Chapter 1. Literature Review

1.1. Introduction.

Table eggs are an important part of the human diet (Lublin and Sela, 2008); they are a cheap, readily available (Aboonajmi, et al. 2010) and generally a safe food source. They are, however, periodically implicated in cases of food borne illness in Australia and around the world (OzFoodNet Working Group, 2010). Eggs in Australia are considered to constitute a low to medium risk of food borne illness (Food Standards Australia New Zealand, 2009). The most significant bacterial risk associated with eggs are bacteria of the genus *Salmonella;* there are thousands of *Salmonella* serovars with different impacts on animal and human health. *Salmonella* serovar Enteritidis is not endemic in Australian poultry flocks; however serotypes such as *S.* Typhimurium are of concern. In 2010, the incidence of *Salmonella* infection in Australia was 53.4 people per 100,000 people, with food poisoning costing the Australian economy approximately $1.2 billion (OzFoodNet Working Group, 2010). The eggshell is a significant physical barrier to penetrating microorganisms, providing both contamination protection and a suitable container for the egg contents.

1.2. Microbiology of the Egg.

Eggs are a safe, nutritious and available food source; however, there are risks associated with consuming eggs contaminated with pathogenic bacteria. Table eggs made up approximately 12% of confirmed human salmonellosis cases in the European Union in 2008 (European food safety report, 2010) while, in Australia in 2010, *Salmonella* infection accounted for 49% of reported human food poisoning cases (OzFoodNet Working Group, 2010). Overall contamination rates of the egg even when exposed to *Salmonella* are low (Kretzschmar-Mccluskry, et al. 2009); however due to the large number of eggs consumed, even a very low rate of infection is important for population health. The egg yolk is an ideal growth medium for microorganisms which are hazardous to humans (Chousalkar, et al. 2010) and contamination of the yolk quickly results in spoilage and rapid growth of bacteria, due to the nutritious environment and lack of anti-bacterial agents in the yolk (Ayres and Taylor, 1956).

Eggs are contaminated by pathogenic microorganisms in a number of ways, and there is a range of microorganisms that have the ability to penetrate the egg and survive until consumption. However, the single largest food safety threat comes from the different serotypes of *Salmonella enterica*. Microorganisms can contaminate eggs at a number of different stages, from production to processing, preparation and consumption (De Reu, et al. 2006). However, this review will focus on penetration of the shell by bacteria and the eggshell structures that facilitate bacterial penetration. Wall, et al. (2008) found that, regardless of housing system, eggs will always come into contact with bacteria from the environment when laid, and to a varying degree become contaminated with bacteria. However, one of the benefits of the conventional cage

system is that faeces and eggs are adequately separated immediately post lay (Wall, et al. 2008). Another potential source of shell contamination is cross contamination in cooking and processing centres (Lublin and Sela, 2008).

In the last 20 years, the consumers' opinion of a 'good' egg has changed from one with a clean shell and stable albumen (Board, 1980) to one that has strong microbial integrity (Jones, et al. 2004). The egg as the natural incubator for the developing chick has a number of defensive mechanisms, both physical and chemical, with the purpose of preventing bacterial colonization of the egg.

1.2.1. Egg Contamination by Microorganisms

There are two methods of microbial contamination of the egg. Vertical (trans-ovarian) contamination occurs when the eggs are infected during their formation, either in the ovary or oviduct. Horizontal transmission occurs after lay when the egg is exposed to bacteria post lay and the bacteria enter through the shell (De Reu, et al. 2006; Dolman and Board, 1991; Gantois, et al. 2008; Gantois, et al. 2009; Miyamoto, et al. 1996). Bacterial contamination is often not apparent; Ayres and Taylor (1956) found that visual examination of the eggs failed to reveal any evidence of incipient spoilage until the bacterial loads exceeded several hundred million cells per egg.

Shell penetration by bacteria or horizontal transmission is the focus of this project. Stokes, et al. (1956) surmised that eggs are more commonly contaminated post lay and Gast, et al. (2007) found that the natural deposition of bacteria within the yolk was relatively uncommon. The egg is exposed to a number of contaminants post lay; in a commercial shed, eggs in a contaminated nest box can lead to outer shell contamination (Gantois, et al. 2009) and post lay

processing may also contaminate eggs. As the hen has a common opening for intestinal, urinary and reproductive tracts, this contributes to faecal exposure (Chousalkar, et al. 2010). Miyamoto, et al. (1996), however, dispute this commenting that, because the terminal end of the vagina protrudes outside the cloaca during oviposition, the egg rarely touches the contaminated cloaca. Faecal contaminated by bacteria is a potential risk factor as faeces provide both the nutrients for the bacteria to grow and some level of physical protection (Gantois, et al. 2009; Schoeni, et al. 1995).

Several researchers have commented that, immediately after the egg is laid, it cools from the hen's body temperature (42°C) to room temperature, causing the contents of the egg to contract, effectively 'sucking' bacteria in from the outside of the shell (Berrang, et al. 1990; Berrang, et al. 1999; Cox, et al. 2000_a; De Reu, et al. 2008a). There are a number of potential sources of bacterial contamination of the freshly laid egg; the hatchery environment, trucks, nest boxes and cold storage (Cox, et al. 2000_b). Commercial production facilities attempt to limit interactions between eggs and any pathogenic bacteria and effective biosecurity is in itself a partly effective control measure. Wright and Frank (1956) found that the post lay cooling of the egg had no effect on the penetration rates of *Salmonella* Typhimurium. However, they only cooled the egg for a short period of time, potentially not allowing time for bacterial entry. Temperature is the most important factor in controlling bacterial penetration of the egg; *Salmonella* growth is restricted at 4°C. However, Schoeni, et al. (1995) and Wright and Frank (1956) found that *Salmonella* could survive at 4°C and grow to large numbers over even a short time of 25 days. Jones, et al. (2004) suggested that storage of eggs at room temperature may lead to accelerated degradation of the antimicrobial systems of the egg, leading to increased

bacterial loads. There has been a lot of research into the time taken for bacteria to penetrate the shell. Taylor (1956) and De Reu, et al. (2006) concluded that eggshell penetration occurred often within zero to two days of inoculation and Jones, et al. (2004) found that penetration could occur in as little as 24 hours. Stokes, et al. (1956), however, commented that a week is required for *Salmonella* to penetrate the shell and the membranes and appear within the egg. There is debate about the effect of hen age on the rate of bacterial penetration. Eggshell penetration was found to remain almost constant during the entire laying period of the hen from 34 weeks to 74 weeks by De Reu, et al. (2006); this is contested by the results of Messens, et al. (2005_b), who found significantly more penetration in a young flock than an older one.

Chilled storage post lay is a key factor in preventing the contamination of eggs (Schoeni, et al. 1995); Clay and Board, (1991) comment that the chilled storage of eggs can be part of the protective barrier between the laying flock and the consumer. However, *Salmonella* retained in the membrane do not necessarily die out during chilled storage (Clay and Board, 1991). Schoeni, et al. (1995) also found that certain *Salmonella* serovars including Typhimurium are able to persist and even grow at ≤10°C. Schoeni, et al. (1995) found that, after as little as one day at 25°C, there was an increase of three to five log units. The egg shell is the most significant physical barrier to invading microorganisms, and Guard-Bouldin and Burh, (2006) advocate targeting eggs with detrimental physical characteristics (such as microcracks) and removing them from the commercial supply chain.

1.2.2. *Salmonella*

Salmonella is a gram negative rod in the family enterobacteriaceae; they are relatively small bacteria 0.5 µm to 2 µm in length and most strains are motile with peritrichous flagella (Cox, et al. 2000_a). *Salmonella* is divided into two species, *enterica* and *bongori*; *enterica* is a group of over 2,500 serotypes that efficiently contaminates the internal contents of the egg (Guard-Bouldin and Burh, 2006). *Salmonella* has often been associated with raw or undercooked table eggs and egg products, among other foods (Jones, et al. 2004). There are a number of *Salmonella* serovars that can penetrate eggs and, within each serovar, there are a number of phage types, each with different virulence, pathogenicity and characteristics (Okamura, et al. 2001). The *Salmonella* serotype most likely to cause food borne illness worldwide is *Salmonella* Enteritidis (Miller, et al. 2010). However, the Australian poultry industry is considered to be free of *Salmonella* Enteritidis (Chousalkar, et al. 2010; OzFoodNet Working Group, 2010) due to geographic isolation and tight import controls on food and animal products.

