs were then allowed to dry after which shell colour (L*a*b) and shell reflectivity were measured on the eggs without cuticle. Eggs were re stained as described earlier and shell colour (L*a*b) and shell reflectivity (%) were again measured.

Egg internal contents were removed by making a small hole at the blunt end of the egg using a rotary tool (Dremel High Speed rotary tool, 300 series). A small piece from around the equator of the shell was cut out, mounted on an aluminium stub using silver paint (1005 aqueous conductive silver liquid-SEM adhesive, ProSciTech, Australia), photographed under a light microscope at 12x magnification, sputter coated with gold for 5 min in a Neocoater (Nikon, Japan) and viewed under a JCM-5000 Neoscope benchtop scanning electron microscope (SEM) at various magnifications.

7.2.2 Protoporphyrin IX quantification from eggshell by spectrophotometry

7.2.2.1 Sample preparation for Protoporphyrin IX extraction

Eggs from the 33, 50 and 67 week flocks were analyzed for protoporphyrin IX in the egg cuticle and whole eggshell (including the cuticle). All eggs analyzed were free from any extraneous calcareous

material. A method described by the Poole (1965) and used by Ito *et al.* (1993) and Wang *et al.* (2009, 2007) was used with some modification. Egg contents were removed as described above and the inner shell walls rinsed with tap water to remove the adherent albumen. A clean thin stick was passed across the long axis of the eggshell in such a way as to immerse one longitudinal half of the shell in a glass container containing 0.34 M EDTA (pH 7.5) for 5 minutes at the same time as maintaining the other half outside the solution. The cuticle of the soaked longitudinal half side of the eggshell was washed away in running tap water by scrubbing with a small soft brush. After removal of the cuticle on one side, the eggshell was cut off longitudinally into two equal halves; one having cuticle and one without cuticle. From both halves, shell membranes were carefully peeled off and the shells were allowed to dry thoroughly. The procedure was repeated for each eggshell individually. After drying shells completely, 0.250 g from the equator of each shell was measured in a clean 10 mL plastic centrifuge tube into which 4 mL of Methanol - concentrated HCl (2:1) solvent was added. For each flock, 30 tubes were prepared with whole eggshell and 30 tubes from the shell without cuticle. Thus, the total number of tubes from all the three flocks for both whole eggshell and shells without cuticle was 180. All the tubes were wrapped in aluminium foil and placed in a refrigerator for 12 hours, avoiding exposure to light. The samples were centrifuged at 3000 rpm for 1 hr. After centrifugation, the supernatant solution was decanted into spectrophotometer (Shimadzu, UV-1201) cuvettes (4mL) and the absorbance of the supernatant read at 412 nm. In order to confirm that the shell had dissolved completely, the sediment remaining in the bottom of the centrifuge tubes was viewed under a light microscope at various magnifications.

7.2.2.2 Protoporphyrin IX standard preparation

A stock standard solution was prepared by dissolving 0.0018 g of powder protoporphyrin IX disodium salt (Sigma Aldrich Australia) in 30 mL Methanol - concentrated HCl (2:1) solvent. Serial dilutions were prepared until a 1:128 dilution was reached. The absorbance of all the dilutions was read in the spectrophotometer at 412 nm after calibrating to zero with distilled water and blank (Methanol-HCl). A standard curve was constructed by plotting the concentrations of protoporphyrin in the standards: 1:16 (6.87×10^{-6} mM), 1:32 (3.43×10^{-6} mM), 1:64 (1.72×10^{-6} mM), and 1:128 (8.59×10^{-7} mM), dilutions of the stock standard solution against the absorbance reading for each standard.

7.2.2.3 Calculation of PP IX in per gram of shell

The absorbance values were converted into concentration of PP IX in the sample solvent in mmol/L and the amount or protoporphyrin in 1 g of eggshell (with and without cuticle present) was calculated. For determination of the amount of protoporphyrin IX in the cuticle, the values of the eggshell samples without cuticle were subtracted from the values of the eggshell samples with the cuticle still present.

Data were analyzed using Statview Software (SAS Institute Inc., Version 5.0.1.0) as explained in Chapter 2, section 2.5.

7.3 Results

In experiment 7.2.1, there was no significant effect ($P=0.1477$) of flock age on shell reflectivity (%) when the cuticle was present (with and without MST cuticle blue staining) (Table 7.3.1). However, after the cuticle had been removed with EDTA, there were significant differences amongst flocks with and without cuticle blue staining with the 67 wk flock having the lowest reflectivity. There was a significant main effect of cuticle treatment on shell reflectivity ($P<0.0001$). Shell reflectivity decreased following staining of eggs with cuticle intact. Shell reflectivity increased after the cuticle was removed from the eggs and was not significantly different following cuticle blue staining except for the 33 wk flock.

Table 7.3.1 Shell reflectivity (%) values of eggshells at various stages of shell treatment

Flocks age (wk)	+ Cuticle - Stain	+ Cuticle + Stain	- Cuticle - Stain	- Cuticle + Stain	All values	P Value
33	^{c}29.07±0.75	^{d}24.37±0.85	^{a}35.17±0.77^{xy}	^{b}34.03±0.78^{x}	30.66±0.55	<0.0001
50	^{b}29.80±0.69	^{c}23.83±0.82	^{a}36.30±0.63^{x}	^{a}36.47±0.57^{x}	31.60±0.59	<0.0001
67	^{b}27.80±0.55	^{c}25.23±0.63	^{a}33.50±0.55^{y}	^{a}32.97±0.57^{y}	29.87±0.43	<0.0001
All values	28.89±0.39	24.48±0.45	34.99±0.39	34.49±0.40		
P Value	0.1091	0.4364	0.0124	0.0009		

+ Cuticle: - reading of eggshell having cuticle; - Cuticle: - reading of eggshell have cuticle removed

+ Stain: - reading of stained eggshells; - Stain: - reading of unstained eggshells

Values are Mean ± SE

$^{a, b, c, d}$ Across a row, values with different superscripts are significantly different from each other

x, y, z Different superscripts within a column indicate significant differences

The SCI L* component of the L*a*b space system was not significantly (P=0.1860) affected by flock age when the cuticle was intact. However, following the removal of the cuticle, there were differences among flocks with values being highest for the 50 wk flock. There was a significant effect of cuticle treatment on mean values of SCI L* among each age group as shown in Table 7.3.2. Staining of eggs with intact cuticle resulted in lower L* values whereas cuticle removal increased these values.

Table 7.3.2 Spectrophotometry (SCI L*) values of eggshells at various stages of treatment

Flocks age (wk)	+ Cuticle - Stain	+ Cuticle + Stain	- Cuticle - Stain	- Cuticle + Stain	All values	P Value
33	[b]60.82±0.63	[c]55.29±0.83	[a]65.36±0.59 [y]	[a]64.65±0.60 [y]	61.53±0.49	<0.0001
50	[c]60.59±0.62	[d]55.34±0.85	[a]67.47±0.49 [x]	[b]66.26±0.46 [x]	62.41±0.54	<0.0001
67	[b]59.70±0.48	[c]55.98±0.65	[a]64.62±0.43 [y]	[a]63.84±0.42 [y]	61.04±0.40	<0.0001
All values	60.37±0.37	55.54±0.45	65.81±0.32	64.92±0.30		
P Value	0.3567	0.7858	0.0005	0.0033		

+ Cuticle: - reading of eggshell having cuticle; - Cuticle: - reading of eggshell have cuticle removed

+ Stain: - reading of stained eggshells; - Stain: - reading of unstained eggshells

Values are Mean ± SE

[a, b, c, d] Across a row, values with different superscripts are significantly different from each other

x, y, z Different superscripts within a column indicate significant differences

There was a significant effect of flock age on the SCI a* reading for all cuticle treatments with the 67 wk flock having the highest values. There was also a significant effect of cuticle treatment on values for SCI a* for all flocks, as shown in Table 7.3.3. The a* reading decreased following staining in eggs with intact cuticle. Removal of the cuticle with EDTA resulted in lower readings than for shells with intact cuticle. Staining with cuticle blue dye of eggs with cuticle removed resulted in reduction in the a* value for all flocks.

Table 7.3.3 Spectrophotometry (SCI a*) values of eggshells at various stages of treatment

Flocks age (wk)	+ Cuticle - Stain	+ Cuticle + Stain	- Cuticle - Stain	- Cuticle + Stain	All values	P Value
33	$^{a}16.66\pm0.38^{y}$	$^{d}0.21\pm0.77^{y}$	$^{b}14.74\pm0.41^{y}$	$^{c}12.05\pm0.42^{y}$	10.92±0.64	<0.0001
50	$^{a}16.71\pm0.35^{y}$	$^{d}-1.37\pm0.93^{y}$	$^{b}13.95\pm0.31^{y}$	$^{c}11.80\pm0.37^{y}$	10.27±0.69	<0.0001
67	$^{a}17.89\pm0.28^{x}$	$^{d}6.67\pm0.77^{x}$	$^{b}16.28\pm0.26^{x}$	$^{c}13.92\pm0.28^{x}$	13.67±0.45	<0.0001
All values	17.08±0.20	1.84±0.60	14.99±0.22	12.59±0.23		
P Value	0.0183	<0.0001	<0.0001	0.0001		

+ Cuticle: - reading of eggshell having cuticle; - Cuticle: - reading of eggshell have cuticle removed

+ Stain: - reading of stained eggshells; - Stain: - reading of unstained eggshells

Values are Mean ± SE

$^{a, b, c, d}$ Across a row, values with different superscripts are significantly different from each other

x, y, z Different superscripts within a column indicate significant differences

There was a significant effect of flock age on SCI b* values with the 67 wk flock having the highest values. There was also a significant effect of cuticle treatment for all flocks as shown in Table 7.3.4. Staining of eggs with intact cuticle resulted in higher b* readings whereas removal of cuticle resulted in lower readings with or without cuticle blue staining.

Table 7.3.4 Spectrophotometry (SCI b*) values of eggshells at various stages of treatment

Flocks age (wk)	+ Cuticle - Stain	+ Cuticle + Stain	- Cuticle - Stain	- Cuticle + Stain	All values	P Value
33	$^{b}28.38\pm0.34^{y}$	$^{a}30.96\pm0.42^{y}$	$^{c}27.60\pm0.36^{y}$	$^{c}27.00\pm0.34^{x}$	28.48±0.23	<0.0001
50	$^{b}28.02\pm0.34^{y}$	$^{a}30.86\pm0.24^{y}$	$^{c}26.50\pm0.38^{z}$	$^{c}25.82\pm0.36^{y}$	27.80±0.24	<0.0001
67	$^{b}29.80\pm0.24^{x}$	$^{a}32.19\pm0.24^{x}$	$^{c}28.58\pm0.29^{x}$	$^{d}27.83\pm0.30^{x}$	29.60±0.20	<0.0001
All values	28.73±0.19	31.33±0.19	27.56±0.22	26.88±0.21		
P Value	0.0002	0.0047	0.0003	0.0003		

+ Cuticle: - reading of eggshell having cuticle; - Cuticle: - reading of eggshell have cuticle removed

+ Stain: - reading of stained eggshells; - Stain: - reading of unstained eggshells

Values are Mean ± SE

$^{a, b, c, d}$ Across a row, values with different superscripts are significantly different from each other

x, y, z Different superscripts within a column indicate significant differences

A high correlation between the presence of cuticle blue stain and the amount of cuticle as viewed under the scanning electron microscope was recorded. Eggs with good quality intact cuticle stained well; eggs with patchy cuticle acquired patchy stain whereas, in the absence of the cuticle, the eggs showed almost no stain. SEM observations of the re stained eggshell after cuticle was removed by EDTA confirmed that, in the absence of cuticle, eggshells did not stain with cuticle blue dye. However, in some cases, small amounts of cuticle remained in crevices and pores so that there was some staining with cuticle blue dye.

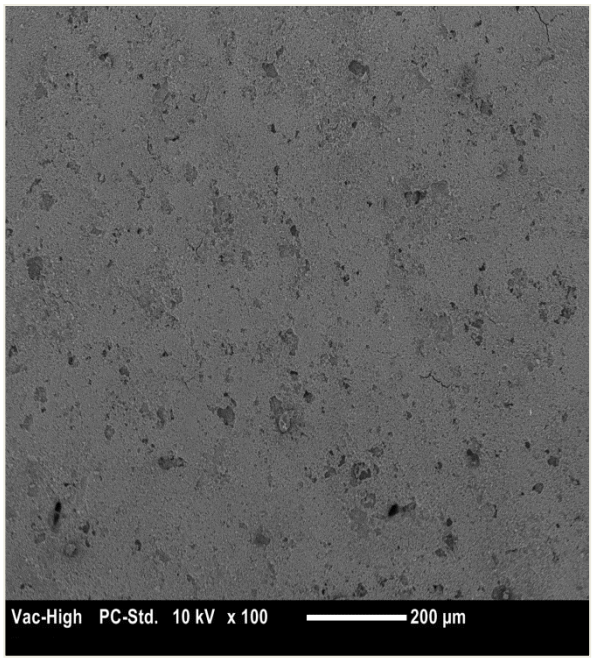

Plate 7.3.1 SEM image of eggshell having no cuticle

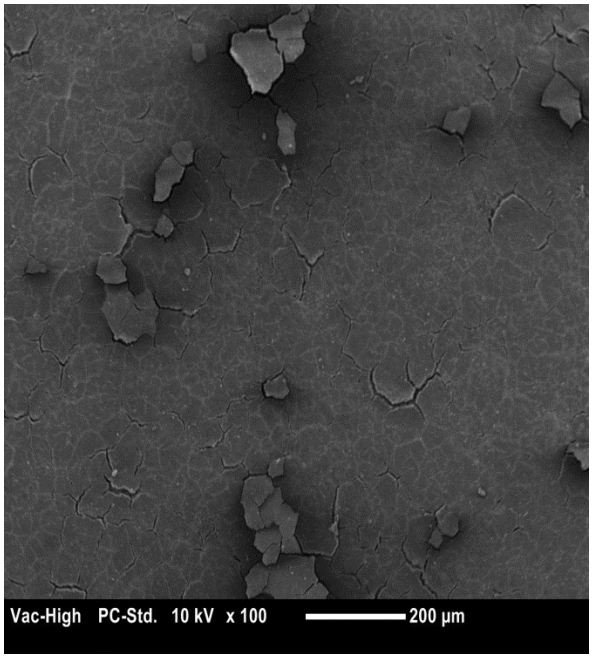

Plate 7.3.2 SEM image of eggshell having good intact cuticle

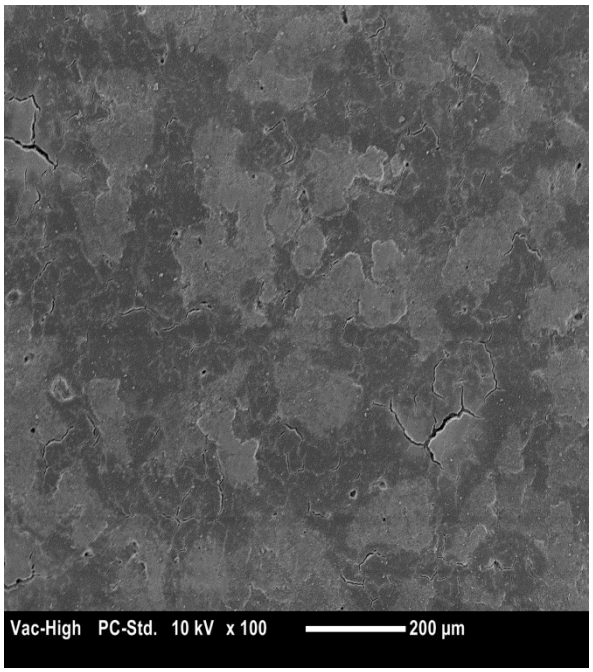

Plate 7.3.3 SEM image of eggshell having patchy cuticle

In Experiment 7.2.2, for a 1 g piece of eggshell, there was more protoporphyrin IX (PP IX) in the shell with cuticle intact, as compared with a piece of shell from the same egg with cuticle removed. When the difference between the two was calculated, there was more protoporphyrin present in the true (calcareous) shell than in the cuticle from the same amount of shell, as shown in Table 7.3.5. The total amount of PP IX in 1 g of shell with cuticle intact was not significantly different among the flocks. The total amount of PP IX in 1 g of shell without cuticle was not significantly different among the three flocks. However, when the amount of PP IX in the cuticle alone of 1 g of total eggshell was calculated, it was significantly higher ($P<0.02$) in the 50 and 67 week flocks as compared with the 33 week flock. For a given weight of whole eggshell, the percentage of total PP IX found in the cuticle was 13, 20 and 18% in 33, 50 and 67 wk flock eggs, respectively. Microscopic observations of the digested shell precipitates showed only shell membranes which confirmed that all shells had been dissolved in the solvent.