Human salmonellosis is usually characterised by acute onset of fever, abdominal pain, nausea and sometimes vomiting (Lin, et al. 1997). Symptoms are usually mild and self-limiting (OzFoodNet Working Group, 2010); however, serious effects can be both short term, such as dehydration, or long term resulting in blood infection (European Food Safety Report, 2010). The number of confirmed cases of human salmonellosis in the EU has decreased between 2004 and 2008 as shown in Figure 1.2.2. (European Food Safety Report, 2010). However, in Australia in 2010, there was an increase in salmonellosis cases compared to the average of the previous 5 years (OzFoodNet Working Group, 2010). In the US, between 696,000 and 3,840,000 cases of food borne salmonellosis occur annually. The results of the European Food Safety Report (2010)

and the work of Lin, et al. (1997) showed that *Salmonella* disproportionally affected young people; however, this may be explained by the increased severity of the disease in young people, resulting in greater reporting of the illness. Minor cases of food born salmonellosis often go unreported; therefore, there are limits to our understanding of the *Salmonella* serotypes responsible for minor cases.

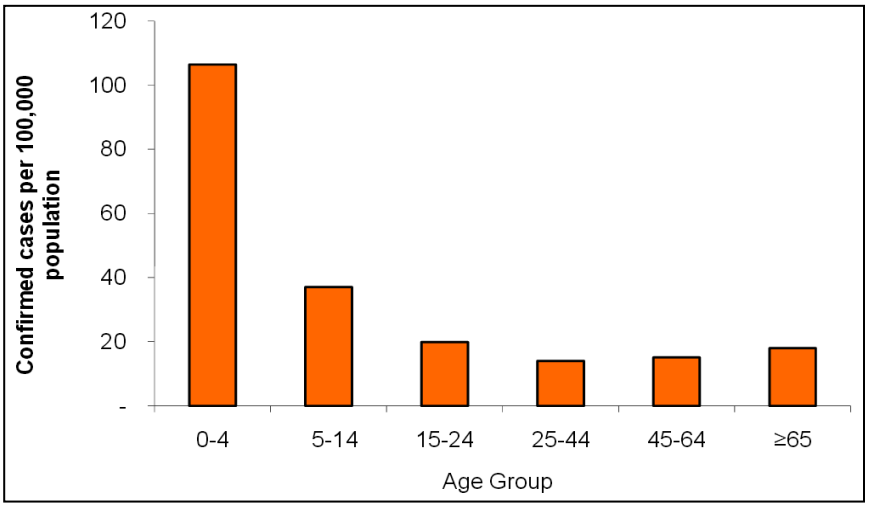


Figure 1.2.1. Age specific distribution of human salmonellosis EU 2004-2008 (Sourced from European Food Safety Report, 2010).

Eggs may become infected with *Salmonella* either as a result of infection of the oviduct or by faecal contamination (De Reu, et al. 2008). It is a feature of all *Salmonella* infections that there is a colonization of the gastro intestinal tract associated with faecal shedding (Morgan, et al. 2004).

1.2.3. *Salmonella* Enteritidis

Salmonella Enteritidis is considered the most common *Salmonella* serovar implicated in egg-borne food poisoning around the world; this is mainly due to the ability of this serovar to colonise the upper oviduct of the hen. Other *Salmonella* serovars including *S*. Typhimurium

generally contaminate eggs horizontally and are usually found in the egg following shell penetration (Martelli and Davies, 2012; European Food Safety Report, 2010). *S.* Enteritidis is considered the serotype of greatest concern worldwide, accounting for >62% of reported food poisoning cases in Europe and approximately 80% of reported cases in the US; within the EU >90% of all farm egg *Salmonella* isolations proved to be *S*. Enteritidis (Gantois, et al. 2008). *S*. Enteritidis is not endemic in Australian layer flocks and, of the 587 confirmed cases in Australia in 2008, 547 had a confirmed travel history (OzFoodNet Working Group, 2009). The strict quarantine rules surrounding the import of animals and animal products have helped prevent *S*. Enteritidis from becoming endemic in Australia (Martelli and Davies, 2012). There are over 30 phage types of *S.* Enteritidis; however it is generally accepted that PT4 is the most dangerous strain to humans (Board and Fuller, 1994).

In the UK, vaccination of larger laying flocks began in 1998 and, since the introduction of these vaccinations and other control mechanisms in breeding flocks, the number of *Salmonella* Enteritidis strain PT4 isolates from breeder flocks decreased from 15,564 in 1990 to 581 in 2009 (Martelli and Davies, 2012).

1.2.4. Salmonella Typhimurium

Salmonella Typhimurium has been associated with several egg-*Salmonella* out-breaks including incidents in France and Italy (European food safety report, 2010). In Australia, where *S.* Enteritidis is not endemic; *S.* Typhimurium is most commonly associated with egg-borne *Salmonella* food poisoning (41%) (OzFoodNet Working Group, 2009), which appears to be caused by contamination of the shells. In Europe, *S.* Typhimurium is most commonly associated with

pork meat out-breaks (Martelli and Davies, 2012). *S.* Typhimurium is also the predominant serovar in other regions including northern India and Iran (Martelli and Davies, 2012).

It has been demonstrated that *S.* Typhimurium can survive in the egg albumen during egg formation, which may be due to *S.* Typhimurium's apparent ability to resist the effects of lysozyme (Martelli and Davies, 2012). Schoeni, et al. (1994) state that *S.* Typhimurium can grow at 10°C, and persist at 4°C if allowed to infect the albumen. Olesiuk (1969) states that *S.* Typhimurium infections can be present in the intestines without producing a serological response or clinical signs, potentially leaving it undetected in flocks. Olesiuk (1969) also stated that the most accurate culture for identifying *S.* Typhimurium in flocks is litter swabs, which proved more dependable than cultures of cloaca, water, nest litter, egg yolks or embryos. Gantois, et al. (2008) found that *S.* Typhimurium has the ability to colonise the reproductive tract, similar to *S.* Enteritidis.

Padron (1990) exposed recently laid eggs to a spray of *S.* Typhimurium designed to mimic the bacterial contamination by faeces; the results showed that 100% of eggs were infected in the albumen and 83% of yolks were shown to be infected. Padron (1990) also concluded that, in order to prevent infection with *S.* Typhimurium, the most important factor is to improve nest sanitation. Gast, et al. (2007), however, propose rapid refrigeration of eggs as the critical step in controlling *Salmonella*.

1.2.5. Pathogenicity of *Salmonella*

There has been no single experiment that has compared every *Salmonella* serovar, although *S.* Enteritidis and *S.* Typhimurium are included in many studies. *S.* Typhimurium was found to result in greater bird mortality than *S.* Enteritidis, *S*. Virchow, *S*. Heidelberg and *S.* Hadar (Gantois, et al. 2008), while *Salmonella* Enteritidis has been the major cause of a food-borne salmonellosis pandemic in humans over the last 20 years (Gantois, et al. 2009; Jones, et al. 2004). In Australia, 44% of salmonellosis cases were reported as *Salmonella* Typhimurium; however *Salmonella* Infantis was found to be increasing, producing 2.2 times as many cases in 2010 as 2009. Bauer-Garland, et al. (2006) made two determinations concerning *S*. Typhimurium; firstly, *S.* Typhimurium appears to be more capable of developing resistance to anti-microbial drugs and secondly, *Salmonella* Typhimurium shed more bacteria into the environment than other *Salmonella* serovars which may explain the increased rate of *S.* Typhimurium infection among flocks.

1.2.6. Shell Penetration by *Salmonella* Serovars

There are significant differences in the shell penetration and survivability in the egg of different *Salmonella* serovars (Gantois, et al. 2008). Flagella were thought to be required for *Salmonella* to migrate through the albumen towards the vitelline membrane, and flagella are common to most *Salmonella* serotypes (Gantois, et al. 2008); De Reu, et al. (2006), commented that the motile, non clustering nature of *Salmonella* serovars favoured penetration. However, Stokes, et al. (1956) found that non-motile serovars *Salmonella* Pullorum and *Salmonella* Gallinarum penetrate eggs as readily as motile *Salmonella* serotypes. Stokes, et al. (1956)

concluded that the enzymatic, proteolytic activities of the bacteria are of far greater importance than bacterial motility.