Table 7.3.5 Values of protoporphyrin IX in cuticle and true eggshell of Hy Line brown eggs

Variable	**Flocks age (Weeks)**			P Value
	33	**50**	**67**	
PP IX in cuticle of 1 g of eggshell mM	$^{b}1.304 \times 10^{-8}$	$^{a}1.898 \times 10^{-8}$	$^{a}1.806 \times 10^{-8}$	0.0195
PP IX in 1 g of shell without cuticle mM	8.406×10^{-8}	7.569×10^{-8}	8.164×10^{-8}	0.0581

[a, b] Across a row, values with different superscripts are significantly different from each other

Values are Mean

7.4 Discussion

In Experiment 7.2.1, as expected, a significant change in shell reflectivity (%) and L*a*b components of the colour space system with the various cuticle treatments confirmed the effectiveness of the procedure used for cuticle removal. For unstained eggs with cuticle intact, there was no difference

among the flocks for either shell reflectivity or L* values. Following cuticle removal (but prior to staining), reflectivity and L* values were highest for the 50 wk flock. From the current experiments it is concluded that although shell reflectivity and SCI L component of the L*a*b colour space system provide similar, but not always identical, information about overall shell colour. The SCI a* value of eggshells stained with MST cuticle blue dye is the most important indicator of the amount of cuticle present on an eggshell. The higher SCI a* values for +cuticle, +stain eggs in the 67 wk flock indicated the presence of less cuticle. When cuticle blue stain was applied to shells with cuticle removed, all flocks showed slight reductions in a* values, indicating a small amount of staining which was occurring where cuticle was present in crevices and pores. Experiment 7.2.1 also verified that the MST cuticle blue dye is a reliable indicator of the presence of cuticle on eggshells. SEM of shells with cuticle removed confirmed that the EDTA treatment reliably removed the cuticle without eroding the calcium carbonate of the eggshell.

The results of the experiment 7.2.2 showed that there is more pigment (Protoporphyrin IX) in the calcareous components of the eggshell than in the cuticle of commercial brown eggs. These results are in contrast to previous research that reports more pigment in the cuticle than in the calcareous layers of the eggshell (Baird *et al.,* 1975; Miksik *et al.,* 2007; Schwartz *et al.,* 1975; Wang *et al.,* 2007, 2009). However, the results of the present study are consistent with the findings of Nys *et al.* (1991) who reported that 75% of the protoporphyrin was found in the calcareous layers of the shell of brown egg layers. In the present study, the amount of protoporphyrin IX in the eggshell with the cuticle removed (P=0.0581) and shell with the cuticle (P=0.4363) was not significantly different among the three age group eggs which supports the suggestion of Odabasi *et al.* (2007) of a constant rate of secretion of PP IX in the shell gland throughout the production cycle of laying hen. However, there was a statistically significant (P=0.0195) difference among the three flocks in the amount of PP IX in the cuticle alone. However, a reduced amount of PP IX in the cuticle of the shell does not necessarily indicate reduced cuticle cover as, in the current study, the 33 week flock eggs, which had the lower amount of PP IX in per gram of shell, showed more cuticle followed by 50 and 67 week flock age eggs.

Campo *et al.* (2007) have linked the variation in shell colour within a brown egg laying breed as time dependent and mentioned that eggs laid in the afternoon were more white or tinted compared to eggs laid in the morning. Nys *et al.* (1991) have extracted more porphyrin from an egg laid more than 24

hr after the previous egg, compared to an egg laid 20 hr after the previous oviposition. A decrease in the amount of egg pigments with increasing flock age is reported to be affected by egg size (Odabasi *et al.,* 2007). A number of factors affecting the amount of pigment in eggshell have been described (Butcher and Miles, 1995; Lang and Wells, 1987). Our finding that there is a greater percentage of total pigment in the calcareous part of the shell than in the cuticle raises questions about the stages of eggshell formation and the times at which protoporphyrin deposition is maximal. Most authors have suggested that pigment is secreted in the last hour of oviposition and deposited in the cuticle (Kennedy and Vevers, 1976; Poole, 1965; Schwartz *et al.,* 1975).

From the present study, it was concluded that MST cuticle blue stain has a high degree of reliability with the cuticle. It was also confirmed that more eggshell pigment (Protoporphyrin IX) in brown eggs is in the calcareous components of the eggshell than in the cuticle.

Chapter 8

Whole Egg and Agar Egg Penetration Studies-*Salmonella* Infantis

8.1 Introduction

The table egg is an important component of human diets all around the world. However, eggs contaminated with bacteria such as *Salmonella* can cause diseases of public health concern. An egg can be contaminated during its formation in the reproductive tract of a *Salmonella* spp. infected hen (vertical transmission). It can also be contaminated horizontally (after the shell has formed either during oviposition or following oviposition) depending upon the nature of the pathogen and architecture of the eggshell. Not all *Salmonella* serovars are transmitted vertically. *Salmonella* Enteritidis is the only serovar that primarily follows the vertical transmission route (Barrow and Lovell, 1991; Humphrey, 1994, Keller *et al.,* 1995) whereas *Salmonella* Infantis is transmitted horizontally (Okamura *et al.,* 2001). Out of the 6 main serovars, *Salmonella* Enteritidis and Typhimurium are the only serovars that colonize the reproductive tract of the hen (Okamura *et al.,* 2001). Eggshell characteristics like shell thickness, amount of cuticle, thickness of individual layers (mammillary and palidsade), pore distribution and mammillary ultrastructural variables have been shown to influence eggshell quality and microbial penetration (Berrang *et al.,* 1998; Miyamoto *et al.,* 1998; Nascimento *et al.,* 1992; Sauter and Petersen, 1974; Schoeni *et al.,* 1995; Solomon, 1992). Egg translucency has also been shown to be positively correlated with microbial penetration (Chousalkar *et al.,* 2010; Solomon, 1986). An understanding of the influence of eggshell quality parameters on bacterial penetration can be useful for planning effective strategies to prevent the introduction of bacteria particularly *Salmonella* spp. into eggs by means of eggshell penetration (Messens *et al.,* 2005). In Australia, the prevalence of *Salmonella* Typhimurium egg farms is very high (Tribe *et al.,* 2002). *Salmonella* Enteritidis has not been reported in the Australian egg industry as yet but *Salmonella* Infantis associated with the egg and chicken meat industry is of significant public health concern (Cox *et al.,* 2002). A high prevalence of *Salmonella* Infantis was observed in layer flocks and in raw egg pulp in Australia but the overall frequency of isolation was low compared to the other prevalent serovars (Cox *et al.,* 2002). *Salmonella* Infantis has also been isolated more frequently from pigs than any other food animal in Australia (Cox *et al.,* 2002).

8.2 Materials and methods

Fresh eggs were collected from a cage system at 42 and 44 wk of flock age. Eggs were prepared and processed for whole egg penetration assay and for agar moulding technique as outlined in Tables 8.2.1.1 & 8.2.1.2.

8.2.1 Whole egg penetration assay

Salmonella Infantis previously isolated from the eggshell wash was used for the agar and whole eggshell penetration studies. In order to make serial dilutions and select desired colony forming units (cfu), *Salmonella* Infantis was streaked onto MacConkey agar plate and incubated overnight at 37°C. Standard dilutions were prepared by dissolving the grown colonies in 20 mL phosphate buffered saline (PBS) and matching its turbidity with 0.5 McFarland standards (bioMerieux France). Enumeration of viable bacteria was performed by serial dilution and plating 100 µL each solution on MacConkeys agar plates and incubated overnight at 37°C.

Table 8.2.1.1 Whole egg penetration sample preparation and treatment in *Salmonella* Infantis dilutions

	Group I			**Group II**			**Group III**		
	Washed eggs (n=30)			Unwashed eggs (n=30)			Unwashed eggs (n=30)		
70% Ethanol treatment	30 Sec			30 Sec			30 Sec		
S. Infantis (Aliquot) treatment for 90 Sec	Control (n=10) in PBS	10^3cfu (n=10)	10^5cfu (n=10)	Control (n=10) in PBS	10^3cfu (n=10)	10^5cfu (n=10)	Control (n=10) in PBS	10^3cfu (n=10)	10^5cfu (n=10)
Incubation	**20°C for 21 days**			**20°C for 21 days**			**37°C for 21 days**		

PBS- Phosphate buffered saline; cfu- colony forming units

In order to recover *Salmonella* Infantis, from infected eggshell surface and internal contents following 21 days of incubation, the pooled egg samples (n=2) were washed in a sterile whirl pak bag (Nasco, USA) containing 20 mL of PBS. The eggs were gently rubbed for 90 seconds in order to detach surviving bacteria (if any) and a 100 µL from the aliquot was plated on Xylose Lysine

Deoxycholate (XLD), the plates were incubated overnight at 37°C and checked for colony growth of *Salmonella* Infantis. Media was prepared as described in the appendix.

For processing internal contents of the incubated eggs, eggs were dipped in 70% ethanol for 30 seconds. Pooled samples (n=2) were emptied into the whirl pack bags by opening the egg on the edge of sterile container. The contents were mixed thoroughly and 2 mL was added into 8 mL buffered peptone water (BPW) and the enrichment media was incubated overnight at 37°C. After incubation, 100 µL from the enriched BPW was plated on XLD plates and incubated overnight at 37°C. Plates were examined for colony growth of *Salmonella* Infantis.

8.2.2 Agar egg moulding technique

The agar moulding technique developed the by Board and Board (1967) and used by Berrang *et al.* (1998); Chousalkar *et al.*, (2010); Messens *et al.* (2005a) and De Reu *et al.*, (2006) was adapted for studying microbial penetration across the eggshell. Freshly collected eggs (44wk flock age) were divided into 2 groups based on the translucency score as explained below in Table 8.2.2.1.

Eggs were emptied by making a small hole at the blunt end using an 18 G Needle (BD, Australia). The egg contents were sucked out after making a hole and using a sterile syringe. Eggs were then washed with sterile phosphate buffered saline (PBS; pH 7.2) to remove all the albumen adhering to the membrane. Each egg was filled with 35 to 37 mL MacConkeys agar. After hardening of the agar, the eggs were sealed with cellophane tape as shown in Plate 8.2.2.2.

A *Salmonella* Infantis aliquot was prepared as described in section 8.2.1. A bacterial dose of 10^5cfu was selected for the agar egg penetration method.

Table 8.2.2.1 Egg samples preparation for the XLD agar moulding method of infection

		Group I		**Group II**	
		Washed eggs (n=32)		Unwashed eggs (n=32)	
70% Ethanol treatment		30 Sec		30 Sec	
Filling all eggshells with XLD agar		35mL/egg		35mL/egg	
Agar filled egg treatment with S. Infantis aliquot for 90 Sec		Control (n=12) In PBS	10^5cfuconcentration (n=20)	Control (n=12) In PBS	10^5cfu concentration (n=20)
Translucency Score	**0**	3 eggs	5 eggs	3 eggs	5 eggs
	1	3 eggs	5 eggs	3 eggs	5 eggs
	2	3 eggs	5 eggs	3 eggs	5 eggs
	3	3 eggs	5 eggs	3 eggs	5 eggs
Incubation		**20°C for 21 days**		**20°C for 21 days**	

PBS- Phosphate buffered saline; XLD- xylose lysine deoxycholate; cfu- colony forming units

Agar filled eggs were dipped in 70% ethanol. After drying, all the control group eggs were dipped into PBS for 90 seconds and eggs from the treatment group were immersed for 90 seconds in *Salmonella* Infantis with an approximate dilution of 10^5 CFU per ml. After incubation (19 days), the eggs were broken aseptically at the edge of a petri dish and penetration for *Salmonella* Infantis was recorded as “Yes” or “No” by observing the growth of *Salmonella* Infantis on the selective agar (XLD). Penetrated areas were marked and correlated with egg translucency and the amount of cuticle present in both groups of eggs. A score of “1” was assigned to non penetrated and “2” to penetrated eggs and the values were analyzed using two way ANOVA.

Plate 8.2.2.1 Eggshell preparation for agar media filling

Plate 8.2.2.2 Agar filled egg properly sealed with cellotape

8.2.3 Correlation of shell features with *Salmonella* Infantis penetration

Penetrated areas were marked and correlated with egg translucency, the amount of cuticle present, shell thickness and mammillary layer ultrastructural scoring in both groups of eggs. For translucent spots match with bacterial penetration, all the penetrated points were marked and counted in a single ½ shell from which the penetration points through translucent spots percentage was calculated. For cuticle estimation eggshells were stained as mentioned in chapter 2, section 2.2 cuticle staining. Stained pieces were cut off, mounted on aluminium stubs and viewed under SEM as explained in chapter 2, section 2.2. Shell thickness was measured in the same way as described in chapter 2 section 2.1. Penetrated areas were marked and prepared for the ultra structure scoring of the mammillary layer of the shell similarly as explained in chapter 2, section 2.3.

8.3 Results

8.3.1 Whole egg penetration assay

Of all 40 unwashed infected eggs (20 pooled samples) incubated at 20°C and 37°C, *Salmonella* was isolated from only the shell surface of 1 sample incubated at 37°C. The total number of colonies on

the plate was 95 cfu. *Salmonella* was not isolated from the 20 washed eggs (10 pooled samples). All 30 eggs from control group were negative for *Salmonella* Infantis. *Salmonella* was not isolated from egg internal contents from washed or unwashed eggs.

8.3.2 Examination of Agar filled eggs for *Salmonella* Infantis penetration

As shown in Table 8.3.2.1, there was a significant effect ($P<0.05$) of washing and all the washed eggs were positive for *Salmonella* Infantis penetration irrespective of translucency score. From the unwashed eggs, 3 eggs each from translucency scores 2 and 1 showed no *Salmonella* Infantis penetration. All the control group eggs were negative.

Table 8.3.2.1 Agar eggs penetrated with *Salmonella* Infantis (10^5 cfu)

Category	Mean value	Translucency score P value	Category P value
Washed	2	0.0159	0.0015
Unwashed	1.7		

8.3.3 Correlation of shell features with *Salmonella* Infantis penetration

Cuticle cover tended to be more complete in unwashed compared to washed eggs although this was not statistically significant ($P>0.05$). There was no significant effect of shell thickness on the incidence of bacterial penetration. Shell thickness tended to be lower in unwashed shells with translucency scores of 0 and 3 compared to translucency scores 1 and 2 as shown in Table 8.3.3.1.

Table 8.3.3.1 Eggshell characteristics and *Salmonella* Infantis penetration

Treatment	Translucency score	Mean Cuticle cover score	Mean shell thickness	% eggs positive
Washed eggs	0	4	401.66	100
	1	3.8	409.46	100
	2	3.8	414.46	100
	3	4	402.53	100
Unwashed eggs	0	3.6	344.60	100
	1	3.2	410.86	40
	2	3	412.53	40
	3	3.4	365.46	100

A positive correlation was found between the presence of translucent areas in the eggshell and penetration points of bacteria into the egg. Most of the penetration points were through the translucent spots and were greater in eggs with a higher incidence of translucency for both washed and unwashed eggs. The exception to this trend was the unwashed eggs with “0” translucency score.

Table 8.3.3.2 Correlation of translucent eggshell regions with microbial penetration

Washed eggs		Unwashed eggs	
Translucency score	**% penetrated translucent shell regions**	**Translucency score**	**% penetrated translucent shell regions**
3	95.5	3	68.8
2	84.2	2	19.4
1	75.8	1	13.8
0	43.3	0	70.9

There was no significant difference ($P>0.05$) between the washed and unwashed groups of eggs for any of the mammillary layer ultrastructural variables except depression. A slightly higher incidence of depression was recorded in washed groups. SEM of the penetrated points in both groups (washed and unwashed) showed a higher incidence of alignment, late fusion, cubic, open pores, Type B bodies, Type A bodies, depression and erosion of the mammillary caps. The non penetrated areas in both groups (washed and unwashed eggs) showed good cap quality with higher incidence of confluence as shown in Plate 8.3.3.4.