The degree of *Salmonella* deposition on the shell surface may not be sufficiently high to result in albumen penetration, although Stokes, et al. (1956) found that as few as 100 bacterial cells could result in infection and growth of bacteria within the egg. Gast, et al. (2007) showed that 8 different *Salmonella* serotypes were able to penetrate the shell readily at 30°C; however, penetration of the yolks occurred infrequently at 20°C, but significantly more commonly at 30°C. When incubated at 1°C, five *Salmonella* serotypes (*S.* Typhimurium, *S*. Oranienburg, *S.* Montevideo, *S*. Pullorum and *S*. Gallinarum) all failed to penetrate the shell and appear in the albumen, even over extended storage times of up to 168 days (Stokes, et al. 1956). At 1°C, not only did the *Salmonella* fail to penetrate the shell, but when inoculated into the shell, no bacteria remained after 6 months storage at 1°C. De Reu, et al. (2006) showed that, despite the anti-microbial defences of the egg, a number of bacterial strains were able to penetrate the membranes and remain viable in the albumen for 21 days. Stokes, et al. (1956) showed that, after 4 weeks, 5 serovars of *Salmonella* (*S*. Typhimurium, *S.* Oarnienburg, *S*. Montevideo, *S*. Pullorum and *S*. Gallinarum) had penetrated the eggshells and multiplied significantly within the egg. Within the *Salmonella* serotypes, *S*. Enteritidis and *S*. Typhimurium are considered the most important serovars for human illness (Barrow, 1992). Ibarra and Steele-Mortimer (2009) comment that *S.* Typhimurium is one of the most common causes of food borne gastroenteritis in humans, and also an important pathogen in food producing animals, such as cattle, pigs, and chickens. Examination of chickens naturally infected with *S.* Enteritidis PT4 has shown that infection consistently involved the colonization of the body tissues (Chart, et al. 1992).

The course of microbial infection of shell eggs can be considered in stages, 1. penetration of the cuticle and shell, 2. colonization of the underlying membranes, and 3. contamination of the albumen leading eventually to generalised infection of egg contents (Clay and Board, 1991). Sparks and Board (1984) found that water either as a vapour or as a liquid appears to be essential for microbial penetration, thus a high 95% humidity in the atmosphere surrounding the egg is a prerequisite for growth of mould or bacteria on the cuticle. Messens, et al. (2006) found that, while temperature played a role in the rate of bacterial shell penetration, humidity did not significantly affect penetration rates. Again, rapid cooling has been shown to prevent growth of *Salmonella* on the outside of the shell (Lublin and Sela, 2008). Although the egg is naturally equipped with barriers that prevent microorganisms from penetrating, these barriers have been shown to fail (Kretzschmar-Mccluskry, et al. 2009). The exact mechanism of shell contamination by *Salmonella* remains unclear (Schoeni, et al. 1995). Carnarious, et al. (1996) suggested that variation in the shell quality may lead to infection by *Salmonella*.

1.2.7. Colonisation of the oviduct by *Salmonella* Serovars

Vertical transmission to eggs of *Salmonella* requires the organism to colonize parts of the oviduct, and different *S.* serotypes have different affinities for oviduct colonization. Gantois, et al. (2009) concluded that it is not yet clear which is the most important entry method of *S.* Enteritidis, horizontal or vertical. When eggs are developing in a *S.* Enteritidis positive oviduct, with a high *Salmonella* population, they are likely to be contaminated with the organism (Miyamoto, et al. 1996). *S.* Enteritidis was found to colonize the reproductive tract of the hen to a greater extent than *S.* Typhimurium, *S.* Virchow, *S.* Heidelberg and *S.* Hadar (Gantois, et al. 2008). However, Gantois, et al. (2008) were unable to conclude why *S.* Enteritidis is the dominate

serovar over *S.* Typhimurium. Okamura, et al. (2001), however, found that *S.* Enteritidis had a greater ability to colonize the reproductive tract than other serovars. A key factor in preventing Salmonella contamination of the egg is maintaining a flock that is free from clinical and subclinical *Salmonella* infections.

1.2.8. Anti-Microbial Defences of the Egg

The anti-microbial defences of the egg can be roughly divided into physical and chemical defences. The physical defences of the egg include the shell, the shell membranes and the vitelline membrane, although physical defences may also include the viscosity change in the albumen post lay, reducing bacterial motility (Brown, et al. 2006). Chemical defences are predominantly proteins; however they also include the pH change in the albumen post lay. The physical and chemical defences of the shell, including the thickness and porosity of the shell, shell membrane thickness and the level of different anti-microbial proteins, all affect different bacterial serotypes differently (Stokes, et al. 1956).

1.2.9. Physical Defences of the Egg

The physical defences of the egg are made up of the cuticle filling the pores of the shell, the shell itself, the shell membranes and the physical attributes of the egg albumen (Lifshitz, et al. 1963). The eggshell is the first barrier to pathogenic microorganisms (Bain, et al. 2011) and the shell has been reported as being an ineffective barrier to bacterial penetration as the pores are wide enough to allow entry of bacteria (Berrang, et al. 1999). Jones, et al. (2004) identified the shell membranes as the primary physical barrier to microbial penetration of the egg contents. When testing the inner membranes alone, in the absence of albumen, the membranes resisted

bacteria for up to 2-3 days; however most were penetrated within 1-2 days (Brown, et al. 2006) which shows that, while the membrane can slow the rate of bacterial growth, it is not an impermeable barrier. Lifshitz, et al. (1963) found that the inner membrane was the most important physical barrier to bacterial penetration; significantly more so than the exterior structures. This was tested by a series of models that attempted to test each structure separately. Following infection of the albumen, the vitelline membrane is the final barrier to bacteria penetrating the egg yolk and gaining access to the wealth of nutrients it contains. When exposed to *E. coli* by Haigh and Betts (1991) the inner shell membranes were found to pose no significant barrier to bacterial entry. Leleu, et al. (2009) comment that a stronger vitelline membrane provided greater anti-bacterial protection to the yolk.

Riley and Betts (2014) comment that the frequency and volume of pores affect the susceptibility of the egg to microorganisms. De Reu, et al. (2006) found that the shell thickness, number of pores and area of the eggshell had no statistical effect on bacterial eggshell penetration, and Bain, et al. (2011) found that the presence of microcracks did not make a significant difference in the rate of penetration by *Salmonella* Enteritidis. Messens, et al. (2005_b) found that penetration was not dependent on the extent of cuticle disposition. While the cuticle acts to plug the pores through the shell, its coverage is not complete and therefore the defence of pores is not perfect (Berrang, et al. 1999). Stokes, et al. (1956) found that, while bacteria are 'sucked' into the shell post lay, they are not pulled through the shell membrane. Berrang, et al. (1999) added that, while the shell membranes do not have any inherent antimicrobial properties, they do add some protective value to the shell. This is disputed by Gantois, et al. (2009) who found a number of antimicrobial proteins in the shell membrane. The fibrous nature of the

membrane (when observed by Scanning Electron Microscope) may act to increase surface area contact with bacteria assisting the membranes inherent antimicrobial proteins. The physical consistency of the egg albumen is another factor in preventing bacteria reaching the yolk, a thicker albumen is expected to slow the growth of penetrating bacteria.

1.2.10 Chemical Defences of the Egg

The chemical defences of the egg are found predominantly in the shell membranes and egg albumen. While these anti-microbial proteins are effective at preventing the growth of some bacteria, Messens, et al. (2004) showed that *S*. Enteritidis and *S.* Typhimurium are able to grow following inoculation into the albumen. Gantois, et al. (2009) found a number of proteins in the shell membrane that have been previously associated with anti-bacterial roles. The chemical defences of the albumen can be divided into antimicrobial proteins and the chemical conditions in the albumen. The albumen has a neutral pH at oviposition; however, as the egg ages and some of the antimicrobial proteins break down, the pH increases (Jones and Musgrove, 2005; Leeson and Caston, 1997; Silverside and Scott, 2001). A higher pH will kill some bacteria and retard the growth of others (Messens, et al. 2004). Messens, et al. (2004) also reported a pH change from 8.16 at lay to 9.26 after 22 days unrefrigerated storage.