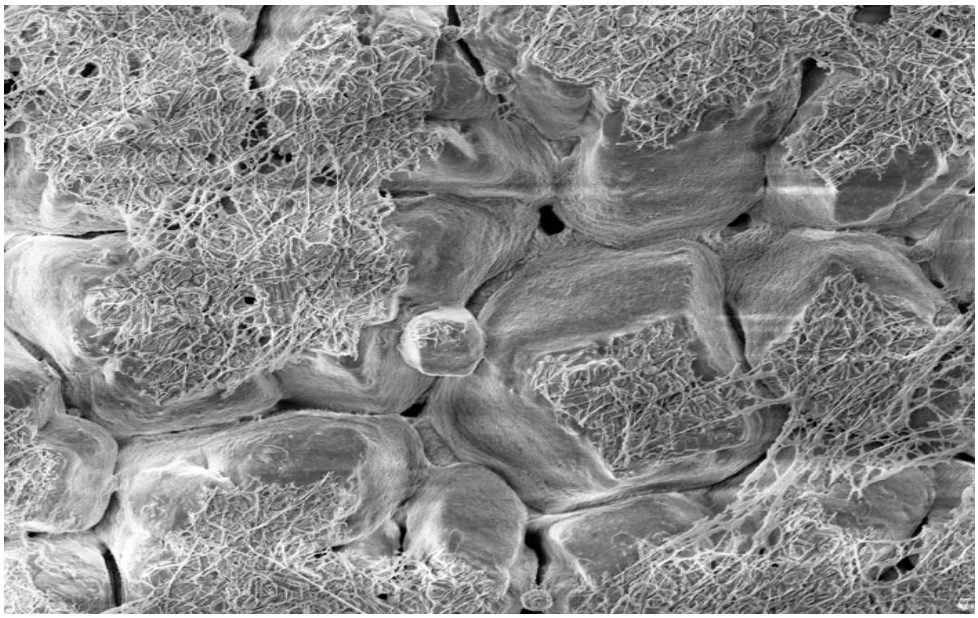

Plate 8.3.3.1 SEM of the mammillary layer of penetrated area showing exposed pores, and B body
Magnification=X240; Scale bar 100µm

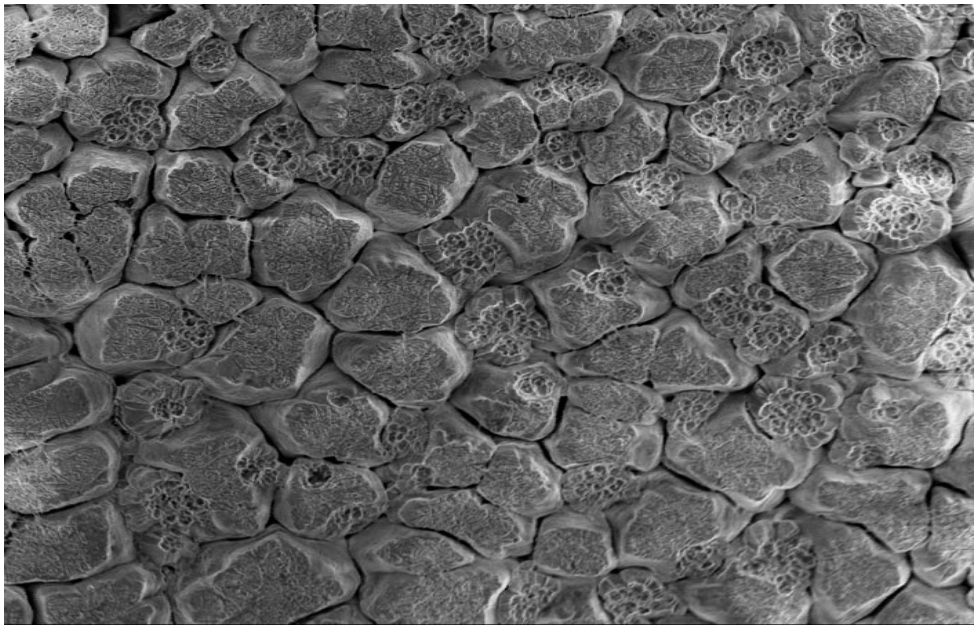

Plate 8.3.3.2 SEM of the mammillary layer of penetrated area showing eroded caps
Magnification=X100; Scale bar 200µm

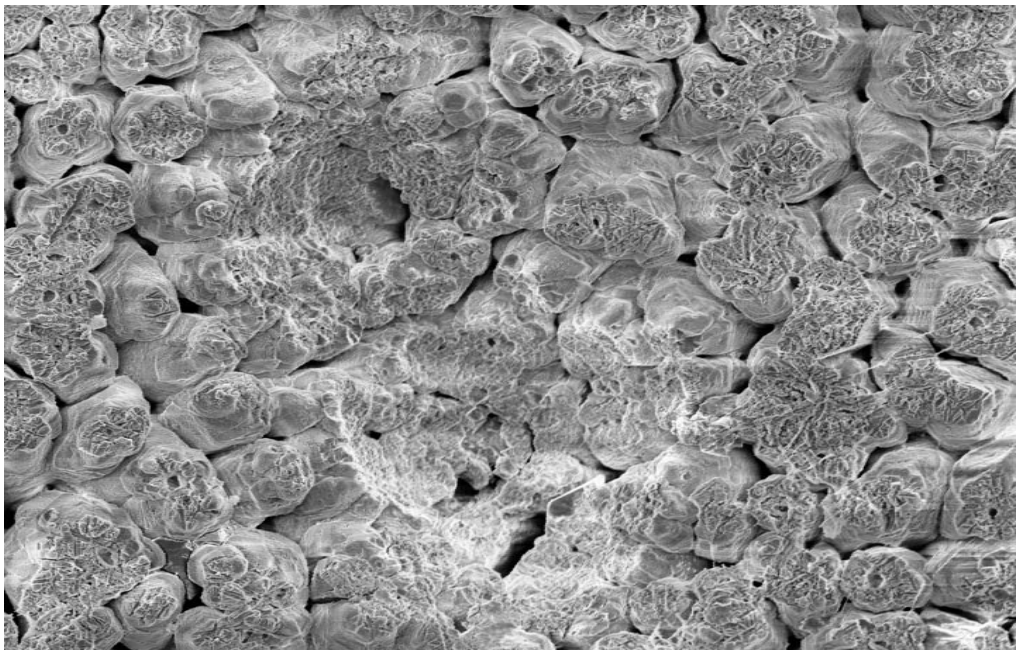

Plate 8.3.3.3 SEM of the mammillary layer of penetrated area showing depression
Magnification=X200; Scale bar 100µm

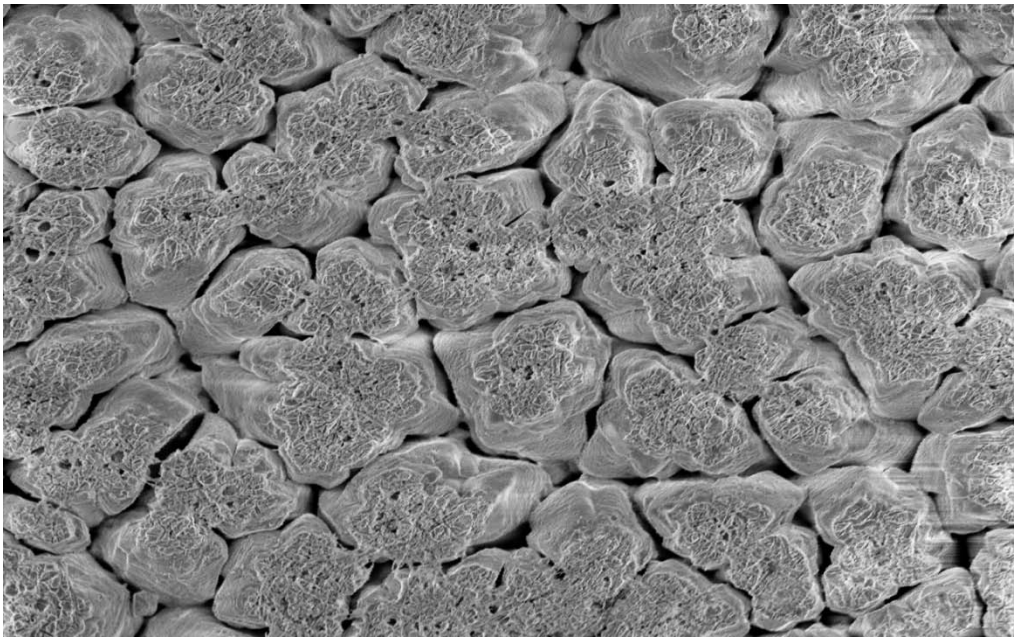

Plate 8.3.3.4 SEM of the mammillary layer of non penetrated area showing good cap quality
Magnification=X170; Scale bar 100µm

8.4 Discussion

In the present study, *Salmonella* Infantis was isolated from only one pooled shell wash sample. It can be assumed that *Salmonella* Infantis did not survive on the egg shell surface. None of egg internal contents were positive for *Salmonella* which could be attributed to the antimicrobial properties of albumen. It appeared that the *Salmonella* Infantis used in this study was able to penetrate across the eggshell but could not survive in the albumen. A number of factors such as the pH of albumen, albumen viscosity and antimicrobial enzymes and proteins inhibit bacterial proliferation in egg contents (Mayes and Takeballi, 1983). Trans-shell penetration was confirmed using agar moulding technique in which all the washed eggs were penetrated while penetration rate in the unwashed group was 70% as shown in Table 8.3.2.1. Earlier studies have shown that *Salmonella* serovars other than *Salmonella* Infantis penetrate across the eggshell (Sauter and Peterson, 1974; Neill *et al.,* 1985; Williams *et al.,* 1968). Murase *et al.* (2006) suggested that *Salmonella* serovars including *Salmonella* Infantis which enter the albumen are unlikely to show active motility through the albumen towards the yolk. However in the present study, it appeared that *Salmonella* Infantis could not survive in egg albumen after penetration through the shell. Some albumen proteins like ovotransferrin have antimicrobial peptides (OTAP-92) that could kill gram negative bacteria by crossing the outer membrane of bacteria and thus altering the biological function of its cytoplasmic membrane (Ibrahim *et al.,* 2000; Schoeni *et al.,* 1995). In the studies of Hughey and Johnson (1987) *Salmonella* Typhimurium was resistant to the antibacterial properties of albumen lysozyme but how lysozyme acts against *Salmonella* Infantis need to be investigated. In the present whole egg penetration study, washed eggs were only incubated at 20°C as washed eggs are not used in hatchery where incubation temperature is 37°C. The effect of incubation temperature (20 vs 37°C) on microbial penetration across shell can not be clearly explained as *Salmonella* Infantis was not recovered from all the incubated whole infected eggshell surface except 1 sample incubated at 37°C.

Egg translucency had a positive correlation with microbial penetration in the present studies except for the unwashed translucency score “0” eggs. Higher translucency incidence may enhance microbial penetration across the eggshell and Chousalkar *et al.* (2010) positively correlated higher incidence of translucency with microbial penetration. The 100% penetration is eggs from unwashed group with translucency score of “0” might have been due to a translucency score which increased significantly following egg collection as the eggs were scored as soon as they were collected. Although

translucency was not measured after 21 days of post inoculation/incubation period in this experiment, hence further investigation is required to provide this hypothesis. It can also be assumed that lower shell thickness was a contributing factor to the 100% penetration in this group. Washing greatly reduced the total bacterial load on the eggshell surface, while humidity increased the chances of bacterial replication or survival on shell surface after washing (Musgrove *et al.,* 2005). A 100% penetration in the washed eggs might explain the importance of cuticle in microbial prevention as the washed eggs had less cuticle compared to unwashed eggs as shown in Table 8.3.3.1. The hydrophobic proteinaceous outermost layer (cuticle) of the eggshell hinders bacterial penetration (Haigh and Betts, 1991). However the function of the cuticle as a microbial defense tool has been questioned (Nascimento *et al.,* 1992). In the egg penetration studies of Messens *et al.* (2007), a high correlation was found between absence of cuticle and *Salmonella* penetration, but the amount of cuticle did not affect bacterial penetration in the studies of Messens *et al.* (2005). In the present study, mean cuticle deposition was lower in the penetrated areas while shell thickness was non significantly lower for the unwashed group translucency score “0” and “3”. Cuticle removal increased bacterial penetration from 20 to 60% in the studies of Alls *et al.* (1964). In the studies of De Reu *et al.* (2006f, 2010) and Messens *et al.* (2005), eggshell characteristics such as shell pores, shell thickness, area of the shell did not affect bacterial penetration of whole eggs and agar eggs while the mean cuticle deposition was higher for non penetrated eggs versus penetrated eggs (De Reu *et al.,* 2006f). Similarly, in the egg penetration studies of Williams *et al.* (1968), Messens *et al.* (2005) and Smeltzer *et al.* (1979), bacterial penetration was independent of shell thickness. However, in the studies of Orel (1959) and Sauter and Peterson (1974) a significant effect of shell thickness on bacterial penetration was recorded. In the present studies, scanning electron microscopy (SEM) of the penetrated areas of outer shell surface showed open pores unplugged by the cuticle. Open pores with larger diameters than the bacterial size is the primary route of bacterial entrance to intact eggs (Tyler, 1956; Board and Halls, 1973). Nascimento *et al.* (1992) and Nascimento and Solomon (1991) reported bacterial penetration to be independent of pore numbers. Poles were highly penetrated areas in the studies of Haigh and Betts (1991) but, in the present study bacterial penetration was observed equally in all regions of the shell, including the equator of agar filled shells. The blunt pole was not included in the current study as eggs were only filled with 35 to 37 mL of agar and the blunt pole was not exposed to the agar. In the present study, the higher incidence of ultrastructural variables of the mammillary layer such as late fusion, Type B bodies, Type A bodies, poor cap quality, alignment, depression,

erosion and cubic appeared to enhance bacterial penetration. Similarly, Solomon (1992) positively correlated all the ultrastructural variables that negatively affect mammillary layer quality with bacterial penetration. However more research is needed to correlate the shell mammillary layer ultrastructural features with microbial penetration as, on the basis of a higher incidence of a single variable, a true baseline cannot be established. The eroded mammillary caps observed at penetration sites (see Plate 8.3.3.2) are unusual. They may have been present in the shell and facilitates the entry of bacteria or the erosion may have resulted from the activity of the bacteria after they penetrated the shell.

In the studies of Schoeni *et al.* (1995), *Salmonella* other than Infantis either remained stable or steadily increased in albumen. It is likely that the motile and non-clustering properties of most of *Salmonella enterica* serovars enhance its penetration across the eggshell more frequently compared to other bacteria (De Reu *et al.,* 2006).

It can be concluded from the current experiments that *Salmonella* Infantis penetrated across the eggshells but cannot survive inside the egg due to the antimicrobial properties of albumen. Extensive research is needed to investigate the effect of the agar method on the properties of shell membranes as shell membrane plays an important role in prevention of microbial entrance into egg.

Chapter 9

General Discussion and Conclusions

9.1 Traditional eggshell and egg internal quality

9.1.1 Eggshell quality

The current study formed part of a larger project which is investigating the relationship between egg quality and the food safety of table eggs. The main objective of the current study was to evaluate the effect of age and production system on egg quality parameters by following individual flocks throughout their production cycle. Eggshell quality and egg internal quality parameters were significantly affected by flock age and production system as described in Chapters 3, 4, 5 and 6. Translucency score was affected by flock age but was also affected by the time that had elapsed between egg collection and translucency scoring of eggs. The higher translucency score in the free range eggs, as compared with the cage eggs would be due, in part, to the fact that these eggs were scored 3-4 days post collection whereas the cage eggs were scored on the day of collection. Generally, shell reflectivity (%) increased with increasing flock age, indicating that shell colour became lighter, as has been described by previous studies. It can be concluded from chapters 3, 4, 5 and 6 that shell reflectivity was significantly higher in free range eggs, and increased with increasing flock age. In the horizontal study, the effect of flock age was more similar for shell reflectivity with shell colour being lighter in the older flocks. Egg weight (g) increased with increasing flock age in both production systems with a higher egg weight in cage versus free range production. Similarly, an increase in egg weight with flocks age was also recorded in the horizontal study. From chapters 3, 4, 5 and 6 it can be concluded that both age and production system both had a significant effect on egg weight. Shell breaking strength (BSN) and shell deformation (μm) were more affected by flock age than by production system. From chapters 3, 4, 5 and 6 it can be concluded that BSN and shell deformation decreased with flock age and there was a possible positive effect of hen molting on eggshell strength in the caged flock. Shell weight (g) increased with increasing egg weight and was significantly higher in the cage system as compared with free range, in line with the higher egg weight in the cage eggs.. It can be concluded from chapters 3, 4, 5 and 6 that shell weight (g) increased with flock age, egg weight and was influenced by production system. Percentage shell

tended to be higher (P=0.0083) in cage eggs versus free range eggs. In the present study, a negative correlation of percentage shell to egg weight in both production systems indicates that the increase in shell weight that occurs as birds age is not necessarily proportional to the increase in egg weight such that percentage shell may decrease. From chapters 3, 4, 5 and 6 it can also be concluded that percentage shell decreases with flock age. From chapters 3 and 4 it can be concluded that cage eggshells are thicker than the free range eggs and that shell thickness increased with hen age. Shell thickness (μm) from the horizontal study produced different results, with shell thickness being higher for younger (22 week) and older (79 week) flocks compared to the 39 and 55 week flocks.

9.1.2 Egg internal quality

A relatively higher albumen height (mm) and Haugh unit in the cage eggs compared to free range was due, at least in part, to the longer time interval between oviposition and analysis for the free range eggs. From chapters 3, 4, 5 and 6 it can be concluded that albumen height and Haugh unit decreased with hen age and thus older hen eggs are inferior in egg internal quality to younger flock eggs. Yolk colour varied with hen age and production system. In commercial production systems, whether they are cage or free range, the colour of the egg yolks is directly related to the amount of pigment included in the diets.

9.2 Estimation of the amount of cuticle

As outlined in Chapters 3, 4, 5 and 6, the amount of cuticle present on the eggs varied significantly with hen age and production system with a higher amount of cuticle in cage eggs. Increased values of SCI L* with flock age indicated that the eggs were lighter in colour at older flock ages and free range eggs were lighter in colour than cage eggs. The horizontal study found a similar effect of lighter coloured shells with increasing flock age. From the combined results of SCI a* and scanning electron microscopy (SEM) of cuticle, it can be concluded that MST cuticle stain had a strong affinity with the cuticle and that the amount of cuticle was higher in the mid lay period in both production systems. It can be also concluded that cage eggs had a significantly higher amount of cuticle as compared with free range.. Similarly in the horizontal study, the highest amount of cuticle was found in eggs from the 55 wk flock.

9.3 Ultrastructural scoring of the shell mammillary layer

The higher incidence of mammillary layer variables that negatively affect shell quality, in free range eggs, suggests that free range eggs were inferior to cage eggs in terms of shell ultrastructure. In the present longitudinal and horizontal studies, the overall incidence of negative ultrastructural variables was low irrespective of the production system and changed significantly with flock age. The decrease in the incidence of positive shell ultrastructural variables (confluence, early fusion, mammillary cap size) with increasing flock age indicated that shell ultrastructural quality decreased as the flock advanced in age.

9.4 Egg microbiology

A significantly lower total bacterial count (TBC) for cage eggs supports the previous findings that reported lower levels of bacterial contamination for cage eggs (De Reu *et al.,* 2005b; Harry, 1963; Quarles *et al.,* 1970). The total bacterial load in shell crush, total Enterobacteriaceae load (TEC) on the shell and in the shell crush support the view that Australian table eggs are relatively safe as a food commodity. Egg internal contents negative for *Salmonella* and negative swabs sampling from egg belts and manure further indicate good hygiene and proper quarantine procedures on the farms studied in the present study.

9.5 Protoporphyrin IX extraction

Chapter 7 reports the quantification of Protoporphyrin IX (PP IX) from the cuticle and true eggshell (without cuticle). Some previous research claimed that there was more pigment in the cuticle rather than the true eggshell of brown eggs (Miksik *et al.,* 2007). However, the study of Nys *et al.* (1991) suggested that more protoporphyrin was found in the calcareous components of the egg shell. Spectrophotometric quantification showed that the amount of pigment (PP IX) is greater in the true eggshell (without cuticle) rather than in the cuticle. It was also concluded that the amount of protoporphyrin in the eggshell was greater in the shells from hens in the mid lay period, compared to early and late lay periods.

9.6 Egg penetration studies

Chapter 8 reports the effect of washing and egg translucency on *Salmonella* Infantis penetration across the eggshell. The whole egg penetration studies indicated that *Salmonella* Infantis penetration occurs, but that the organisms could not survive in the albumen due to its microbial properties. Among all the washed and unwashed eggs in the whole egg penetration studies, the recovery of *Salmonella* from the eggshell of only 1 pooled (2 eggs) sample suggests that, during incubation, most of the bacteria on the shell surface either penetrated across the shell or died on the shell surface. Washing had a significant effect on microbial penetration as all washed eggs were penetrated (100%) compared to the incidence of penetration of unwashed eggs (70%). Washing might have removed cuticle as the SEM of penetrated eggshells showed a lower amount of cuticle in the washed group. Translucency had a positive correlation with bacterial penetration except for the unwashed group translucency "0" group. The 100% penetration of eggs with translucency score "0" cannot be explained at this stage and further studies are needed in this field. It is possible that some of the eggs in this group might have increased in translucency score with time, as translucency develops after storage time. A positive correlation of translucency with bacterial penetration was also found when the number of translucent spots and penetrated points were matched and correlated. From the current penetration study, it can be concluded that there is a significant effect of egg washing and egg translucency on *Salmonella* Infantis penetration.

The studies presented in this thesis indicate that changes occur in egg shell quality and egg internal quality as flocks increase in age. The egg quality features of completeness of cuticle cover and the incidence of shell translucency appear to influence the ease with which bacteria can penetrate through the egg shell and enter the egg contents. However, further studies are necessary to clearly elaborate these correlations. In general, the eggs produced in the Australian egg industry are relatively free from bacterial contamination. High standards of management and biosecurity and regular monitoring will ensure that Australian table eggs remain a safe food product for the consumer.

References

Aabo, S., Christensen, J.P., Chadfield, M.S., Carstensen, B., Olsen, C., and Bisgaard, M. (2002). Quantitative comparison of intestinal invasion of zoonotic serotypes of *Salmonella enterica* in poultry. *Avian Pathology,* 31:41–47.

Abrahamsson, P., and Tauson, R. (1998). Performance and egg quality of laying hens in an aviary system. *Journal of Applied Poultry Research,* 7:225-232.

AECL annual report 2010/2011. Retrieved from http://www.aecl.org/system/attachments/484/original/Annual%20Report%202010.2011.pdf?1321843838

AECL annual report 2010. Retrieved from http://www.aecl.org/system/attachments/347/original/Annual_Report_2010.pdf?1289462015

Ahmed, A.M.H., Rodriguez-Navarro, A.B., Vidal, M.L., Gautron, J., Garcia-Ruiz, J.M., and Nys, Y. (2005). Changes in eggshell mechanical properties, crystallographic texture and in matrix proteins induced by moult in hens. *British Poultry Science,* 46(3), 268–279.

Aitken, R.N.C., and Johnston, H.S. (1963). Observations on the fine structure of the infundibulum of the avian oviduct. *Journal of Anatomy,* 97:87-99.

Alakomi, H.L., and Saarela, M. (2009). *Salmonella* importance and current status of detection and surveillance methods. *Quality Assurance and Safety of Crops and Foods*. Retrieved online from: http://onlinelibrary.wiley.com/doi/10.1111/j.1757-837X.2009.00032.x/pdf

Alls, A.A., Cover, M.S., Benton, W.J., Krauss, W.C. (1964). Treatment of hatching eggs for disease prevention - factors affecting permeability and a visual inspection of drug absorption. *Avian Diseases,* 8:245-246.

Al-Natour, M.Q., Alaboudi, A.R., Al-Hatamelh, N.A., and Osaili, T.M. (2011). *Escherichia coli* O157:H7 facilitates the penetration of *Staphylococcus aureus* into table eggs. *Journal of Food Science,* 77(1), M29-M34.

Anonymous (2012). *Salmonella* Typhimurium outbreak linked to chickens. Government of Western Australia, Department of Health. Online retrieved from: http://www.health.wa.gov.au/diseasewatch/vol16_issue2/*Salmonella*_typhimurium_outbreak_linked_to_chickens.cfm

Arias, J.L., and Fernandez, M.S. (2003). Biomimetic process through the study of mineralized shells. *Material Characterization,* 50:189-195.

Arias, J.L., Fernandez, M.S., Dennis, J.E., and Caplan, A.I. (1991). The fabrication and collagenous substructure of the eggshell membrane in the isthmus of the hen oviduct. *Matrix,* 11(5):313-320.

Arpasova, H., Halaj, M., and Halaj, P. (2010). Eggshell quality and calcium utilization in feed of hens in repeated laying cycles. *Czech Journal of Animal Science,* 55(2):66–74.

Asmundson, V.S., Baker, G.A., and Emlen, J.T. (1943). Certain relation between the parts of bird's eggs. *The Auk,* 60(1):34-44.

Australian Bureau of Statistics Cat. No. 7503.0 - Value of Agricultural Commodities Produced, Australia, 2010-11. Retrieved from: http://www.abs.gov.au/ausstats/abs@.nsf/latestProducts/7503.0Media%20Release12010-11

Bain, M.M. (2004). Recent advances in the assessment of eggshell quality and their future application. *XXII World's Poultry Congress,* Istanbul Turkey, June, 8-13.

Bain, M.M. (1992). Eggshell strength: a relationship between the mechanism of failure and the ultrastructural organization of the mammillary layer. *British Poultry Science,* 33:303-319.

Baird, T., Solomon, S.E., and Tedstone, D.R. (1975). Localization and characterization of eggshell porphyrins in several avian species. *British Poultry Science,* 16:201-208.

Baker, J.R., and Balch, D.A. (1962). A study of the organic material of hen's eggshell. *Biochemistry Journal,* 82:352-361.

Banga-Mboko, H., Mabas, J.S., and Adzona, P.P. (2010). Effect of housing system (battery cages versus floor pen) on performance of laying hens under tropical conditions in Congo Brazaaaville. *Research Journal of Poultry Sciences,* 3(1):1-4.

Barrow, P.A., Bumstead, N., Marston, K., Lovell, M.A., and Wigley, P. (2004). Faecal shedding and intestinal colonization of *Salmonella enterica* in in-bred chickens: the effect of host-genetic background. *Epidemiology and Infection,* 132(1):117-126.

Barrow, P.A. (1994). The microflora of the alimentary tract and avian pathogens: translocation and vertical transmission. In R. G. Board and R. Fuller (Eds.). *Microbiology of the Avian Egg* (pp.117-138). London, U.K.: Chapman and Hall.

Barrow, P.A. and Lovell, M. A. (1991). Experimental infection of egg-laying hens with *Salmonella* Enteritidis phage type 4. *Avian Pathology,* 20:335-348.

Barua, A., and Yoshimura, Y. (2004). Ovarian cell-mediated immune response to *Salmonella* Enteritidis in laying hens (*Gallus domesticus*). *Poultry Science,* 83:997–1002.

Bejaei, M., Wiseman, K., and Cheng, K.M. (2011). Influences of demographic characteristics, attitudes, and preferences of consumers on table egg consumption in British Columbia, Canada. *Poultry Science,* 90:1088–1095.

Bellairs, R., Harkness, M., and Harkness, R.D. (1963). The vitelline membrane of the hen egg: a chemical and electron microscopical study. *Journal of Ultrastructure Research,* 8:339-359.

Bellairs, R. (1961). The structure of the yolk of the hen egg as studied by electron microscopy. I. The yolk of the unincubated egg. *Journal of Biophysical and Biochemical Cytology,* 11:207-225.

Belyavin, C.G., and Boorman, K.N. (1980). The influence of the cuticle on eggshell strength. *British Poultry Science,* 21:295-298.

Berndt, A., Wilhelm, A., Jugert, C., Pieper, J., Sachse, K., and Methner, U. (2007). Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness. *Infection and Immunity,* 75(12):5993-6007.

Bennett, T., and Malmfors, T. (1970). The adrenergic nervous system of the domestic fowl. *Zeitscrift fur Zellforschung und Mikroskopische Anatomie,* 106(1):22-50.

Berrang, M.E., Cox, N.A., Frank, J.F., and Buhr, R.J. (1999). Bacterial penetration of the eggshell and shell membranes of the chicken hatching egg: A Review. *Journal of Applied Poultry Research,* 8:499-504.

Berrang, M.E., Frank, J.F., Buhr, R.J., Bailey, J.S., Cox, N.A., and Mauldin, J. (1998). Eggshell characteristics and penetration by *Salmonella* through the productive life of a broiler breeder flock. *Poultry Science,* 77:1446–1450.

Berrang, M.E., Cox, N.A., Bailey, J.S., and Blankenship, L.C. (1991). Methods for inoculation and recovery of *Salmonella* from chicken eggs. *Poultry Science,* 70, 2267–2270.

Board, R.G., and Halls, N.A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *British Poultry Science,* 14:69-97.

Board, P.A., and Board, R.G. (1967). A method of studying bacterial penetration of the shell of the hen's egg. *Laboratory Practice,* 16(4):471-472.

Bolton, D.J., O'Neill, C.J., and Fanning, S. (2011). A preliminary study of *Salmonella,* Verocytotoxigenic *Escherichia coli*/*Escherichia coli* O157 and *Campylobacter* on four mixed farms. *Zoonoses and Public Health,* 59(3):1-11. Doi: 10. 1111/j. 1863-2378.2011.01438.x

Botteldoorn, N., Coillie, E.V., Goris, J., Werbrouck, H., Piessens, V., Godard, C., and Scheldeman, P. (2010). Limited genetic diversity and gene expression differences between egg- and non-egg-related *Salmonella* Enteritidis strains. *Zoonoses and Public Health,* 57:345-357.

Brackpool, C. E. (1995). Eggshell ultrastructure as an indicator of eggshell quality in laying hens. PhD Thesis. The University of New England Armidale Australia.

Brown, W.E., Baker, R.C., and Naylor, H.B. (1965). Comparative susceptibility of chicken, duck and turkey eggs to microbial invasion. Retrieved from onlinelibrary.wiley.com/doi/10.1111/j.1365-2621.1965...x/pdf

Bruce, J., and Drysdale, E.M. (1994). Trans-shell transmission. In R.G. Board and R. Fullar (Eds.) *Microbiology of the Avian Egg* (pp. 63-91. London, U.K.: Chapman and Hall.

Bruce, J., and Johnson, A.L. (1978). The bacterial flora of unhatched eggs. *British Poultry Science,* 19:681-689.

Burke, W.H., Ogasawara, F.X., and Fuqua, C.L. (1972). A study of the ultrastructure of the uterovaginal sperm-storage glands of the hen, *Gallus domesticus* in relation to a mechanism for the release of spermatozoa. *The Journal of the Society for Reproduction and Fertility,* 29:29-36.

Burley, R.W., and Vadehra, D.V. (1989). The Avian Egg. Chemistry and Biology. *Wiley Interscience.* New York.

Butcher, G.D., and Miles, R. (1995). Factors causing poor pigmentation of brown shelled eggs. *Cooperative Extension Service Fact Sheet* VM94. Institute of Food and Agricultural Sciences, University of Florida, Gainesville, 32611. Retrieved from: http://edis.ifas.ufl.edu/vm047

Butcher, G.D., and Miles, R. (1994). *Salmonella* control and molting of egg laying flocks- Are they compatible? University of Florida Cooperative Extension Service, Gainesville, 1-3. Retrieved from: http://edis.ifas.ufl.edu/vm017

Campo, J.L., Gil, M.G., and Davila, S.G. (2007). Differences among white, tinted and brown egg laying hens for incidence of eggs laid on the floor and for oviposition time. *Archiv fur Geflugelkunde,* 71(3):105-109.

Caudill, A.B., Curtis, P.A., Anderson, K.E., Kerth, L.K., Oyarazabal, O., Jones, D.R., and Musgrove, M.T. (2010). The effects of commercial cool water washing of shell eggs on Haugh unit, vitelline membrane strength, aerobic microorganisms and fungi. *Poultry Science,* 89:160-168.

Charlton, B.R., Tiwary, A.K., Bickford, A.A., and Filigenzi, M. (2005). Acute depigmentation of fertile brown eggs in a commercial layer operation. *Journal of Veterinary Diagnostic Investigation,* 17:286-288.

Chavez, C., Knape, K.D., Coufal, C.D., and Carey, J.B. (2002). Reduction of eggshell aerobic plate count by ultraviolet irradiation. *Poultry Science*, 81:1132-1135.

Chousalkar, K.K., and Roberts, J.R. (2012). Recovery of *Salmonella* from eggshell wash, eggshell crush, and egg internal contents of unwashed commercial shell eggs in Australia. *Poultry Science,* 91:1739-1741.

Chousalkar, K.K., Flynn, P., Sutherland, M., Roberts, J.R., and Cheetham, B.F. (2010). Recovery of *Salmonella* and *Escherichia coli* from commercial eggshells and effect of translucency on bacterial penetration in eggs. *International Journal of Food Microbiology*, 142:207-213.

Clavijo, R.I., Loui, C., Andersen, G.L., Riley, L.W., and Lu, S. (2006). Identification of genes associated with survival of *Salmonella enterica* serovar Enteritidis in chicken egg albumen. *Applied and Environmental Microbiology,* 72:1055–1064.

Clay, C.E., and Board, R.G. (1991). Growth of *Salmonella* Enteritidis in artificially contaminated hen's eggs. *Epidemiology and Infection,* 106(2):271-281.

Clerici, F., Casiraghi, E., Hidalgo, A., and Rossi, M. (Online). Evaluation of eggshell quality characteristics in relation to the housing system of laying hens. Retrieved from: http://www.cabi.org/animalscience/Uploads/File/AnimalScience/additionalFiles/WPSAVerona/10732.pdf

Cogan, T.A., Jorgensen, F., Lappin-Scott, H.M., Benson, C.E., Woodward, M.J., and Humphrey, T.J. (2004). Flagella and curli fimbriae are important for growth of *Salmonella enterica* serovars in hen eggs. *Microbiology,* 150:1063-1071.

Cook, M.I., Beissinger, S.R., Toranzos, G.A., Rodriguez, R.A., and Arendt, W.J. (2003). Trans-shell infection by pathogenic microorganisms reduces the shelf life of non-incubated bird eggs: a constraint on the onset of incubation? *Proceedings of Royal Society London,* 270:2233-2240.

Cooke, A.S., and Balch, D.A. (1970). Studies of membranes, mammillary core and cuticle of the hen eggshell. *British Poultry Science,* 11:345-352.

Cotter, P.F., Murphy, J.E., Klinger, J.D., and Taylor, Jr. R.L. (1995). Identification of *Salmonella* Enteritidis from experimentally infected hens using a calorimetric DNA Hybridization method. *Avian Diseases,* 39:873-878.

Coufal, C.D., Chavez, C., Knape, K.D., and Carey, J.B. (2003). Evaluation of a method of ultraviolet light sanitation of broiler hatching eggs. *Poultry Science*, 82:754-759.

Cox, J.M., Woolcock, J.B., and Sartor, A.L. (2002). The significance of *Salmonella*, particularly *Salmonella* Infantis, to the Australian egg industry. Report for the *Rural Industries Research and Development Corporation Australia.*

Cox, N.A., Berrang, M.E., and Cason, J.A. (2000). *Salmonella* penetration of eggshells and proliferation in broiler hatching eggs – a review. *Poultry Science,* 79:1571-1574.

Cudjoe, K.S., Krona, R., Gron, B., and Olsen, E. (1994). Use of ferrous sulphate and innunomagnetic separation to recover *Salmonella* Enteritidis from raw eggs. *International Journal of Food Microbiology,* 23:149-158.

Curtis, P.A. (Online). Changes in eggs over a production cycle. From proceedings of the "Midwest poultry federation convention", *St. Paul, Minnesota,* USA. Retrieved from http://www.zootecnicainternational.com/article-archive/management/50-changes-in-eggs-over-a-production-cycle-.html

Curtis, P.A., Gardner, F.A., and Mellor, D.B. (1985). A comparison of selected quality and compositional characteristics of brown and white shell eggs. II. Interior quality. *Poultry Science,* 64:302-306.

Dahl, E. (1971). Studies of the fine structure of ovarian interstitial tissue 1. A comparative study of the fine structure of the ovarian interstitial tissue in the rate and the domestic fowl. *Journal of Anatomy,* 108 (2):275-290.

Daughtry, B., Sumner, J., Hooper, G., Thomas, C., Grimes, T., Horn, R., Moses, A., and Pointon, A. (2005). National food safety risk profile of eggs and egg products. A report submitted to Australian Egg Corporation Limited, Sydney, Australia.

Dennis, J.E., Xiao, S.Q., Agarwal, M., Fink, D.J., Heuer, A.H., and Caplan, A.I. (1996). Microstructure of matrix and mineral components of eggshell from white leghorn chickens (*Gallus gallus*). *Journal of Morphology,* 228:287-306.