There are a number of antimicrobial proteins and chemicals in the albumen including lysozyme (Ayres and Taylor, 1956; Berrang, et al. 1999; Brown, et al. 2006; Messens, et al. 2004; Schoeni, et al. 1995; Stokes, et al. 1956), conalbumen (Ayres and Taylor, 1956; Berrang, et al. 1999; Brown, et al. 2006), Ovotransferrin (Schoeni, et al. 1995), aviden, ovomucoid, ovomucin as well as carbon dioxide (Ayres and Taylor, 1956; Stokes, et al. 1956). These proteins and chemicals

act differently to prevent the growth of bacteria or attack the cells of invading bacteria. Lysozyme acts by lysing the cell wall of bacteria, while conalbumen is an iron binding enzyme that inhibits the growth of bacteria by binding the available iron (Berrang, et al. 1999; Board and Fuller, 1994). *Salmonella* are severely inhibited and in some cases killed by conalbumen and ovotransferrin which are of high concentration in the albumen (Kim, et al. 2012). Messens, et al. (2004) examined the possibility that nutrients or minerals leaked out of the yolk during storage and may result in a corresponding increase in albumen bacterial growth; however they disproved this over 3 weeks at 20°C storage temperature.

1.2.11 Microbiological Experimental Procedures

Comparison between different research studies can be difficult, as a result of the different methodologies used. The age of hens and eggs is often different or not provided in publications. Incubation times and bacterial loads are also not common among studies. However, in order to examine the bacterial penetration rates, there are a number of well established experimental methods. Dolman and Board (1991) described the age of the eggs used in their experiments but failed to describe the storage conditions; they used 2 day old eggs in their penetration experiments. Carnarius, et al. (1996) used shell samples that were stored in a desiccator room before analysis and Clay and Board, (1991) stored their eggs for less than 2 days before conducting the experiment; however, neither article describes the temperature of storage prior to experimentation and many other articles do not describe storage at all.

Board and Board (1967) pioneered the use of the moulded agar egg microbiology experiment; this technique examines the mirobial integrity of the shell without the complicating

factors of the albumen and other egg internal defences. The agar egg technique was also used by Bain, et al. (2011), Berrang, et al. (1998), Chousalkar, et al. (2010), De Reu, et al. (2006) and Schoeni, et al. (1995). Wright and Frank (1956) used the same method; however, they attempted to not disrupt the internal membrane structures of the shell. The experiment involves removing the contents of the egg via a needle and syringe, washing the inside of the shell and replacing the egg contents with agar. Eggs are then sealed with tape or paraffin and dipped in a bacterial solution. Another version of the agar egg experiment involves adding 0.1% 2,3,5 triphenyl tetrazolium chloride to the agar. When bacteria penetrate the shell, the triphenyl tetrazolium chloride is reduced to formazen and there is a colour change to red which is easily observed in the agar (Berrang, et al. 1998; Berrang, et al. 1999; De Reu, et al. 2006). De Reu, et al. (2006) stated that the agar approach seems most suited to studying the influence of the eggshell characteristics but it fails to give an estimation of the effect of the albumen. Whole egg studies involve disinfecting the egg and then exposing it to bacterial solutions; this has the advantage of recording the effect of the albumen on bacterial penetration (Chousalkar, et al. 2010).

Relatively few studies have aimed to determine the natural incidence of *Salmonella* in shell eggs, probably because of the very large amount of laboratory work required to analyse a significant number of eggs (Stokes, et al. 1956). Humphrey, et al. (1991) found a contamination incidence of 0.6% from 5700 eggs (15 flocks), however contamination was low in all but three eggs. In an effort to reproduce commercial environments, Gast and Beard (1990) used a 4 day delay from the time the eggs were laid before experimenting, to simulate the handling and packaging time for normal commercial eggs. In addition, De Reu, et al. (2006), Chousalkar, et al. (2010) and Gantois, et al. (2008) used only bacterial serovars isolated from commercial poultry

flocks to ensure that the serovar studied was relevant to the industry. It is difficult to infect eggs in a natural way as naturally infected flocks will produce few infected eggs and potentially infect their eggs both vertically and horizontally. After artificial infection by intravenous injection of bacteria, hens showed a high rate of *Salmonella* positive cloacal samples (Miyamoto, et al. 1996). Gast and Beard (1990) found that experimentally infected hens can produce a large number of infected eggs, but only for a short period of time following hen infection.

1.3. The Egg.

1.3.1. Formation of the Egg and Shell

While the shell is designed to protect the developing embryo, for farmers, retailers and consumers the appearance of the egg and its contents is also important (Ar and Rahn, 1985; Jones and Musgrove, 2005). The structural quality of the shell egg is important to the producer because eggs that are structurally sound will reach the consumer in the best condition (Jones and Musgrove, 2005). The egg is formed in a number of steps from the development of the yolk lipids in the liver through the stages occurring in the ovary and oviduct (Nys and Guyot, 2011; Solomon, 1991).

1.3.2. The Ovary

A sexually mature hen contains a single usually left ovary. At any one time, the ovary contains four to six developing follicles at different stages of formation (Nys and Guyot, 2011; Solomon, 1991).

1.3.3. The Oviduct

The oviduct can be functionally divided into six parts, the infundibulum, magnum, isthmus, tubular shell gland, shell gland pouch and vagina (Solomon, 1991). The infundibulum is an 8-9cm long structure, whose main role is to guide the yolk into the oviduct. Fertilization also occurs within the infundibulum (Nys and Guyot, 2011; Solomon, 1991). The magnum is the longest section of the oviduct, and is responsible for the addition of the albumen and membranes to the egg (Nys and Guyot, 2011; Solomon, 1991). The isthmus is smaller and

narrower than the magnum and it plays an important role in shell development. It functions specifically to produce the paired shell membranes (Solomon, 1991). The ends of shell membrane fibres are chemically modified to produce mammillary cores and these mammillary cores along with the deposited calcium salts form the mammillary bodies (Solomon, 1991). The tubular shell gland is functionally responsible for the deposition of calcium salts onto the shell membrane; the mammillary cores are the targets of the salt deposition (Nys and Guyot, 2011; Solomon, 1991).

The shell gland pouch is responsible for the addition of calcium and cuticle to the shell. The egg leaves the shell gland pouch approximately 15 g heavier than it enters. (Solomon, 1991). Towards the end of calcification, the acidity in the shell gland pouch changes and the palisade layer is overlain with a narrow band of vertically orientated calcium carbonate crystals which become the surface crystal layer. On top of this layer, the cuticle is deposited, immediately preceding oviposition (Solomon, 1991). Many shell defects are initialised during the process of shell mineralisation. The nature of these defects and the degree of their presence will affect the structural integrity of the egg (Solomon, 1986). Oviposition is a result of hormone-mediated muscular contraction as the egg passes through the vagina and exits the cloaca (Nys and Guyot, 2011). The vagina, however, is host to glands that produce nutrients; these sperm host glands ensure that any sperm present remain viable for longer periods of time (Solomon, 1991). Immediately after oviposition (release of the egg through the cloaca), the egg continues to develop. The cuticle is soft immediately after oviposition and hardens over time (Solomon, 1991).

The age of the laying hen has a number of effects on the egg. Messens, et al. (2005_a) found that, as flock age increased, so did shell abnormalities and Roberts and Ball (2003) found

that egg weight increased up until approximately 50 weeks of age where it reached a plateau. The egg changes due to increased flock age can be reduced by the induction of moult; moulting of birds also increases overall flock productivity (Ahmed, et al. 2005). Ahmed, et al. (2005) also found that the moult did not influence shell weight, or thickness.

1.3.4. Structures of the Egg and Shell

The avian egg shell consists of about 94% calcium carbonate (Board and Scott 1980; Bradfield, 1951) as well as small quantities of magnesium carbonate and organic material (Cain and Heyn, 1964). Tullett (1983) also mentions traces of aragonite and octacalcium phosphate. The egg is made up of distinct layers, each with a specific function for a developing chick and the table egg consumer (Tullett, 1983). There are several objective measures of egg quality such as conductance, specific gravity (Berrang, et al. 1998), shell thickness and Haugh unit.

There are a number of factors which influence shell structure; eggshell characteristics such as shell thickness and shell area are significantly influenced by hen age (De Reu, et al. 2006). However, Berrang, et al. (1998) found that eggs increased in size as the flock aged without a decrease in shell thickness. Samli, et al. (2005) reported that increased laying hen productivity has been reported to lead to a reduction in eggshell quality. The mechanical properties of the egg depend on geometric variability such as the shape and thickness of the eggshell combined with the eggshell's fundamental physical properties (Bain, et al. 2006; Carnarius, et al. 1996). Ahmed, et al. (2005) comment that shell thickness is the main variable contributing to the strength of the shell and that features such as cracks and microcracks affect both the physical

and microbial integrity of the shell. Microcracks originate from the small crevices in the mammillary layer that form on the inner surface of the eggshell (Bain, et al. 2006).