De Reu, K., Messens, W., Grijspeerdt, K., Heyndrickx, M., Uyttendaele, M., and Herman, L. (2010). Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella* Enteritidis. *Proceedings of the Australian Poultry Science Symposium,* 21:126-129.

De Reu, K., Messens, W., Heyndrickx, M., Rodenburg, T.B., Uyttendaele, M., and Herman, L. (2008). Bacterial contamination of table eggs and the influence of housing systems. *World Poultry Science Journal,* 64:5-19.

De Reu, K., Grijspeerdt, K., Heyndrickx, M., Uyttendaele, M., Debevere, J., and Herman, L. (2006c). Bacterial eggshell contamination in the egg collection chains of different housing systems for laying hens. *British Poultry Science,* 47:163-172.

De Reu, K., Grijspeerdt, K., Messens, W., Heyndrickx, M., Uyttendaele, M., Debevere, J., and Herman, L. (2006f). Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella* Enteritidis. *International Journal of Food Microbiology,* 112:253–260.

De Reu, K., Grijspeerdt, K., Heyndrickx, M., Zoons, J., De Baere, K., Uyttendaele, M., Debevere, J., and Herman, L. (2005b). Bacterial eggshell contamination in conventional cages, furnished cages and aviary housing systems for laying hens, *British Poultry Science*, 46(2):149-155.

De Reu, K., Heyndrickx, M., Grijspeerdt, K., Rodenburg, T.B., Tuyttens, F., Uyttendaele, M., Debevere, J., and Herman, L. (Online). Estimation of the vertical and horizontal bacterial infection of hen's eggs. Retrieved from http://www.cabi.org/animalscience/Uploads/File/AnimalScience/additionalFiles/WPSA2007/7_De%20Reu%20Koen.pdf

Dieckert, J.W., Dieckert, M.C., and Creger, C.R. (1989). The calcium reserve assembly: a basic structural unit of the calcium reserve system of the hen eggshell. *Poultry Science,* 68:1569-1584.

Dukic-Stojcic, M., Peric, L., Bjedov, S., and Milosevic, N. (2009). The quality of table eggs produced in different housing systems. *Biotechnology in Animal Husbandry*, 25(5-6):1103-1108.

Dyda, A., Hundy, R., Moffatt, C.R.M., and Cameron, S. (2009). Outbreak of *Salmonella* Typhimurium 44 related to egg consumption. *Communicable Diseases Intelligence,* 33(4):414-418.

Englmaierova, M., and Tumova, E. (Online). The effect of housing system and storage time on egg quality characteristics. Retrieved from www.cabi.org/.../File/.../51_eggmeat2009_englmaierova_EP6.pdf dated 04/07/2012

European Food Safety Authority (EFSA). The European Union Summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. *EFSA Journal* 2012 10(3):2597 retrieved from http://www.efsa.europa.eu/en/efsajournal/doc/2597.pdf

Fernandez, M.S., Escobar, C., Lavelin, I., Pines, M., and Arias, J.L. (2003). Localization of Osteopontin in oviduct tissue and eggshell during different stages of the avian egg laying cycle. *Journal of Structural Biology,* 143:171-180.

Fernandez, M.S., Moya, A., Lopez, L., and Arias, J.L. (2001). Secretion pattern, ultrastructural localization and function of extracellular matrix molecules involved in eggshell formation. *Matrix Biology,* 19:793-803.

Fernandez, M.S., Moya, A., and Arias, J.L. (1997). Eggshells are shaped by a precise spatio-temporal arrangement of sequentially deposited macromolecules. *Matrix Biology,* 16:13-20.

Ferrante, V., Lolli, S., Vezzoli, G., and Cavalchini, L.G. (2009). Effects of two different rearing systems (organic and barn) on production performance, animal welfare traits and egg quality characteristics in laying hens. *Italian Journal of Animal Science,* 8:165-174.

Fisher, C. (1969). The effects of a protein deficiency on egg composition. *British Poultry Science,* 10:149-154.

Fraser, A.C., Bain, M.M., and Solomon, S.E. (1999). Transmission electron microscopy of the vertical crystal layer and cuticle of the eggshell of the domestic fowl. *British Poultry Science,* 40:626-631.

Freedman, S.L. (1968). The innervation of the suprarenal gland of the fowl. *Acta Anatomica,* 69(1):18-25.

Fullerton, K. (2008). Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the OzFoodNet Network, 2007. *Communicable Diseases Intelligence,* 32(4):400-424.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R., Humphrey, T.J., and Van Immerseel, F. (2009). Mechanisms of egg contamination by *Salmonella* Enteritidis. *FEMS Microbiology Reviews,* 33:718-738.

Garlich, J., Brake, J., Parkhurst, C.R., Thaxton, J.P., and Morgan, G.W. (1984). Physiological profile of caged layers during one production year, molt, and postmolt: egg production, eggshell quality, liver, femur, and blood parameters. *Poultry Science,* 63(2):339-343.

Gast, R.K. (1993). Recovery of *Salmonella* Enteritidis from inoculated pools of egg contents. *Journal of Food Protection,* 56:21-24.

Gast, R.K., and Beard, C.W. (1990). Production of *Salmonella* Enteritidis contaminated eggs by experimentally infected hens. *Avian Diseases,* 34:438-446.

Gautron, J., Hincke, M.T., Garcia-Ruiz, J.M., Vidal, M.L., and Nys, Y. (online). Relationship between eggshell matrix proteins and egg quality. Retrieved online from http://www.cabi.org/animalscience/Uploads/File/AnimalScience/additionalFiles/WPSA_files/gautron.pdf

Gautron, J., Hincke, M.T., and Nys, Y. (1997). Precursor matrix proteins in the uterine fluid change with stages of eggshell formation in hens. *Connective Tissue Research,* 36(3):195-210.

Gautron, J., Bain, M., Solomon, S., and Nys, Y. (1996). Soluble matrix of hen's eggshell extracts changes *in vitro* the rate of calcium carbonate precipitation and crystal morphology. *British Poultry Science,* 37(4):853-866.

Gentry, R.F., and Quarles, C.L. (1972). The measurement of bacterial contamination on eggshells. *Poultry Science,* 51(3):930-933.

Gilbert, A.B. (1971). The egg: Its physical and chemical aspects. In D. J. Bell and B. M. Freeman (Eds.). *Physiology and Biochemistry of the domestic fowl,* Vol. 3, pp. 1379-1399.

Gilbert, A.B. (1969). Innervations of the ovary of the domestic hen. *Quarterly Journal of Experimental Physiology,* 54:404-411.

Gilbert, A.B., Reynolds, M.E., and Lorenz, F.W. (1968b). Distribution of spermatozoa in the oviduct and fertility in domestic birds. VII. Innervations and vascular supply of the uterovaginal sperm-host glands of the domestic hen. *The Journal of the Society for Reproduction and Fertility,* 17:305-310.

Gilbert, A.B., and Lake, P.E. (1963). Terminal innervations of the uterus and vagina of the domestic hen. *The Journal of the Society for Reproduction and Physiology,* 5:41-48.

Graves, R.C., and Maclaury, D.W. (1962). The effects of temperature, vapor pressure and absolute humidity on bacterial contamination of shell eggs. *Poultry Science,* 41:1219-1225.

Gregory, D.W. (1948). *Salmonella* infections of turkey eggs. *Poultry Science,* 27:359-366.

Guan, J., Grenier, C., and Brooks, B.W. (2006). *In vitro* study of *Salmonella* Enteritidis and *Salmonella* Typhimurium definitive type 104: survival in egg albumen and penetration through the vitelline membrane. *Poultry Science,* 85:1678-1681.

Guerin-Dubiard, C., Pasco, M., Molle, D., Desert, C., Croguennec, T., and Nau, F. (2006). Proteomic analysis of hen egg white.Journal of Agricultural and Food Chemistry, 54(11):3901-10.

Gupta, L. (2010). Optimum eggshell quality. Retrieved on 27 Nov, 2010 from http://www.thepoultrysite.com/books/b180/new-optimum-egg-quality-a-practical approach

Guesdon, V., Ahmed, A.M.H., Mallet, S., Faure, J.M., and Nys, Y. (2006). Effects of beak trimming and cage design on laying hen performance and egg quality. *British Poultry Science,* 47(1):1-12.

Guesdon, V., and Faure, J.M. (2004). Laying performance and egg quality in hens kept in standard or furnished cages. *Animal Research,* 53:45-57.

Haigh, T., and Betts, W.B. (1991). Microbial barrier properties of hen eggshells. *Microbios,* 68:137-146.

Hald, T., Vose, D., Wegener, H.C., and Koupeev, T. (2004). A Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. *Risk Analysis,* 24(1):255-269.

Harry, E.G. (1963). The relationship between egg spoilage and the environment of the egg when laid. *British Poultry Science,* 4:91-100.

Heil, G., and Hartmann, W. (1997). Combined summaries of European random sample egg production tests completed in 1995 and 1996. *World Poultry Science Journal,* 53:291-296.

Hidalgo, A.M., Rossi, M., Clerici, F., and Ratti, S.M. (2008). A market study on the quality characteristics of eggs from different housing systems. *Food Chemistry,* 106:1031–1038.

Hincke, M.T., Gautron, J., Nys, Y., Rodriguez-Navarro, A.B., and McKee, M.D. (2011). The eggshell: structure and productive function. In Yves Nys, Maureen Bain and Filip Van Immerseel (Eds.) *Improving the safety and quality of eggs and egg products.* Woodhead Publishing Limited, Volume, 1:151-182.

Hincke, M.T., Nys, Y., and Gautron, J. (2010). The role of matrix protein in eggshell formation (Review). *Journal of Poultry Science,* 47: 208-219.

Hincke, M.T., Chien, Y.C., Gerstenfeld, L.C., and McKee, M.D. (2008b). Colloidal-gold immunocytochemical localization of osteopontin in avian eggshell gland and eggshell. *Journal of Histochemistry and Cytochemistry,* 56:467-476.

Hincke, M.T., Gautron, J., Panheleux, M., Carcia-Ruiz, J., McKee, M.D., and Nys, Y. (2000). Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. *Matrix Biology,* 19:443-453.

Hincke, M.T., Tsang, C.P., Courtney, M., Hill, V., and Narbaitz, R. (1995). Purification and immunochemistry of a soluble matrix protein of the chicken eggshell (Ovocleidin 17). *Calcified Tissue International,* 56:578-583.

Hodges, R.D. (1974). The Histology of the Fowl. Academic Press Inc. London.

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

Holt, P.S. (2003). Molting and *Salmonella enterica* serovar Enteritidis infection: The problem and some solutions. *Poultry Science,* 82:1008-1010.

Holt, P.S. (1995). Horizontal transmission of *Salmonella* Enteritidis in molted and unmolted laying chickens. *Avian Diseases,* 39:239-249.

Honkatukia, M., Tuiskula-Haavisto**,** M., De Koning, D.J., Virta, A., Mäki-Tanila, A., and Vilkki, J. (2005). A region on chicken chromosome 2 affects both egg white thinning and egg weight. *Genetics Selection Evolution,* 2005, 37:563-577.

Hooge, D.M. (2007). *Bacillus subtilis* spores improve brown egg colour. *World Poultry,* Volume 23(3):14-15. Retrieved from www.worldpoultry.net

Hughes, B.O., Dun, P., and McCorquodale, C.C. (1985). Shell strength of eggs from medium-bodied hybrid hens housed in cages or on range in outside pens. *British Poultry Science,* 26(1):129-136.

Hughey, V.L., and Johnson, E.A. (1987). Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Applied and Environmental Microbiology,* 53(9):2165-2170.

Humphrey, T.J. (1994). Contamination of eggshell and contents with *Salmonella* Enteritidis: A review. *International Journal of Food Microbiology,* 21:31-40.

Humphrey, T.J., Whitehead, A., Gawler, A.H.L., Henley, A., and Rowe, B. (1991). Numbers of *Salmonella* Enteritidis in the contents of naturally contaminated hens' eggs. *Epidemiology and Infection,* 103:489-496.

Humphrey, T.J., Baskerville, A., Mawer, S., Rowe, B., and Hopper, S. (1989). *Salmonella* Enteritidis phage type 4 from the contents of intact eggs: a study involving naturally infected eggs. *Epidemiology and Infection,* 103:415-423.

Hunton, P. (2005). Research on eggshell structure and quality: An historical overview. *Brazilian Journal of Poultry Science,* 7(2):67-71.

Hunton, P. (1995). Understanding the architecture of the egg-shell. *World Poultry Science Journal,* 51:141-147.

Huneau-Salaun, A., Michel, V., Huonnic, D., Balaine, L., and Le Bouquin, S. (2010). Factors influencing bacterial eggshell contamination in conventional cages, furnished cages and free range systems for laying hens under commercial conditions. *British Poultry Science,* 51(2):163-169.

Hutchison, M.L., Gittins, J., Walker, A., Spark, N., Humphrey, T.J., Burton, C., and Moore, A. (2004). An assessment of the microbiological risks involved with egg washing under commercial conditions. *Journal of Food Protection,* 67(1):4-11.

Hutchison, M.L., Gittins, J., Walker, A., Moore, A., Burton, C., and Spark, N. (2003). Washing table eggs: a review of the scientific and engineering issues. *World Poultry Science Journal,* 59:233-248.

Ibarra, J.A., and Steele-Mortimer, O. (2009). *Salmonella* – the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. *Cellular microbiology,* 11:1579-1586.

Ibrahim, H.R., Sugimoto, Y., and Aoki, T. (2000). Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1523(2–3):196-205.

Ingram, D.R., Hatten, L.F., and Homan, K.D. (2008). A study on the relationship between eggshell colour and eggshell quality in commercial broiler breeders. *International Journal of Poultry Science,* 7:700-703.

Ishikawa, S., Suzuki, K., Fukuda, E., Arihara, K., Yamamoto, Y., Mukai, T., and Itoh, M. (2010). Photodynamic antimicrobial activity of avian eggshell pigments. *FEBS Letters,* 584:770-774. doi:10.1016/febslet.2009.12.041

Ito, S., Tsudzuki, M., Komori, M., and Mizutani, M. (1993). Celadon: An eggshell colour mutation in Japanese Quail. *The Journal of Heredity,* 84(2):145-147.

Izat, A.L., Gardner, F.A., and Mellor, D.B. (1986). The effects of age of bird and season of the year on egg quality. II. Haugh unit and compositional attributes. *Poultry Science,* 65 (4):726-728.

Jacob, J.P., Miles, R.D., and Mather, F.B. (2011). Egg quality. University of Florida. Retrieved from http.//edis.ifas.ufl.edu

Jacob, J., Pescatore, T., and Cantor, A. (2011). Avian female reproductive system. UK University of Kentucky. Online retrieved from: http://www2.ca.uky.edu/afspoultry-files/pubs/Anatomy_Female_reproductive.pdf

Johnson, A.L. (2000). Reproduction in the female. In G. Causey Whittow (Eds.) Sturkie Avian Physiology (pp. 569-596). Academic Press, USA.

Johnston, S.A., and Gous, R.M. (2007). Modelling the changes in the proportions of the egg components during a laying cycle. *British Poultry Science,* 48:347-353.

Johnston, H.S., Aitken, R.N.C., and Wyburn, G.M. (1963). The fine structure of uterus of the domestic fowl. *Journal of Anatomy,* 97:333–344.

Jonchere, V., Rehault-Godbert, S., Hennequet-Antier, C., Cabau, C., Sibut, V., Cogburn, L.A., Nys, Y., and Gautron, J. (2010). Gene expression profiling to identify eggshell proteins involved in physical defense of the chicken egg. *BMC Genomics,* 11:57.

Jones, D.R., Lawrence, K.C., Yoon, S.C., and Heitschmidt, G.W. (2010). Modified pressure imaging for egg crack detection and resulting egg quality. *Poultry Science,* 89:761-765.

Jones, D.R., Musgrove, M.T., Anderson, K.E., and Thesmar, H.S. (2010). Physical quality and composition of retail shell eggs. *Poultry Science,* 89:582-587.

Jones, D.R., and Musgrove, M.T. (2008). Identification of Enterobacteriaceae on vacuum loaders in shell egg processing. *Poultry Science,* 87:1678-1681.

Jones, D.R., Musgrove, M.T., and Northcutt, J.K. (2004). Variations in external and internal microbial populations in shell eggs during extended storage. *Journal of Food Protection,* 67(12):2657-2660.

Jones, D.R., Tharrington, J.B., Curtis, P.A., Anderson, K.E., Keener, K.M., and Jones, F.T. (2002). Effects of cryogenic cooling of shell eggs on egg quality. *Poultry Science,* 81:727-733.

Jung, J.G., Lim, W., Park, T.S., Kim, J.N., Han, B.K., Song, G., and Han, J.Y. (2011). Structural and histological characterization of oviductal magnum and lectin-binding patterns in Gallus domesticus. *Reproductive biology and Endocrinology,* 9:62.

Karoui, R., Nicolai, B., and Daerdemaeker, J.D. (2008). Monitoring the egg freshness during storage under modified atmosphere by fluorescence spectroscopy. *Food Bioprocess Technology,* 1:346-356.

Kawasaki, T., Musgrove, M.T., Murata, M., Tominaga, N., and Kawamoto, S. (2008). Comparative study of shell swab and shell crush methods for the recovery of *Salmonella* from shell eggs. *Journal of Food Safety,* 28:482-498.

Keller, L.H., Benson, C.E., Krotec, K., and Eckroade, R.J. (1995). *Salmonella* Enteritidis colonization of the reproductive tract and forming and freshly laid eggs of chickens. *Infection* and *Immunity,* 63:2443-2449.

Kennedy, G.Y., and Vevers, H.G. (1976). A survey of avian eggshell pigments. *Comparative Biochemistry and Physiology,* Vol. 55B, 117-123.

Kennedy, G.Y., and Vevers, H.G. (1973). Eggshell pigments of the Araucano fowl. *Comparative Biochemistry and Physiology,* Vol. 44B, 11-25.

Kim, J.W., and Slavik, M.F. (1996). Changes in eggshell surface microstructure after washing with cetylpyridinium chloride or trisodium phosphate. *Journal of Food Protection,* 59 (8):859-863.

Kim, C.J., Emery, D.A., Rinke, H., Nagaraja, K.V., and Halvorson, D.A. (1989). Effect of time and temperature on growth of *Salmonella* Enteritidis in experimentally inoculated eggs. *Avian Diseases,* 33:735-745.

Kirk, M.D., McKay, I., Hall, G.V., Dalton, C.B., Stafford, R., Unicomb, L., and Gregory, J. (2008). Foodborne Disease in Australia: The OzFoodNet Experience. *Food Safety,* CID 2008:4 (1):392-400.

Knape, K.D., Carey, J.B., and Ricke, S.C. (2001). Response of food borne *Salmonella* spp. marker strains inoculated on eggshell surfaces to disinfectants in a commercial egg washer. *Journal of Environmental Science and Health,* B36(2):219-227.

Knape, K.D., Carey, J.B., Burgess, R.P., Kwon, Y.M., and Ricke, S.C. (1999). Comparison of chlorine with iodine-based compound eggshell surface microbial populations in a commercial egg washer. *Journal of Food Safety,* 19:184-194.

Kraft, A.A., McNally, E.H., and Brant, A.W. (1958). Shell quality and bacterial infection of chicken eggs. *Poultry Science,* 37:638-644.

Kretzschmar-McCluskey, V., Curtis, P.A., Anderson, K.E., Berry, W.D., and Derth, L.K. (2009). Influence of hen age and strain on eggshell exterior, eggshell interior with membranes, and egg contents of microflora, and on *Salmonella* incidence during a single production cycle. *Journal of Applied Poultry Research,* 18:665-670.

Kretzschmar-McCluskey, V., Curtis, P.A., Anderson, K.E., Kerth, L.K., and Berry, W.D. (2008). Influence of hen age and strain on eggshell exterior, interior and contents of microflora and

Salmonella prevalence during a second production cycle. *The Journal of Applied Poultry Research,* 18(4):665-670.

Kusuda, S., Iwasawa, A., Doi, O., Ohya, Y., and Yoshizaki, N. (2011). Diversity of the cuticle layer of avian eggshells. *Journal of Poultry Science,* 48:119-124.

Lang, M.R., and Wells, J.W. (1987). A review of eggshell pigmentation. *World Poultry Science Journal,* 43:238-246.

Lapuz, R.R., Umali, D.V., Suzuki, T., Shirota, K., and Katoh, H. (2012). Comparison of the prevalence of *Salmonella* infection in layer hens from commercial layer farms with high and low rodent densities. *Avian diseases,* 56:29-34.

Lavelin, I., Meiri, N., and Pines, M. (2000). New insight in eggshell formation. *Poultry Science,* 79:1014–1017.

Lay, Jr., D.C., Rulton, R.M., Hester, P.Y., Karcher, D.M., Kjaer, J.B., Mench, J.A., Mullens, B.A., Newberry, R.C., Nicol, C.J., O'Sullivan, N.P., and Porter, R.E. (2011). Hen welfare in different housing systems. *Poultry Science,* 90(1):278-94. doi:10.3382/ps.2010-00962

Ledvinka, Z., Zita, L., Hubeny, M., Tumova, E., Tyller, M., Dobrovolny, P., and Hruska, M. (2011). Effect of genotype age of hens and K/k allele on eggshell quality. *Czech Journal of Animal Science,* 56(5):242-249.

Leleu, S., Bain, M., Herman, L., Heyndrickx, M., De Baerdemaeker, J., Michiels, C.W., Perianu, C., and Messens, W. (2011). The effect of microcracks and the presence of the cuticle on trans-shell penetration of table eggs by *Salmonella* Enteritidis. *Proceedings of the European Symposium on the Quality of Eggs and Egg Products, Leipzig, Germany,* 2011.

Leleu, S., Messens, W., De Reu, K., De Preter, S., Herman, S., Heyndrickx, M., DE Baerdemaeker, J., Michiels, C.W., and Bain, M. (2011). Effect of egg washing on the cuticle quality of brown and white table eggs. *Journal of Food Protection,* 74(10):1649–1654.

Lichovnikova, M., and Zeman, L. (2008). Effect of housing system on the calcium requirements of laying hen and eggshell quality. Czech Journal of Animal Science, 53:162-168.

Lifshitz, A., Baker, R.C., and Naylor, H.B. (1964). The relative importance of chicken egg exterior structures in resisting bacterial penetration. *Journal of Food Science,* 29:94-99.

Lock, J.L., Dolman, J., and Board, R.G. (1992). Observations on the mode of bacterial infection of hen's egg. *FEMS Microbiological Letters,* 100:71-74.

Lofts, B., and Murton, K. (1973). Reproduction in birds. In Donald S. Farner and Fames R. King (Eds.) *Avian Biology* Vol. III, (pp. 1-107). Academic Press New York and London.

Lubin, A., and Sela, S. (2008). The impact of temperature during the storage of table eggs on the viability of *Salmonella enterica* Serovars Enteritidis and Virchow in the eggs. *Poultry Science,* 87:2208-2214.

Mallet, S., Guesdon, V., Ahmed, A.M.H., and Nys, Y. (2006). Comparison of eggshell hygiene in two housing systems: Standard and furnished cages. *British Poultry Science,* 47(1):30-35.

Manfreda, G., Cevoli, C., Lucchi, A., and Pasquali, F. (2010). Hot air treatment for surface decontamination of table eggs experimentally infected with *Salmonella, Listeria* and *Escherichia coli*. *Veterinary Research Communications,* 34(1):S179-S182.

Mann, K., and Mann, M. (2011). In-depth analysis of the chicken egg white proteome using an LTQ Orbitrap Velos. *Proteome Science 2011,* 9:7. http://www.proteomesci.com/content/pdf/1477-5956-9-7.pdf

Mann, K., and Mann, M. (2008). The chicken egg yolk plasma and granule proteomes. Proteomics, 8:178-191. Retrieved from: http://onlinelibrary.wiley.com/doi/10.1002/pmic.200700790/pdf

March, B.E. (1969). Bacterial infection of washed and unwashed eggs with reference to *Salmonella*. *Applied Microbiology,* 17(1):98-101.

Marks, A.R. (2003). Calcium and the heart: a question of life and death. *The Journal of Clinical Investigation.* 111(5): 597–600.

Martinez-de la Puente, J., Merino, S., Moreno, J., Tomas, G., Morales, J., Lobato, E., Garcia-fraile, S., and Martinez, J. (2007). Are eggshell spottiness and colour indicators of health and condition in blue tits Cyanistes caeruleus? *Journal of Avian Biology,* 38-377-384.

Maurer, V., Amsler, Z., Perler, E., and Heckendorn, F. (2009). Poultry litter as a source of gastrointestinal helminthes infections. *Veterinary Parasitology*, 161:255–260.

Mayes, F.J., and Takeballi, M.A. (1983). Microbial contamination of the hen egg: a review. *Journal of Food Protection,* 46:1092-1098.

McKee, M.D., and Nanci, A. (1996). Osteopontin: An interfacial extracellular matrix protein in mineralized tissues. *Connective Tissue Research,* 35(14):197-205.

Mertens, K., Vaesen, I., loffel, J., Kemps, B., Kamers, B., Perianu, C., Zoons, J., Darius, P., Decuypere, E., De Baerdemaeker, J., and De Ketelaere, B. (2010). The transmission colour value: A novel egg quality measure for recording shell colour used for monitoring the stress and health status of a brown layer flock. *Poultry Science,* 89:609-617.

Mertens, K., F. Bamelis, F., Kemps, B., Kamers, B., Verhoelst, E., De Ketelaere, B., Bain, M., Decuypere, E., and De Baerdemaeker, J. (2006). Monitoring of eggshell breakage and eggshell strength in different production chains of consumption eggs. *Poultry Science,* 85:1670–1677.

Messens, W., Leleu, S., De Reu, K., De Preter, S., Herman, L., De Baerdemaeker, J., and Bain, M. (2009). Effect of egg washing on the cuticle of table eggs. *Proceedings of the XIII European Symposium on the quality of eggs and egg products.* Turku Finland, June 21-25.

Messens, W., Grijspeerdt, K., De Reu, K., De Ketelaere, B., Mertens, K., Bamelis, F., Kemps, B., De Baerdemaeker, J., Decuypere, E., and Herman, L. (2007). Eggshell penetration of various types of hens eggs by *Salmonella enterica* serovar Enteritidis. *Journal of Food Protection,* 70(3):623-628.

Messens, W., Grijspeerdt, K., and Herman, L. (2006). Eggshell penetration of hens eggs by *Salmonella enterica* serovar Enteritidis upon various storage conditions. *British Poultry Science,* 47:554-560.

Messens, W., Grijspeerdt, K., and Herman, L. (2005a). Eggshell penetration by *Salmonella*: a review. *World Poultry Science Journal,* 61:71-85.

Messens, W., Grijspeerdt, K., and Herman, L. (2005b). Eggshell characteristics and penetration by *Salmonella enterica* serovar Enteritidis through the production period of a layer flock. *British Poultry Science,* 46(6):694-700.

Messens, W., Grijspeerdt, K., and Herman, L. (2005). Intrinsic and extrinsic factors influencing eggshell penetration by *Salmonella* Enteritidis. *Proceedings of the XIth European Symposium on the Quality of Eggs and Egg Products,* Doorwerth, The Netherland. 23-26 May, 2005.

Messens, W., Duboccage, I., Grijspeerdt, K., Heyndrickx, M., and Herman, I. (2004). Growth of *Salmonella* serovars in hen's egg albumen as affected by storage prior to inoculation. *Food Microbiology,* 21:25-32.

Miksik, I., Eckhardt, A., Sedlakova, P., and Mikulikova, K. (2007). Proteins of insoluble matrix of avian (*Gallus gallus*) eggshell. *Connective Tissue Research,* 48:1-8.

Miksik, I., Holan, V., and Deyl, Z. (1996). Avian eggshell pigments and their variability. *Comparative Biochemistry and Physiology,* 113B (3):607-612.

Miksik, I., Holan, V., and Deyl, Z. (1994). Quantification and variability of eggshell pigment content. *Comparative Biochemistry and Physiology,* 109A (3):769-772.

Mine, Y. (2007). Egg Proteins and Peptides in Human Health-Chemistry, Bioactivity and Production. *Current Pharmaceutical Design,* 13(9):875-884.

Mine, Y., Oberle, C., and Kassafy, Z. (2003). Eggshell matrix proteins as defense mechanism of avian eggs. *Journal of Agricultural and Food Chemistry,* 51:249-253.

Miyamoto, T., Horie, T., Baba, E., Sasai, K., Fukata, T., and Arakawa, A. (1998). *Salmonella* penetration through eggshell associated with freshness of laid eggs and refrigeration. *Journal of Food Protection,* 61:350-353.

Miyamoto, T., Baba, E., Tanaka, T., Sasai, K., Fukata, T., and Arakawa, A. (1997). *Salmonella* Enteritidis contamination of eggs from hens inoculated by vaginal, cloacal and intravenous routes. *Avian Diseases,* 41:296-303.

Moats, W.A. (1978). Egg washing - a review. *Journal of Food Protection,* 41(11):919-925.

Moreno, J., and Osorno, J.L. (2003). Avian egg colour and sexual selection: does eggshell pigmentation reflect female condition and genetic quality? *Ecology Letters,* 6:803-806.

Mostert, B.E., Bowes, E.H., and Van der Walt, J.C. (1995). Influence of different housing systems on the performance of hens of four laying strains. *South African Journal of Animal Science,* 25(3):80-86.

Murase, T., Fujimoto, K., Nakayama, R., and Otsuki, K. (2006). Multiplication and motility of *Salmonella* enterica serovars Enteritidis, Infantis and Mentervideo in *in vitro* contamination models of eggs. *Journal of Food Protection,* 69(5):1012-1016.

Musgrove, M.T., Northcutt, J.K., Jones, D.R., Cox, N.A., and Harrison, M.A. (2008). Enterobacteriaceae and related organisms isolated from shell eggs collected during commercial processing. *Poultry Science,* 87:1211-1218.

Musgrove, M.T., Jones, D.R., Northcutt, J.K., Harrison, M.A., Cox, N.A., Ingram, K.D., and Hinton, A.J. (2005). Recovery of *Salmonella* from commercial shell eggs by shell rinse and shell crush methodologies. *Poultry Science,* 84:1955-1958.

Musgrove, M.T., Jones, D.R., Northcutt, J.K., Harrison, M.A., and Cox, N.A. (2005). Impact of commercial processing on the microbiology of shell eggs. *Journal of Food Protection,* 68(11):2367-2375.

Musgrove, M.T., Jones, D.R., Northcutt, J.K., Cox, N.A., and Harrison, M.A. (2004). Identification of Enterobacteriaceae from washed and unwashed commercial shell eggs. *Journal of Food Protection,* 67(11):2613-2616.

Nascimento, V.P., Cranstoun, S., and Solomon, S.E. (1992). Relationship between shell structure and moment of *Salmonella* Enteritidis across the eggshell wall. *British Poultry Science,* 33:37-48.

Nascimento, V.P., and Solomon, S.E. (1991). The transfer of bacteria (*Salmonella* Enteritidis) across the eggshell wall of eggs classified as “poor” quality. *Animal Technology,* 42(3):157-166.

Novo, R.P., Gama, L.T., and Soares, M.C. (1997). Effects of oviposition time, hen age and extra dietary calcium on egg characteristics and hatchability. *Journal of Applied Poultry Research*, 6:335-343.

Neill, S.D., Campbell, J.N., and O’brien, J.J. (1985). Egg penetration by Campylobacter jejuni. *AvianPathology*, 14(3):313-320.

Nys, Y., and Guyot, N. (2011). Egg formation and chemistry. In Yves Nys, Maureen Bain and Filip Van Immerseel (Eds.) *Improving the safety and quality of eggs and egg products.* Woodhead Publishing Limited, Volume 1, pp. 83-132.

Nys, Y., Gautron, J., Garcia-Ruiz, J.M., and Hincke, M.T. (2004). Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. *Comptes Rendus Palevol,* 3:549-562.

Nys, Y., Gautron, J., McKee, M.D., Garcia-Ruiz, J.M., and Hincke, M.T. (2001). Biochemical and functional characterization of eggshell matrix proteins in hens. *World Poultry Science Journal,* 57:401-413.

Nys, Y., Hincke M.T., Arias J.L., Garcia-Ruiz J.M., and Solomon, S. (1999). Avian eggshell mineralization. *Poultry and Avian Biology Reviews,* 10:143-166.

Nys, Y., Zawadzki, J., Gautron, J., and Mills, A.D. (1991). Whitening of brown-shelled eggs: mineral composition of uterine fluid and rate of protoporphyrin deposition. *Poultry Science,* 70:1236-1245.

Nys, Y. (1986). Relationship between age, shell quality and individual rate and duration of shell formation in domestic hens. *British Poultry Science,* 27:253-259.

Odabasi, A.Z., Miles, R.D., Balaban, M.O., and Porier, K.M. (2007). Changes in brown eggshell colour as the hen ages. *Poultry Science*, 86:356-363.

Okamura, M., Kamijima, Y., Miyamoto, T., Tani, H., Sasai, K., and Baba, E. (2001). Differences among six *Salmonella* serovars in abilities to colonize reproductive organs and to contaminate eggs in laying hens. *Avian Diseases,* 45:61-69.

Onbasilar, E.E., and Erol, H. (2007). Effects of different forced molting methods on postmolt production, corticosterone level, and immune response to sheep red blood cells in laying hens. *Journal of Applied Poultry Research,* 16:529–536.

Orel, V. (1959). The *Pseudomonas* spoilage of eggs laid by individual hens. *Poultry Science,* 38:8-12.

Palmiter, R.D., and Wrenn, J.T. (1971). Interaction of estrogen and progesterone in chick oviduct development. 3. Tubular gland cell cytodifferentiation. *Journal of Cell Biology,* 50:598-615.

Pang, E., Tien-Lin, C., Selvaraj, M., Chang, J., and Kwang, J. (2011). Deletion of the aceEgene (encoding a component of pyruvate dehydrogenase) attenuates *Salmonella enterica* serovar Enteritidis. *FEMS Immunology and Medical Microbiology,* 63:108-118.

Panheleux, M., Nys, Y., Williams, J., Gautron, J., Boldicke, T., and Hincke, M. T. (2000). Extraction and quantification by ELISA of eggshell organic matrix proteins (ovocleidin-17, ovalbumin, ovotransferrin) in shell from young and old Hens. *Poultry Science,* 79:580–588.

Panheleux, M.., Bain, M., Fernandez, M.S., Morales, I., Gautron, J., Arias, J.L., Solomon, S.E., Hincke, M., and Nys, Y. (1999). Organic matrix composition and ultrastructure of eggshell: A comparative study. *British Poultry Science,* 40:240-252.