1.3.5. Layers of the Shell

The cuticle is the thin tough waxy layer on the outside of an egg (Baker and Balch, 1961). Nys and Guyot (2011) commented that the cuticle is responsible for the colour of the shell; however, recent research by Samiullah and Roberts (2013) found that over 80% of the protoporphyrin pigment is found within the calcareous part of the shell. The cuticle plays an important role in plugging the pores that extend through the shell and this acts to reduce water loss and prevent bacterial entry (Nys and Guyot, 2011). Messens, et al. (2005_b) found that cuticle deposition varied greatly around the shell. De Reu, et al. (2006) found, in their egg penetration experiment, that there were significantly increased levels of cuticle on eggs that were not penetrated as compared with eggs that were penetrated by bacteria. The true shell is made up of the surface crystal layer, the palisade layer and the mammillary bodies; light microscopy studies have shown that there are three clearly distinguishable layers of shell (Cain and Heyn, 1964). The palisade layer provides the bulk of protection. It is made up of an outer thick porous sub-layer and a dense inner sub-layer; this results in a shell that provides a good combination of strength and flexibility. The palisade layer makes up approximately two thirds of the shell's true thickness (Nys and Guyot, 2011). Carnarius, et al. (1996) concluded that the shell strength was directly proportional to the thickness and concentration of vertical columns in the palisade layer. The surface crystal layer is a single layer of specifically oriented calcium crystals on the outside of the shell (Cain and Heyn, 1964; Favejee and Floor, 1965; Carnarius, et al. 1996). The palisade layer of the shell is made up of calcium crystals of random orientation (Cain and Heyn, 1964), and

spreads from where the mammillary knobs fuse to the surface crystal layer (Carnarius, et al. 1996).

The mammillary cores form dome shaped mammillary bodies and adjacent mammillary bodies fuse creating a continuous shell. Each mammillary body contains a core of organic matter which merges into the main body of the shell (Baker and Balch, 1961). Where adjacent mammillary bodies do not fuse, pores develop and may extend either fully or partly through the shell. Cuffing is the build-up of calcium around the mammillary bodies and has been associated with increased shell strength; cuffing is also thought to be responsible for increased resistance to bacterial penetration (Chousalkar, et al. 2010). Type B mammillary bodies are small, nearly spherical, calcium bodies that have limited contact with the outer shell membrane (Solomon, 1991). These mammillary bodies do not increase the strength of the shell and have been associated with shell weakness. There is a range of defects or abnormalities that can occur in the mammillary layer which is not surprising due to the complex nature of shell formation and the different stressors faced by hens (Solomon, 1991). Carnarius, et al. (1996) discuss two mammillary body abnormalities that may affect shell strength, firstly uneven levels of nucleation sites, giving rise to disorganised, poorly interlocking calcite columns and a decrease in overall strength, and secondly areas of the shell where several mammillary knobs are fused together may give rise to calcite columns that are unequal in width which may give rise to interference leading to a weaker palisade layer. Carnarius, et al. (1996) also determined three favourable characteristics of a sound egg, 1. well rounded mammillary knobs in close association, 2. uniform width of mammillary knobs and palisade layer and 3. a non porous palisade layer of sufficient strength.

1.3.6. The Shell Membranes

The shell membranes have three important roles; the first is to protect the egg from bacteria, the second is regulating the transfer of gases from the albumen through the shell (Burley and Vahedra, 1936), and third is to provide nucleation sites for the development of the shells palisade layer. The crystalline structure of the shell is lined on the inside with two membranes (Nys and Guyot, 2011; Tullett, 1983). The shell is attached to the membranes by the mammillary cones (Balch and Tyler, 1964). The two membranes are adjacent except for a small area at the base (blunt end) of the egg where they separate to contain the air cell (Balch and Tyler, 1963; Berrang, et al. 1999; Carnarius, et al. 1996; Tullett, 1983). Each of the shell membranes consists of a network of coarse fibres several layers thick (Tullett, 1983; Baker and Balch, 1961). The fibres are made up of a central protein core surrounded by glycoproteins approximately 0.13 µm thick (Nys and Guyot, 2011). The inner membrane is approximately 20 µm thick and the outer is approximately 50-60 µm (Nys and Guyot, 2011). The outer layer consists of three layers, the outer of keratin fibres and the inner two of mucin fibres (Baker and Balch, 1961). Balch and Tyler (1964) determined that, due to the thickness of shell membranes not correlating with the membrane weight, there must be variations in the packing density of the membrane. While both the shell cuticle and membranes are made up of proteins, these are fundamentally different proteins (Baker and Balch, 1961).

1.3.7. The Egg Albumen

Egg albumen consists of a complex mix of more than 40 proteins. Protein makes up approximately 20% of the albumen while water makes up much of the rest (Solomon, 1991). The egg albumen is made up of three layers which can be identified by their viscosity (Nys and Guyot, 2011). Many of the proteins in albumen are responsible for preventing bacterial contamination (Romanoff and Romanoff, 1949). The amount of albumen was found to be closely associated with egg weight suggesting that it is the major determinant of egg size (Silverside and Scott, 2001). Kemps, et al. (2010) comment that the viscosity of the albumen is functionally related to its characteristics, and that there is significant diversity in the viscosity in albumen over 24 days unrefrigerated storage.

1.3.8. The Vitelline Membrane

The yolk is surrounded by a thin transparent membrane. The role of this membrane is to prevent material exchange between the egg white and yolk (Nys and Guyot, 2011). The vitelline membrane is also the final line of defence against bacterial penetration of the yolk (Nys and Guyot, 2011). There is also no significant difference between the vitelline membrane strength over successive days (Leleu, et al. 2009).

1.3.9. The Yolk

The egg yolk is made up of a complex mix of lipoproteins which are produced primarily in the liver and has a high concentration of cholesterol (480mg/100g) (Solomon, 1991). The yolk contains a number of nutrients, including vitamins, minerals and amino acids, for the developing embryo; however, these nutrients are also conducive to bacterial growth (Ayres and Taylor, 1956). The yolk is highly nutritious and growth and survival of bacteria even at low temperatures is possible here (Schoeni, et al. 1995).

1.3.10. Eggshell Pores

Eggshell pores are essentially empty channels through the calcite shell; these pores allow for respiration in hatching eggs (Hunton, 2005), and allow for the transfer of gases and moisture into or out of the shell in table eggs. The number of pores per shell was calculated to be up to 4953 per shell by Messens, et al. (2007). An earlier study by Messens, et al. (2005_b) found that the number of pores around their experimental shells ranged from 0 to 9360 (per $120mm^2$ of shell) which is a large range indicating significant diversity in the total level of porosity. Haigh and Betts (1991) comment that it is possible to count pores from scanning electron micrographs; however they do not discuss how they compared different regions of the shell. Baker and Balch (1961) comment that shell pores are funnel shaped. However, Board and Scott (1980) discuss a number of pore types including straight and externally branching pores, as well as occluded, plugged, capped, and reticulate cuticle systems, these pores were identified from both domestic fowl as well as other birds. The shell is impervious to gases; therefore, to allow the exchange of O_2 and CO_2 the shell is perforated with microscopic pores. These pores also allow the escape of

water vapour and the possibility of dehydration of the egg (Ar and Rahn, 1985). As the oxygen molecule (O_2) is larger than the water molecule H_20, to allow respiration, the pores must be of a size that also allows the transfer of water out of the shell. Therefore, the pores not only provide for the respiration but also the movement of water (Board, 1980). Tullett (1983) found that eggs lose weight continuously after lay because water can escape from the egg contents. The shell's organic cuticle layer acts to protect the pores of the shell; however, in certain eggs the organic cuticle that protects the pores may be incomplete (Tullett, 1983). Gole, et al. (2014_a) found that egg washing as it is currently conducted in the Australian egg industry, may remove the cuticle and 'open' pores from the outside, potentially increasing rates of horizontal bacterial infection.