Park, C.M., Hung, Y.C., Lin, C.S., and Brackett, R.E. (2005). Efficacy of electrolyzed water in inactivating *Salmonella* Enteritidis and *Listeria monocytogenes* on shell eggs. *Journal of Food Protection,* 68(5):986-990.

Parsons, A.H. (1982). Structure of the eggshell. *Poultry Science,* 61:2013-2021.

Parto, P., Khaksar, Z., Akramifard, A., and Moghisi, B. (2011). The microstructure of oviduct in laying turkey hen as observed by light and scanning electron microscopies. *World Journal of Zoology,* 6(2):120-125.

Pavlovski, Z., Skrbic, Z., and Lukic, M. (2004). Influence of husbandry system on the internal egg quality traits in small flocks hen layers. *Zivinarstvo,* 39(6-7):19-23.

Peebles, E.D., Zumwalt, C.D., Doyle, S.M., Gerard, P.D., Latour, M.A., Boyle, C.R., and Smith, T.W. (2000). Effects of breeder age and dietary fat source and level on broiler hatching egg characteristics. *Poultry Science,* 79:94-96.

Peebles, E.D., Pansky, T., Doyle, S.M., and Smith T.W. (1998). Effects of breeder dietary fat and eggshell cuticle removal on subsequent broiler growth performance. *Journal of Applied Poultry Research,* 7:377-383.

Permin, A., Christensen, J. P., and Bisgaard, M. (2006). Consequences of concurrent *Ascaridia galli* and *Escherichia coli* infections in chickens. *Acta Veterinaria Scandinavica,* 47:43–54.

Permin, A., Bisgaard, M., Frandsen, F., Pearman, M., Kold, J., and Nansen, P. (1999). Prevalence of gastrointestinal helminths in different poultry production system. *British Poultry Science,* 40:439–443.

Petek, M., Fazli, A., Sule Gezen, S., and Cibik, R. (2009). Effects of housing system and age on early stage egg production and quality in commercial laying hens. *Kafkas Universitesi Veteriner Fakultesi Dergisi,* 15(1):57-62.

Petek, M., Sule Gezen, S., Alpay, F., and Cibik, R. (2008). Effects of non-feed removal molting methods on egg quality traits in commercial brown egg laying hens in Turkey. *Tropical Animal Health and Production,* 40:413–417.

Pistekova, V., Hovorka, M. Vecerek, V., Strakova, E., and Suchy, P. (2006). The quality comparison of eggs laid by laying hens kept in battery cages and in a deep litter system. *Czech Journal of Animal Science,* 51(7):318-325.

Poggenpoel, D.G. (1986). Correlated response in shell and albumen quality with selection for increased egg production. *Poultry Science,* 65(9):1633-1641.

Poole, H.K. (1965). Spectrophotometric identification of eggshell pigments and timing of superficial pigment deposition in the Japanese quail. *Proceedings of the Society of Experimental Biology and Medicine,* 119:547-551.

Poppe, C., Johnson, R.P., Forsberg, C.M., and Irwin, R.J. (1992). *Salmonella* Enteritidis and other *Salmonella* in laying hens and eggs from flocks with *Salmonella* in their environment. *Canadian Journal of Veterinary Research,* 56:226-232.

Quarles, C.L., Gentry, R.F., and Bressler, G.O. (1970). Bacterial contamination in poultry houses and its relationship to egg hatchability. *Poultry Science,* 49(1):60-66.

Quinn, P.J., Markey, B.K., Carter, M.E., Donnelly, W.J, Leonard, F.C. (2002). Enterobacteriaceae. Chapter 18, In Veterinary Microbiology and Microbial Disease. Blackwell Science.

Radkowski, M. (2002). Effect of moisture and temperature on survival of *Salmonella* Enteritidis on shell eggs. *Archiv fur Geflugelkunde,* 66(3):119-123.

Rahman, M.A., Moriyama, A., Iwasawa, A., and Yoshizaki, N. (2009). Cuticle formation in quail eggs. *Zoological Science Japan,* 26 (7):496-499.

Rayan, G.N., Galal, A., Fathi, M.M., and El-Attar, A.H. (2010). Impact of Layer Breeder Flock Age and Strain on Mechanical and Ultrastructural Properties of Eggshell in Chicken. *International Journal of Poultry Science,* 9(2):139-147.

Reid, J. (1983). The use of the plasma chemistry unit as a aid to the scanning electron microscope study of avian eggshell structure. *British Poultry Science,* 24:233-235.

Reynolds, A., Moffatt, C.R., Dyda, A., Hundy, R.L., Kaye, A.L., Krsteski, R., Rockliff, S., Kampen, R., Kelly, P.M., and O'Brien, E.D. (2010). An outbreak of gastroenteritis due to *Salmonella* Typhimurium phage type 170 associated with consumption of a dessert containing raw egg. *Communicable Diseases Intelligence,* 34(3):329-33.

Richards, P.D.G., and Deeming, D.C. (2001). Correlation between shell colour and ultrastructure in pheasant eggs. *British Poultry Science,* 42:338-343.

Richardson, K.C. (1935). The secretory phenomena in the oviduct of the fowl, including the process of shell formation examined by the microincineration technique. *Philosophical Transactions of the Royal Society of London*. Series B, Biological Sciences. Vol. 225, No. 522, pp. 149-195.

Rizzi, C., and Chiericato, G.M. (2005). Organic farming production. Effect of age on the productive yield and egg quality of hens of two commercial hybrid lines and two local breeds. *Italian Journal of Animal Science,* 4:160-162.

Roberts, J.R. (2010). Egg quality and food safety. 18th Annual ASAIM SE Asian Feed Technology and Nutrition Workshop Combodia. 24-27, September.

Roberts, J.R. (2004). Factors affecting egg internal quality and eggshell quality in laying hens. *Journal of Poultry Science,* 41:161-177.

Roberts, J.R., and Brackpool, C.E. (1994). The ultra structure of Avian Eggshells. *Poultry Science Review,* 5:245-272.

Roberts-Witteveen, A.R., Campbell, B.A., Merritt, T.D., Massey, P.D., Shadbolt, C.T., and Durrheim, D.N. (2009). Egg-associated *Salmonella* outbreak in an aged care facility, New South Wales, 2008. *Communicable Diseases Intelligence,* 33(1):49-52.

Robley, C. (2010). *Salmonella* Typhimurium in the Australian layer industry. *Health Feature: Poultry Digest,* December/January 2010, Australia.

Rodriguez-Navarro, A., Kalin, O., Nys, Y., and Garcia-Ruiz, J.M. (2002). Influence of the microstructure on the shell strength of eggs laid by hens of different ages. *British Poultry Science,* 43:395–403.

Roland, Sr. D.A. (1979). Factors influencing shell quality of aging hens. *Poultry Science,* 58: 774-777.

Roland, Sr. D.A., Sloan, D.R., and Harms, R.H. (1975). The ability of hens to maintain calcium deposition in the eggshell and egg yolk as the hen ages. *Poultry Science,* 54:1720-1723.

Rose, M.L.H., and Hincke, M.T. (2009). Protein constituents of the eggshell: eggshell-specific matrix proteins. *Cellular and Molecular Life Sciences*, 66:2707-2719.

Ross, I.L., and Heuzenroeder, M.W. (2008). A comparison of three molecular typing methods for the discrimination of *Salmonella enterica* serovar Infantis. *FEMS Immunology and Medical Microbiology*, 53:375-384.

Rossi, M., and Pompei, C. (1995). Changes in some egg components and analytical values due to hen age. *Poultry Science*, 74(1):152-160.

Ruiz, J., and Lunam, C.A. (2000). Ultrastructural analysis of the eggshell: contribution of the individual calcified layers and the cuticle to hatchability and egg viability in broiler breeders. *Brithish Poultry Science*, 41:584-592.

Sato, Y., and Kuwamoto, R. (1999). A case of canine salmonellosis due to *Salmonella* Infantis. *Japan Veterinary Medicine Science*, 61(1):71-72.

Sauter, E.A., Petersen, C.F., Parkinson, J.F., and Steele, E.E. (1977). Effects of pH on eggshell penetration by *Salmonella*. *Poultry Science*, 56:1754-1755.

Sauter, E.A., and Petersen, C.F. (1974). The effect of eggshell quality on penetration by various *Salmonella*. *Poultry Science*, 53:2159–2162.

Scanes, C.G., Mozelic, H., Kavanagh, E., Merrill, G., and Rabii, J. (1982). Distribution of blood flow in the ovary of domestic fowl (*Gallus domesticus*) and changes after prostaglandin F-2α treatment. *The Journal of the Society for Reproduction and Fertility*, 64:227-231.

Schoeni, J., Glass, K.A., McDernott, J.L., and Wong, A.C.L. (1995). Growth and penetration of *Salmonella* Enteritidis, *Salmonella* Heidelberg and *Salmonella* Typhimurium in eggs. *International Journal of Food Microbiology*, 24:385-396.

Schwartz, S., Stephenson, B.D., Sarkar, D.H., and Bracho, M.R. (1975). Red, white and blue eggs as models of porphyrin and heme metabolism. *Annals of the New York Academy of Science*, 244:570-590.

Sekeroglu, A., Sarica, M., Demir, E., Ulutas, Z., Tilki, M., Saatci, M., and Omed, H. (2010). Effects of different housing systems on some performance traits and egg qualities of laying hens. *Journal of Animal and Veterinary Advances,* 9(12):1739-1744.

Sekeroglu, A., and Altuntas, E. (2009). Effects of egg weight on egg quality characteristics. *Journal of Food Agriculture,* 89:379-383.

Sencic, D., Antunovic, Z., Domacinovic, M., Speranda, M., and Steiner, Z. (2006). Egg quality from free range and cage system of keeping layers. *Stocarstvo,* 60:173-179.

Sharan, K., Siddiqui, J.A., Swarnkar, G., and Chattopadhyay, N. (2008). Role of calcium-sensing receptor in bone biology. *Indian Journal of Medical Research,* 127:274-286.

Sharp, P.F., and Powell, C.K. (1931). Increase in the pH of the White and Yolk of hen eggs. *Industrial and Engineering Chemistry,* 23(2):196-199.

Silversides, F.G., and Budgell, K. (2004). The relationships among measures of egg albumen height, pH, and whipping volume. *Poultry Science,* 83:1619-1623.

Silversides, F.G., and Scott, T.A. (2001). Effect of storage and layer age on quality of eggs from two lines of hens. *Poultry Science,* 80:1240-1245.

Silversides, F.G. (1994). The Haugh unit correction for egg weight is not adequate for comparing eggs from chickens of different lines and ages. *Journal of Applied Poultry Research,* 3:120-126.

Silyn-Roberts, H., and Sharp, R.M. (1986). Crystal growth and the role of the organic network in eggshell biomineralization. *Proceedings of the Royal Society,* London B Biological Sciences, 227:303-324.

Simkiss, K., and Taylor, T.G. (1971). Shell Formation. In D. J. Bell and B. M. Freeman (Eds.) Physiology and Biochemistry of the Domestic Fowl. Vol. III, pp. 1331-1343. Academic Press. London. New York.

Simkiss, K., and Taylor, T.G. (1957). A histochemical study of the organic matrix of hen eggshells. *Quarterly Journal of Microscopical Science,* 98(1):19-28.

Simons, P.C.M., and Wiertz, G. (1963). Notes on the structure of membranes and shell in the hen egg: an electron microscopical study. *Zeitschrift fur Zellforschung,* 59:555-567.

Singh, R., Cheng, K.M., and Silversides, F.G. (2009). Production performance and egg quality of four strains of laying hens kept in conventional cages and floor pens. *Poultry Science,* 88:256–264.

Slinko, V.G., McCall, B.J., Stafford, R.J., Bell, R.J., Hiley, L.A., Sandberg, S.M., White, S.A., and Bell, K.M. (2009). Outbreaks of *Salmonella* Typhimurium phage type 197 of multiple genotypes linked to an egg producer. *Communicable Diseases Intelligence,* 44(3):419-425.

Smeltzer, T.I., Orange, K., Peel, B., Runge, G.I. (1979). Bacterial penetration in floor and nest box eggs from meat and layer birds. *Australian Veterinary Journal,* 55:592-593.

Smith, A., Rose, S.P., Wells, R.G., and Pirgozliev, V. (2000). The effect of changing the excreta moisture of caged laying hens on the excreta and microbial contamination of their eggshells. *British Poultry Science,* 41:168-173.

Smith, H.W., and Tucker, J.F. (1980). The virulence of *Salmonella* strains for chickens: their excretion by infected chickens. *Journal of Hygiene (Lond).,* 82(3):479-488.

Solomon, S.E. (2009). Foundation is key for eggshell quality. *World Poultry,* Vol. 25, No. 09, pp. 16-18.

Solomon, S.E. (1992). Eggshell quality and microbial penetration. *Poultry International,* May, 1992, pp. 20-22.

Solomon, S.E. (1992). Can eggshell structure measure quality? *Poultry International,* April, 1992, 31(4):24-27.

Solomon, S.E. (1991). Egg and shell quality. Published by Wolfe publishing ltd. England. P. 149.

Solomon, S.E. (1986). Translucency- Some causative factors. *Poultry International* – July 1986, Vol. 25 (7): 24-28.

Solomon, S.E. (1985). Eggshell quality, a structural variation. *Poultry International* – October 1985, Vol. 24 (11): 58-62.

Solomon, S.E. (1983). Oviduct. In B. M. Freeman (Eds.) Physiology and Biochemistry of the Domestic Fowl, Vol. 4, pp. 379-419. Academic Press. London. New York.

Solomon, S.E. (1975). Studies on the isthmus region of the domestic fowl. *British Poultry Science,* 16(3):255-258.

Solomon, S.E., and Bain, M.M. (online) The normal eggshell. Retrieved online from www.poultryscience.org/docs/pba/1952.../1996%20Solomon.pdf

Sparks, N.H.C. (1994). Shell accessory materials: structure and function. In R.G. Board and R. Fuller (Eds.), *Microbiology of the Avian Egg* (pp. 25-42). London, U.K. : Chapman and Hall.

Sparks, N.H.C., and Board, R.G. (1984). Cuticle, shell porosity and water uptake through hen's eggshells. *British Poultry Science,* 25:267-276.

Stadelman, W.J. (1994). Contaminants of liquid egg products. In R.G. Board and R. Fuller (Eds.), *Microbiology of the Avian* Egg (pp. 139-151). London, U.K. : Chapman and Hall.

Stepien-Pysniak, D. (2010). Occurrence of gram negative bacteria in hens eggs depending on their source and storage conditions. *Polish Journal of Veterinary Sciences,* 13(3):507-513.

Sturkie, P.D., and Mueller, W.J. (1976). Reproduction in the female and egg production. In Avian Physiology, (pp. 303-328), 3rd Eds. Springer-Verlag, New York *Heidelberg* Berlin.

Tanaka, T., and Hurnik, J.F. (1992). Comparison of behavior and performance of laying hens housed in battery cages and an aviary. *Poultry Science,* 71:235-243.

Thomas, C., Daughtry, B., Padula, D., Jordan, D., Arey, G., Davey, K., Holds, G., Slade, J., and Pointon, A. (2006). An egg: *Salmonella* quantitative risk assessment model. A report for the Australian Egg Corporation Limited. March, 2006.

Thompson, M.B., and Goldie, K.N. (1990). Conductance and structure of eggs of adelie penguins, Pygoscelis adeliae, and its implications for incubation. *The Condor,* 92(2):304-312.

Tingari, M.D., and Lake, P.E. (1973). Ultrastructural studies on the uterovaginal sperm-host glands of the domestic hen, *gallus domesticus*. *The Journal of the Society for Reproduction and Fertility*, 34:423-431.

Tribe, I.G., Cowell, D., Cameron, P., and Cameron, S. (2002). An outbreak of *Salmonella* Typhimurium phage type 135 infection linked to the consumption of raw shell eggs in an aged care facility. *Communicable Disease Intelligence,* 26:38-39.

Tuohimaa, P., Joensuu, T., Isola, J., Keinanen, R., Kunnas, T., Niemala, A., Pekki, A., Wallen, M., Ylikomi, T., and Kulomaa, M. (1989). Development of progestin-specific response in the chicken oviduct. *International Journal of Developmental Biology,* 33:125-134.

Tumova, E., Englmaierova, M., Ledvinka, Z., and Charvatova, V. (2011). Interaction between housing system and genotype in relation to internal and external egg quality parameters. *Czech Journal of Animal Science,* 56(11):490-498.

Tumova, E., and Ledvinka, Z. (2009). The effect of time of oviposition and age on egg weight, egg components weight and eggshell quality. *Arch.Geflugelk.,* 73(2):110-115.

Tumova, E. and Ebeid, T. (2005). Effect of time of oviposition on egg quality characteristics in cages and in a litter housing system. *Czech Journal of Animal Science,* 50(3): 129–134.

Tyler, C. (1956). Studies of egg shells. VII. Some aspects of structure as shown by plastic models. *Journal of Science, Food and Agriculture,* 7(7):483-493.

Vadehra, D.V., Baker, R.C., and Naylor, H.B. (1970). Infection routes of bacteria into chicken eggs. *Journal of Food Science,* 61(35):61-62.

Van Den Brand, Dr. H., Parmentier, H.K., and Kemp, B. (2004). Effects of housing system (outdoor vs cages) and age of laying hens on egg characteristics, *British Poultry Science,* 45:(6)745-752.