1.3.11. Conductance and Porosity

Conductance is a measure of shell porosity and is expressed as the volume of water vapour and other gases that can pass through the shell per unit time (Berrang, et al. 1998). Eggshell porosity is determined by three factors, the number of pores, the individual cross section of each pore and their length (Tullett, 1983). The shell thickness provides a useful measure for pore length (Ar, et al. 1974). Wright and Frank (1956) found that there was no significant correlation between shell thickness and shell porosity. Once the shell is formed, the number and size of pores is fixed and therefore, during incubation, the conductance of the shell does not change (Ar and Rahn, 1985). Ar, et al. (1974) comment that, since that water loss of an egg is independent of the metabolic rate, it is likely that the particular pore geometry which determines gas conductance has evolved from the metabolic needs of the embryo. The only mechanism of control over the shell's conductance is the steepness of the diffusion gradient across the shell, controlled by the availability of internal water vapour inside the shell and the

external concentration of water vapour. Tullett (1983) proposed a mechanism by which the hen could control the porosity of the shells by controlling the number of seeding sites where calcification is initiated. These sites determine the number of crystal calcite columns in the shell which, in turn, inversely correlates with the number of pores. Thus, it is probable that the distribution of the oviduct cells which secrete the initial seeding sites ultimately determines the overall porosity of the eggshell (Tullett, 1983). Messens, et al. (2005_a) found that the number of eggshell pores per area, varied between eggs laid by hens of different ages.

The biological consequence of pores and conductance is two conflicting requirements: sufficient gaseous exchange to facilitate embryogenesis, but not to the point where excessive water loss results in dehydration of the embryo. Sparks and Board (1984) also found that the cuticle does not influence conductance appreciably, but does have a role in preventing water ingress (flooding) and preventing the entry of microorganisms into the shell.

1.3.12 Different Pore Structures

In the vast majority of avian species, the shell pores are funnel shaped with a wider orifice facing externally. Two other pore structures have been recognised in species laying larger eggs, including branched pores and complicated reticulate pore systems found in some species (Tullett, 1983). Board (1980) identified a number of pore types which include both straight unbranched pores and externally branching pores, further commenting that the morphology of the pore cannel and especially the composition of the covering material has a marked diversity. All pore abnormalities or structures are created by the conditions in the hen's shell gland (Board and Scott. 1980) and the level of control over the rate of pore deposition remains unclear.

1.3.13. Changes in the Shell Post Lay

At oviposition, there are a number of physical and chemical changes still to be made to the egg. The organic shell cuticle has not yet hardened and the egg is laid at the body temperature of the hen (42°C); as the egg cools, the contents contract. Samli, et al. (2005) found that excess loss of water due to high storage temperature or low humidity is detrimental to the egg. Jones and Musgrove (2005) and Bozkurt and Tekerli, (2009) found that there was a distinct decrease in egg weight during the first 5 weeks of storage and Samli, et al. (2005) report that the deterioration of egg quality increased with increasing storage time in a nonlinear manner. Environmental factors such as temperature, humidity, the presence of CO_2 and storage time are of prime importance in terms of maintenance of egg quality (Samli, et al. 2005). Jones and Musgrove (2005) concluded that the current shelf life of eggs could be extended beyond 30 days, without compromising physical quality factors.

There are a number of changes in the albumen post lay. Samli, et al. (2005) found that a rapid increase in albumen alkalinity occurs even after 2 days storage time, regardless of storage temperature. Time in storage was found not to be related to an increasing ratio of thin albumen to thick ablumen in some studies (Aboonajmi, et al. 2010; Leeson and Caston, 1997; Kim, et al. 2012), which contrasts with the findings of Silverside and Scott (2001) and Jones and Musgrove (2005) who found that time in storage decreased the albumen height and increased the albumen pH of eggs. With increasing egg storage time, the ratio of thick to thin albumen decreases (Aboonajmi, et al. 2010). Several chemical-physical modifications occur inside the egg during the storage period; physical changes include an increase in the size of the air sac, flattening of the yolk and the aforementioned thinning of the albumen. Other changes include an increase in the

pH of the albumen, weakening and stretching of the vitelline membrane and increasing yolk water content (Aboonajmi, et al. 2010; Kim, et al. 2012). The increasing pH is caused by the rapid loss of CO_2 from inside the shell; this CO_2 is lost from the albumen resulting in a decrease in quality until there is a balance of CO_2 on the inside and outside of the shell (Aboonajmi, et al. 2010).

Samli, et al. (2005) found that, with increased storage time and storage temperature, egg weight, specific gravity, albumen height, Haugh unit and yolk index all decreased significantly. They also found that albumen and yolk pH increased significantly. Jones and Musgrove (2005) found that, even in refrigerated storage, there was a decrease of 6% in perivitelline membrane strength. The air-sac inside the shell increased more in unrefrigerated storage when compared to refrigerated storage. Samli, et al. (2005) found that storage at temperatures greater then 5°C resulted in considerable deterioration in yolk index. Aboonajmi, et al. (2010) concluded that temperature was one of the main factors influencing egg quality during storage.

1.4. Eggshell Translucency.

Eggshell translucency is the appearance of lighter coloured regions of the shell that can be seen when an egg is candled over a light source. There is significant diversity in the level of shell translucency (figure 1.4.1.), and this differs between species, flocks, hens, and even in individual hens over time. Normally eggs are uniformly translucent; however, many egg shells have streaks, small spots, large areas, which are more translucent than others (Garlich, et al. 1975). Translucency has been described as glassy by Almquist and Burmester (1934), mottled by Talbot and Tyler (1973b) and higher shell texture by Holst, et al. (1931). Translucency ranges from small pinprick sized spots to larger regions and rarely even over the whole shell (Holst, et al. 1931; Solomon, 1986; Talbot and Tyler, 1973b). Translucency does not appear immediately post lay, but develops over time. The rate of change post lay is not consistent between eggs.

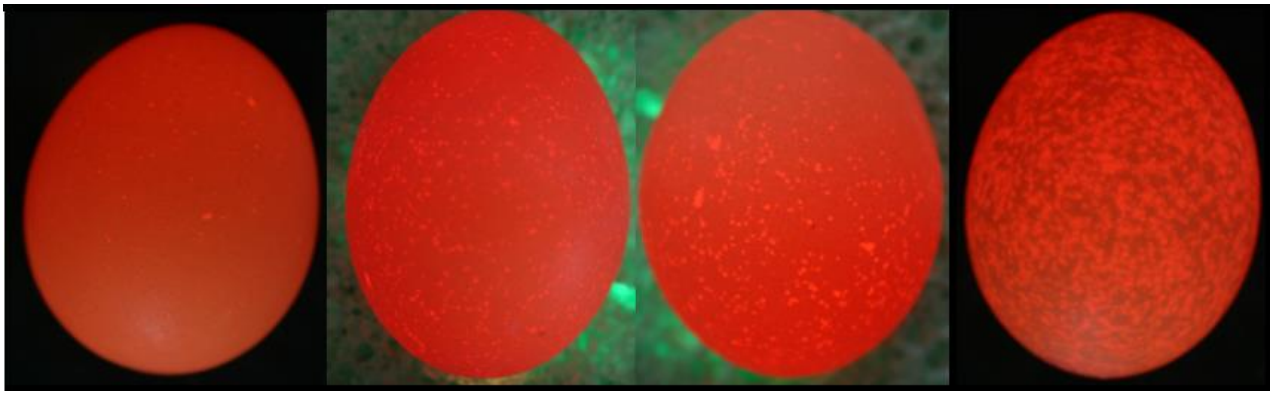


Figure 1.4.1. Candled eggs demonstrating translucency scores (1,2, 3 and 4, left to right respectively).

A number of researchers have reached conclusions about translucency. Talbot and Tyler (1973b) found that translucency was more common at the poles of the egg, Solomon (1986)

reported that translucency was more common in older rather than younger birds and Holst, et al. (1931) concluded that translucency bears no simple relationship to other shell features. Talbot and Tyler (1973) suggested that there are different types of translucency, accidental/artificial translucency and traditional translucency. Accidental/artificial translucency is caused by damage to the cuticle or shell immediately after lay, while traditional translucency is the natural appearance of lighter coloured regions; these different causes may explain some of the shapes and lines observed in shell translucency. The suggestion of shell damage was dismissed by Garlich, et al. (1975) who comments that translucent features are not related to physical damage. Solomon (1986) suggests that the translucent regions are caused by structural change in the shell. It has been suggested that, in translucent shells, the mammillary knobs are larger and more unequally distributed, and that this is due to a reduced number of nucleation sites during the formation of the shell (Leach and Gross 1983).

There is still much that is not known about eggshell translucency; my previous work has confirmed that translucency is caused by the presence of water within the shell structures, and that a range of shell structures result in spaces in the shell for water to occupy (Ray, unpublished thesis, 2013).

Mycoplasma synoviae has been implicated in causing Eggshell Apex Abnormalities (EAA's); these EAA's are characterised by roughened shell, thin shell, cracks and breaks and increased translucency. These abnormalities are confined to the top cone (blunt end) of the egg (Feberwee and Landman, 2010). While the mechanism of action of *Mycoplasma synoviae* causing these shell features is not known, it is likely that most if not all of these effects are potentially caused during

the development of the mammillary layer. Although the EAAs caused by *Mycoplasma synoviae* are a known cause of translucency, they are not currently relevant to this study.

1.4.1. Causes of Eggshell Translucency

Translucency was found to be caused by the presence of water in the shell as, when a shell is dried, the translucency disappears (Tyler and Geake, 1964) and when a shell is saturated with water the translucency reappears (Holst, et al. 1931). When translucency is redeveloping in a dried shell, it first appears in the areas in which it previously existed, which leads to the conclusion that there are specific structures within the shell that act to contain water. Talbot and Tyler (1973b) suggested that the translucent areas of the shell are due to the presence of liquid and Solomon (1986) added that structures within the shell allow for the entry of more water into the shell and that this results in translucency. Solomon (1991) implicated pores as a possible cause of shell translucency due to their ability to transfer water, CO_2 and O_2. Tyler and Geake (1964) found that soaking normal shells in water for 30 minutes did not result in a loss of strength, although after 24 hours there was a noticeable weakening of the shell. The weakening effect of water is not permanent however as, when the shell is dried, the shell strength returns (Tyler and Geake, 1964).

There have been a number of suggested causes for the appearance of shell translucency; however, some have already been disproved. Talbot and Tyler (1973) showed that artificial translucency marks are not due to cuticle damage immediately after lay. Solomon (1986) suggested that the greater nucleation distances involved in larger eggs results in more

translucent patches; however, this does not explain why shell translucency occurs in both larger and smaller eggs.

There have been a number of explanations as to why translucency is distributed unevenly around the shell. These include that translucency indicates thinner portions of the shell, the uneven distribution of fat or oil throughout the shell which may alter the transmission of light through the shell, variation in the crystal calcium structure of the shell and the presence of air spots. Holst, et al. (1931) went on to disprove all of these possible reasons; fat or oil could not be removed from the shell during drying, variations in crystal structure did not change with drying or saturation and air spots between the shell and the membrane are unlikely as translucency remains for hours after the membrane has been removed. X-ray diffraction was used to search for ion impurities, but none were found. Solomon (1986) concluded that the structural variation responsible for translucency was caused by the differential morphology of the mammillary layer. Even though Garlich, et al. (1975) identified that translucent streaks look like shell cracks, they found after hand breaking shells that in only five of 115 did the break follow the translucent streak. Garlich, et al. (1975) also found that highly translucent shells were less heavy than normal shells and had a reduced breaking strength.

1.4.2. Relationship Between Translucency and Shell Features

Many researchers have determined that translucency is related to different shell features. Holst, et al. (1964) found that there was little difference between translucent and non translucent shells when comparing storage capabilities. Solomon (1986), however, commented that translucency was indicative of structural change in the shell. Holst, et al. (1931) indicated

that translucent shells possess poor keeping qualities and show excessive shrinkage of egg contents. Shell strength has been extensively researched and translucent shells were found to be weaker than non translucent shells with this strength difference being more marked in week old than freshly laid eggs (Talbot and Tyler, 1973; Tyler and Geake, 1964). When the shell cuticle was removed by 50% sodium hydroxide, there was no difference in strength between translucent and non translucent shells (Tyler and Geake, 1964).

Talbot and Tyler (1973b) found that translucent and non translucent shells were different when decalcified. There was a clear difference in the distribution of organic matter within the shell. Non translucent shells showed a clear layer high in organic matter just above the mammillary layer. This layer varied in width but was completely absent only in translucent shells. Talbot and Tyler (1973b) also found that the layer of organic material does not make the shell thicker; rather, translucent shells were found to be thicker than non translucent shells. Chousalkar, et al. (2010) found that there was a statistically significantly increased rate of bacterial penetration through the shell of translucent eggshells, as compared with non translucent eggshells, at both 37°C and 20°C; however, this study used only small sample sizes. Chousalkar, et al. (2010) identified a number of shell structural features that were more common in translucent shells than other shells, including lining up (alignment) of mammillary bodies resulting in a continuous groove between the mammillary caps, reduced incidence of cuffing, depression and erosions of the mammillary bodies and the presence of type B mammillary bodies.

1.5. Imaging of the Eggshell.

Candling, Scanning Electron Microscopy (SEM) and Computed Tomography (CT) all have different advantages in the study of eggshells. Candling is unique as it is currently the only method of identifying translucent regions. Computed Tomography and SEM offer high resolution images of the mammillary layer and other structures, which may allow the identification of the structural basis of translucency. The three imaging methods discussed here are complimentary with different strengths, candling can be used to identify the translucent regions, CT can provide detailed transverse sections of the palisade shell later and SEM can provide high resolution images of the mammillary bodies.

1.5.1. Candling

The practice of candling eggs dates back prior to the 1920's (Solomon, 1991) and is the basis of monitoring egg quality at commercial shell-egg operations (Bokhari, et al. 1995). Translucency is only visible when the shell is candled (Holst, et al. 1931). The process of candling for scoring translucency is known to be subjective (Solomon, 1986); therefore, it is important to maintain certain procedures while scoring. The candling process leads to over removal of safe solid eggs; this may be due to the subjective examination process (Bokhari, et al. 1995). Candling also serves to reveal the presence of cracks (Burley and Vahedra, 1936; Solomon, 1986), the detection of inclusions, misplaced yolks, watery albumen and the presence of blood or meat spots (Board and Fuller, 1994). Candling is a non-destructive process that allows the egg to be inspected, prior to sale or further experimentation. Aboonajmi, et al. (2010) comments that candling is a practical way of determining apparent egg quality; however, it is a time consuming

laborious process. Scoring translucency is a complex process and a number of researchers have used different scoring systems, 1 to 3 (Holst, et al. 1931; Solomon, 1991), 1 to 4 (Talbot and Tyler, 1973), 0 to 4 (Chousalkar, et al. 2010) and 0 to 3 (Chousalkar, et al. unpublished data) and 0 to 5 (Roberts, et al. 2013). A complete scoring 'system' includes absolutes for zero and complete translucency along with enough intermediate scores to properly identify translucency. The 0 to 5 scoring method is preferred as it allows for the two extremes and four intermediate scores.

1.5.2. Scanning Electron Microscope

Scanning Electron Microscopy (SEM) is currently the standard method of viewing the mammillary layer of egg shells (Board and Fuller, 1994; Burley and Vahedra, 1936; Chousalkar, et al. 2010). While commonly used on the mammillary layer, SEM can also be used to examine the cuticle layer and transverse shell sections. The unique advantage of the SEM is the high resolution, high magnification images of the mammillary layer, and the readily available equipment over CT. Carnarius, et al. (1996) also used the technique to examine the shells palisade layer in transverse sections. Scanning electron microscopy requires extensive sample preparation. Plasma etching is used to remove the majority of the organic membrane that obscures the view of the mammillary layer and a gold sputter coating is then applied to increase image quality. Scanning Electron Microscopy also necessitates cutting the sample and this may lead to changes around the cut site (Hausherr, et al. 2006). Sample preparation for SEM is a destructive process that involves attaching shell pieces to aluminium stubs with silver conductive paint. Sample processing and mounting prevents a sample being used for other testing methods (Hausherr, et al. 2006).