Varguez-Montero, G., Sarmiento-Franco, L., Santos-Ricalde, R., and Segura-Correa, J. (2012). Egg production and quality under three housings in the tropics. *Tropical Animal Health and Production,* 44:201-204.

Van Toledo, B., Parsons, A.H., and Combs, JR. G.F. (1982). Role of ultrastructure in determining eggshell strength. *Poultry Science,* 61:569-572.

Walden, C.C. Allen, I.V.F., and Trussell, P.C. (1956). The role of the eggshell and shell membranes in restraining the entry of microorganisms. *Poultry Science,* 35:1190-1196.

Wall, H., Tauson, R., and Sorgjerd, S. (2008). Bacterial contamination of eggshells in furnished and conventional cages. *Journal of Applied Poultry Research,* 17:11-16.

Wang, X.L., Zheng, J.X., Ning, Z.H., Qu, L.J., Xu, G.Y., and Yang, N. (2009). Laying hen performance and egg quality of blue-shelled layers as affected by different housing systems. *Poultry Science,* 88:1485-1492.

Wang, X.L., Deng, X.M., Zhao, C.J., Li, J.Y., Xu, G.Y., Lian, L.S., and Wu, C.X. (2007). Study of the deposition process of eggshell pigments using an improved dissolution method. *Poultry Science,* 86:2236-2238.

Wang, H., and Slavik, M.F. (1998). Bacterial penetration into eggs washed with various chemicals and stored at different temperatures and times. *Journal of Food Protection,* 61(3):276-279.

Wellman-Labadie, O., Picman, J., and Hincke, M.T. (2008). Antimicrobial activity of th anseriform outer eggshell and cuticle. *Comparative Biochemistry and Physiology,* Part B, 149:640-649.

Williams, K.C. (1992). Some factors affecting albumen quality with particular reference to Haugh unit Score. *World Poultry Science Journal,* 48:5-16.

Williams, J. E., and Dillard, L. H. (1973). The effect of external shell treatments on *Salmonella* penetration of chicken eggs. *Poultry Science,* 52:1084–1089.

Williams, J.E., Dillard, L.H., and Hall, G.O. (1968). The penetration patterns of *Salmonella* Typhimurium through the outer structures of chicken eggs. *Avian Diseases,* 12(3):445–466.

With, T.K. (1973). Porphyrin in eggshell. *Biochemical Journal,* 137:597-598.

Wong, M., Hendrix, M.J.C., Von Der Mark, K., Little, C., and Stern, R. (1984). Collagen in the eggshell membranes of the hen. *Developmental Biology,* 104:28-36.

Wyburn, G.M., Johnston, H.S., Draper, M.H., and Davidson, M.F. (1973) The ultrastructure of the shell forming region of the oviduct and the development of the shell of *Gallus domesticus*. *Quarterly Journal of Experimental Physiology*, 58:143–151.

Wyburn, G.M., Johnston, H.S., and Draper, M.H. (1970). The magnum of the hen's oviduct as a protein secreting organ. *Journal of Anatomy*, 106:174.

Yakubu, A., Salako, A.E., and Ige, A.O. (2007). Effects of genotype and housing system on the laying performance of chickens in different seasons in the semi humid trophics. *International Journal of Poultry Science,* 6(6):434-439.

Zita, L., Tumova, E., and Stolc, L. (2009). Effects of genotype, age and their interaction on egg quality in brown-egg laying hens. *Acta Veterinaria Brno,* 78:85-91. Doi:10.2754/avb200978010085

Appendix

A.Preparation of media and solutions

Bismuth Sulphate Salt Agar (Oxoid Australia)

A 20 g of Bismuth Sulphate powder was added into 500 ml distilled water and heated in the oven to boiling temperature in order to dissolve it properly. The solution was cooled down to room temperature, poured into sterile Petri dishes in the Bio Safety cabinet to avoid any external contamination and was allowed to solidify properly.

Violet Red Bile Glucose Agar (VRBGA, Oxoid Australia)

VRBGA is one of the differential media used for growth and enumeration of gram negative entero pathogens. VRBGA was prepared by adding 38.5g of the powder into 100 mL distilled water and heated to boiling temperature in an oven. After cooling, the media was poured into sterile Petri dishes in the Bio Safety cabinet and allowed to solidify. This media was used for the culturing and enumeration of total Enterobacteriaceae count.

MacConkeys Agar (Oxoid Australia)

This media was prepared by dissolving 52g of the powder in 1L of distilled water and was autoclaved at 121°C for 15 minutes to sterilize properly. MacConkeys Agar is one of the selective media used for the total bacterial count of Gram negative bacteria.

Rappaport –Vissiliadis Soya Peptone broth (RVS, Oxoid Australia)

It was prepared by adding 26.75g of its powder into 1L of distilled water. After dissolving properly by simply shaking, the broth was poured into test tubes (10mL each) and autoclaved at 121°C for 15 minutes to sterilize. Normally it is deep blue in colour and is one of the selective enrichment medium for the isolation of *Salmonella* spp.

Triple sugar iron agar (TSI, Oxoid Australia)

TSI was prepared by dissolving 65g of powder into 1 litre of distilled water and heating to boiling. After pouring into test tubes, the media was sterilized by autoclaving at 121°C for 15 minutes. Slant was prepared for the culturing of *Salmonella*. In case of *Salmonella* positive samples its colour turns up blackish with the production of H_2S gas.

Buffered peptone water (BPW, Oxoid Australia)

It is considered as one of the pre enrichment medium for the isolation of *Salmonella* from various sources. Sterile solution was prepared by dissolving 20 g of BPW in 1 litre of distilled water and was autoclaved at 121°C for 15 minutes.

Phosphate Buffered Saline (PBS)

Homogenous solution (pH 7.4) was prepared by dissolving 8g Sodium Chloride (NaCl), 0.2 g Potassium Chloride (KCl_2), 1.44 g di-Sodium hydrogen phosphate dodecahydrate (Na_2HPO_4) and 0.22 g Potassium di hydrogen orthophosphate (KH_2PO_4) in 1 litre distilled water and sterilized by autoclaving at 121°C for 15 minutes.

Xylose Lysin Deoxycholate (XLD, Oxoid Australia)

53 g of XLD powder was weighed into sterile bottle into which 1 L water (RO) was added. The solution was gently mixed and heated with frequent agitation until boiled. The boiled media was transferred into water bath of 50°C. Over heating of the media was avoided. 2 g of Agar Bacteriological powder (Oxoid Australia) was added to 43 g of XLD to enhance its solidification power.

EDTA solution preparation

For preparing 1 litre of 0.34 M solution, 126.56g of ethylene di amino tetra acetic acid di sodium salt (EDTA) powder (Molecular weight= 372.24) was dissolved in distilled water. 15 g of NaOH was added bit by bit in order to dissolve the EDTA properly by raising its pH. The pH was adjusted to 7.5 by adding the NaOH.

Molarity = amount of solute dissolved/volume of solution in 1 litre

1 molar solution of EDTA = 372.24/1000 ml

0.34 M solution of EDTA= 0.34 * 372.24 = 126.56 g

Methanol-HCl solvent preparation

A 2 litre of Methanol-HCl (2:1) solvent having pH 7.4 was prepared by adding slowly 500 ml of absolute HCl to 1500 Methanol in a sterile beaker. The Beaker was embedded in ice as the reaction between the two reagents produced heat.

MST cuticle blue stain preparation

A powdered stain (14 g) was dissolved in 1 litre distilled water in a clean bottle and mixed thoroughly. Large volume was prepared accordingly and stored in the fridge until used. MST cuticle blue stain powder was purchased from M S Technologies UK.

B. Eggshell and egg internal quality measurements

Variables	Flocks age (weeks)												P value		
	Conventional Cages						Free Range								
	25	35	45	55	65	75	25	35	45	55	65	75	P	A	P*A
Translucency score	2.23 ±0.20	1.17 ±0.18	1.03 ±0.19	2.40 ±0.21	1.63 ±0.26	1.43 ±0.21	2.77 ±0.27	*2.40 ±0.25	*3.97 ±0.17	*3.13 ±0.19	*2.97 ±0.18	*3.10 ±0.17	<0.0001	0.0002	<0.0001
Shell reflectivity (%)	27.10 ±0.67	30.33 ±0.41	28.87 ±0.59	30.17 ±0.82	29.70 ±0.67	29.40 ±0.57	*29.67 ±0.79	*32.57 ±0.92	*33.37 ±0.52	*37.63 ±1.30	*38.70 ±0.98	*33.97 ±1.2	<0.0001	<0.0001	0.0001
Egg weight (g)	60.35 ±0.87	63.90 ±0.75	63.27 ±0.89	62.97 ±0.78	64.47 ±0.82	68.60 ±0.82	*51.68 ±0.59	62.57 ±0.85	64.30 ±0.58	61.73 ±0.84	*61.97 ±0.85	*63.84 ±1.0	<0.0001	<0.0001	<0.0001
Breaking shell strength (N)	40.07 ±1.4	41.83 ±0.96	39.55 ±0.90	38.19 ±0.99	40.22 ±1.60	36.67 ±1.70	41.31 ±1.60	38.45 ±1.50	*42.74 ±1.30	*41.50 ±1.30	*35.52 ±1.50	37.49 ±1.50	0.9182	0.0173	0.0112
Deformation unit (µm)	328.3 ±12.0	307.3 ±5.45	298.0 ±5.37	299.0 ±5.04	294.0 ±7.62	320.0 ±15.8	334.6 ±7.55	316.6 ±6.58	*322.6 ±8.01	*321.3 ±6.57	294.0 ±12.0	261.6 ±8.04	0.8885	<0.0001	<0.0001
Shell weight (g)	5.40 ±0.07	5.88 ±0.07	5.85 ±0.08	5.67 ±0.08	5.99 ±0.12	6.14 ±0.13	*4.73 ±0.09	*5.20 ±0.11	5.81 ±0.06	5.51 ±0.12	5.70 ±0.09	5.94 ±0.12	<0.0001	<0.0001	0.0009
Percentage shell	9.02 ±0.09	9.21 ±0.09	9.26 ±0.11	9.03 ±0.14	9.29 ±0.13	8.96 ±0.17	9.15 ±0.15	*8.33 ±0.15	9.05 ±0.09	8.92 ±0.13	9.22 ±0.14	9.33 ±0.15	0.0853	0.0048	0.0002
Shell thickness (µm)	370.1 ±3.49	389.4 ±3.82	378.0 ±3.85	376.9 ±5.13	392.4 ±6.02	389.0 ±5.86	*354.8 ±5.41	*352.5 ±5.74	385.3 ±3.55	381.5 ±6.01	387.3 ±4.60	389.3 ±4.72	0.0088	<0.0001	<0.0001
Albumen height (mm)	11.04 ±0.14	10.65 ±0.18	10.24 ±0.21	9.39 ±0.20	10.07 ±0.20	9.95 ±0.28	*9.04 ±0.18	*9.33 ±0.23	*8.53 ±0.20	*6.76 ±0.29	*7.32 ±0.23	*4.81 ±0.24	<0.0001	<0.0001	<0.0001
Haugh Unit	103.5 ±0.62	101.0 ±0.77	99.23 ±0.92	95.40 ±0.96	98.60 ±1.03	96.67 ±1.57	*96.50 ±0.89	*95.17 ±1.14	*90.77 ±1.11	*80.13 ±1.98	*84.20 ±1.38	*63.33 ±2.31	<0.0001	<0.0001	<0.0001
Yolk color	10.07 ±0.09	10.90 ±0.16	11.03 ±0.13	10.50 ±0.15	11.70 ±0.13	11.40 ±0.16	*7.73 ±0.31	*10.03 ±0.15	*9.80 ±0.11	10.07 ±0.19	*9.37± 0.19	11.80± 0.21	<0.0001	<0.0001	<0.0001

* Values indicates significantly different from cage system at the same age; Mean ± SE

P- Production system; A- Age; P*A- Production system & Flock age interaction

C. Ultra structural scoring of shell mammillary layer

Variables	Flocks age (weeks)												P value		
	Conventional Cages						Free Range								
	25	35	45	55	65	75	25	35	45	55	65	75	P	A	P*A
Mammillary cap size	1.48 ±0.11	1.70 ±0.11	2.33 ±0.12	2.13 ±0.18	2.00 ±0.09	1.90 ±0.10	*1.83 ±0.09	*2.10 ±0.12	2.03 ±0.14	2.13 ±0.12	2.27 ±0.11	*2.20 ±0.09	0.0067	<0.0001	0.0090
Confluence	3.00 ±0.11	2.83 ±0.11	2.37 ±0.13	2.23 ±0.14	1.77 ±0.14	1.83 ±0.16	2.70 ±0.14	2.73 ±0.11	2.33 ±0.14	2.30 ±0.17	1.87 ±0.16	*1.40 ±0.12	0.1401	<0.0001	0.3149
Cap quality	1.48 ±0.11	1.67 ±0.15	2.07 ±0.14	2.57 ±0.11	2.37 ±0.18	2.23 ±0.11	1.60 ±0.12	2.03 ±0.18	*2.57 ±0.18	2.73 ±0.16	2.43 ±0.16	2.40 ±0.12	0.0070	<0.0001	0.6687
Early fusion	3.21 ±0.11	2.80 ±0.10	2.77 ±0.08	2.20 ±0.13	2.37 ±0.09	2.40 ±0.09	3.00 ±0.09	*2.37 ±0.10	*2.37 ±0.11	2.13 ±0.12	2.20 ±0.12	2.33 ±0.09	0.0003	<0.0001	0.3285
Late fusion	2.45 ±0.14	3.40 ±0.12	2.67 ±0.09	3.00 ±0.07	3.07 ±0.08	3.07 ±0.07	2.73 ±0.13	3.13 ±0.08	*2.97 ±0.09	3.03 ±0.03	2.97 ±0.03	3.00 ±0.05	0.5407	<0.0001	0.0062
Alignment	2.17 ±0.17	2.00 ±0.14	2.30 ±0.11	2.87 ±0.13	2.77 ±0.12	2.47 ±0.12	1.97 ±0.12	2.33 ±0.10	*2.77 ±0.09	2.77 ±0.10	2.67 ±0.11	*2.87 ±0.12	0.0600	<0.0001	0.0111
Type A body	1.17 ±0.07	1.37 ±0.12	1.23 ±0.08	1.50 ±0.09	1.57 ±0.09	1.47 ±0.09	*1.00 ±0.00	1.27 ±0.08	1.30 ±0.08	1.77 ±0.10	1.70 ±0.11	*1.90 ±0.09	0.0440	<0.0001	0.0082
Type B body	1.52 ±0.12	1.83 ±0.14	2.00 ±0.14	2.13 ±0.12	1.87 ±0.09	1.90 ±0.11	1.40 ±0.12	1.97 ±0.11	*2.20 ±0.16	2.53 ±0.10	*2.63 ±0.14	*2.80 ±0.18	<0.0001	<0.0001	0.0006
Aragonite	1.00 ±0.00	1.10 ±0.06	1.03 ±0.03	1.13 ±0.09	1.07 ±0.05	1.00 ±0.00	1.00 ±0.00	1.20 ±0.11	1.17 ±0.09	1.33 ±0.10	1.07 ±0.05	1.03 ±0.03	0.0381	0.0032	0.5660
Cubic	1.10 ±0.06	1.13 ±0.06	1.17 ±0.08	1.13 ±0.08	1.13 ±0.06	1.07 ±0.05	1.03 ±0.03	1.03 ±0.03	1.20 ±0.07	1.07 ±0.05	1.03 ±0.03	1.10 ±0.07	0.4327	0.1947	0.7456
Cubic cone formation	1.00 ±0.00	1.37 ±0.09	2.90 ±0.12	1.90 ±0.12	2.87 ±0.12	1.93 ±0.13	1.00 ±0.00	*2.10 ±0.15	*2.57 ±0.11	2.13 ±0.11	*2.40 ±0.13	2.13 ±0.13	0.3520	<0.0001	<0.0001
Cuffing	2.86 ±0.12	2.83 ±0.09	1.93 ±0.11	1.47 ±0.12	1.37 ±0.09	1.30 ±0.09	2.90 ±0.14	*2.13 ±0.14	1.97 ±0.11	1.27 ±0.08	1.17 ±0.07	1.20 ±0.07	<0.0001	0.0024	0.0069
Changed membrane	1.86 ±0.22	1.20 ±0.07	1.23 ±0.10	1.40 ±0.10	1.10 ±0.06	1.10 ±0.06	*2.63 ±0.17	*1.53 ±0.10	1.10 ±0.07	*1.00 ±0.00	1.17 ±0.08	1.00 ±0.00	0.1350	<0.0001	<0.0001
Depression	1.07 ±0.05	1.03 ±0.03	1.00 ±0.00	1.10 ±0.06	1.13 ±0.06	1.00 ±0.00	1.00 ±0.00	1.20 ±0.09	1.10 ±0.06	1.07 ±0.05	1.10 ±0.05	1.20 ±0.10	0.0854	0.5658	0.0577
Erosion	1.00 ±0.00	1.00 ±0.00	1.07 ±0.05	1.07 ±0.05	1.10 ±0.06	1.03 ±0.03	1.00 ±0.00	1.03 ±0.03	1.07 ±0.05	1.23 ±0.08	1.10 ±0.06	1.17 ±0.07	0.0393	0.0131	0.2626
Hole	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.03	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.03 ±0.00	1.03 ±0.00	1.00 ±0.03	1.00 ±0.00	1.00 ±0.00	0.5527	.	0.3104

* Values indicates significantly different from cage system at the same age; Mean±SE

P- Production system; A- Age; P*A- Production system & Flock age interaction