1.5.3. Computed Tomography

Prior to the beginning of this project, X-ray Computed Tomography (CT) had not been used to examine eggshells. A paper utilising a small sample size has been published since by Riley and Betts (2014) who also demonstrated the ability of CT to examine shells. Computed Tomography is more commonly associated with development in medical imaging; however, it is increasingly being used to examine the structures of other subjects (Metcher, 2009). X-ray CT imaging systems take two typical forms; the first are lab based which are often self contained and affordable when compared to the second synchrotron source designs. Synchrotrons produce high brilliance X-rays which can be filtered to single wavelength radiation with parallel beam geometry (Wildenschild, et al. 2002). This allows imaging at high resolution, rapid scan times with uniform sample X-ray attenuation by the sample; however it comes at a cost of much higher initial and continuing expenditure (Metcher, 2009). The 3D reconstruction of the CT shell scans allows for viewing of high resolution transverse images of the shell as well as three dimensional renderings. While the magnification of the CT is not as high as SEM, it enables visualisation of the internal structures of the shell. Jones et al. (1998) used CT to examine dinosaur eggs and, while they described the process as non-destructive, the resolution was not sufficient to observe the nature of specific eggshell pores. When completing high resolution CT scans, the scanning process does become dedstructive. Riley and Betts (2014), have examined 'functional pore area' which is the areas of low density within the shell. They did not however, compare their results to established methods.

Laboratory based X-ray systems typically have a divergent beam geometry and as a result, there is a trade-off in sample size as the magnification decreases (Wildenschild, et al. 2002). The

resolution of the final data set is limited by X-ray optics, the resolution of the detector, the sample size and the number of images being recorded (Neues and Epple, 2008). Using a very small sample size, Metcher (2009) achieved an effective voxel size of 1µm. Hausherr et al. (2006) comment that CT can be more effective than SEM when characterizing similar materials, as CT allows for differentiation of materials based on X-ray attenuation. Since each method has its own specific advantages, the combination of all techniques provides the opportunity to produce a complete picture of the shell and its structures.

There are a number of scan parameters that can be altered to influence scan quality. For example exposure time, X-ray wave length (and therefore penetration (keV)), X-ray photon density (or 'brightness' (uA)) and the number of projection angles. For greater details refer to Wildenschild et al (2002) and Helliwell et al (2013). Metcher (2009) found that, by increasing the voltage through the X-ray generator, there was greater sample penetration reducing the required exposure time, resulting in shorter scan times. X-ray Computed tomography is performed by first placing a sample between an X-ray source and detector. The sample is then rotated through 360 degrees and a series of 2D projections are acquired of the sample from a number of angles. The projections are digitally reconstructed to produce a three dimensional representation of the internal composition of the sample (Hausherr, et al. 2006; Neues and Epple, 2008). Computed tomography is based on the different attenuation co-efficient for X-rays in different materials (Neues and Epple, 2008) and the reconstruction program calculates attenuation coefficients for 'voxels' which are effectively three dimensional volume pixels. A drawback of CT scanning is the inability to differentiate between materials of similar electron densities. As scanning technology improves, it may be possible to provide greater differentiation

of similar materials; however, materials with the same electron attenuation will remain undifferentiated (Hausher, et al. 2006).

CT has been used to examine eggshells in a limited extent by Jones, et al. (1998) who identified dinosaur eggs as dendrospherulitic, due to the branching character of formations within the shell cross section. Jones, et al. (1998) produced a series of 2 mm slices and a voxel size (resolution) of 2 mm^3. Computed Tomography was used to produce both a three dimensional representation of the shell piece which can then be divided into a series of transverse images.

1.6. Introduction to the Current Study

The aim of this project was to identify a non-destructive method for identifying eggs that have a greater potential for microbial contamination than others. The appearance of eggshell translucency has previously been associated with increased bacterial penetration and it is therefore important to determine whether highly translucent eggs are more likely to be penetrated by bacteria originating on the outside of the egg shell. If this relationship can be confirmed, highly translucent shells could be directed away from consumers to industry processes that include pasteurisation. Miller, et al. (2010) showed that there are advantages in heat treating liquid whole egg, including extended shelf life, and these processes are already in effect in commercial operations in Australia. Guard-Bouldin and Burh, (2006) also suggest directing potentially dangerous eggs towards pasteurization; however, no method of identifying these eggs was recommended.

While there are a number of potentially dangerous pathogens associated with consumption of eggs, there are two simple activities available to consumers to prevent food borne illness from eggs. Firstly, refrigeration of table eggs is essential for keeping bacterial loads within the egg low (Gast, et al. 2007). Secondly, cooking an egg to the point of a solid yolk will kill most bacteria potentially in the egg (Jones, et al. 2004). However, be it for cultural or taste reasons these two simple steps are often not followed as many people prefer to eat eggs either lightly cooked or raw; raw eggs are often used in products such as aioli and mayonnaise. Uncooked or undercooking is associated with approximately 27% of all egg dishes (Lin, et al. 1997).

This project examines egg shells using a number of different imaging methods. Micro-Computed Tomography (CT) is a novel method that provides the ability to view the shell structures in 3 dimensions allowing identification of shell pores in a way that has not previously been possible. The present study also uses Scanning Electron Microscopy (SEM) and microbiological examinations to better understand the importance of shell structure in the food safety of the table egg.

Chapter 2. General Materials and Methods.

The methods described here are common to all four major experiments. Variation in a number of CT settings among experiments is described in the respective chapters.

2.1. Egg Candling

Whole eggs were candled on a UNE egg candler (UNE workshops) (Figure 2.1.1.) using a zero to five translucency scoring system based on total area. A zero score shell has effectively no translucency , while a five score shell is completely translucent (figure 1.4.1.).

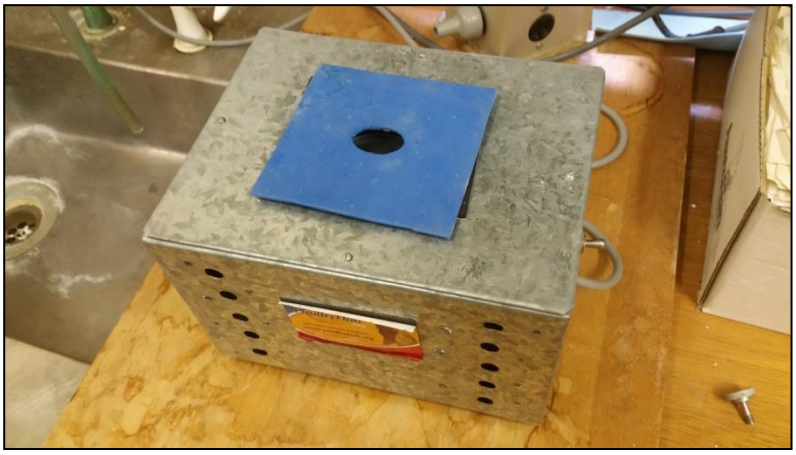


Figure 2.1.1. UNE egg candler, inside the box an incandescent globe produces light which is then transferred through the egg.

2.2. CT Scanning

Samples of shell were hand broken from the midline of the shell and were individually scanned with a 'General Electric Pheonix V|tome|x' micro CT scanner (Figure 2.2.1.). The 180kV transmission type 'Nano' X-ray scanning tube was used with a source energy of 80kV, and current of 180µA. The focal spot size for all experiments was standardised at 4 µm on the tungsten target.

Figure 2.2.1. The GE Pheonix Micro CT scanner used in this work.

Settings that were altered for individual experiments include: the number of projection images per scan, the exposure interval of each projection, and the magnification and therefore the resultant voxel size. Samples were fixed to the sample holder by a small amount of Blu Tack™ and centred using a ruler.

Following completion of CT scanning, three dimensional reconstruction was completed with GE Phoenix datos|x 2 reconstruction Version 2.2.1-RTM reconstruction software. As part of the back-projection algorithm, a digital beam hardening correction index of 2 was applied to compensate for the inhomogeneous attenuation of the polychromatic x-ray source. An automatic geometric calibration was used to compensate for any micro-scale sample movement during the scan. The samples were positioned with the shell's mammillary layer initially facing the X-Ray source.

2.3. CT Image Analysis

VG Studio Max 2.0.5 was used to examine two dimensional slices and three dimensional renderings of the scan. The VG Studio software allows reconstructions to be examined by virtually slicing the volume and allows multiple angles to be observed. To maintain an equal examination area for all samples, a 1 mm radius sphere (4.19 mm^3) was digitally isolated from the rest of the reconstruction and this was used for visual scoring a range of measures (Figure 2.3.2.). The uniform volume was randomly selected from the approximate centre of the sample, ensuring that the shell occupied the entire centremost plane of the sphere. This resulted in CT measurements from ~ 3.14mm2 of the shell surface. The number and type of shell pores (internally branching, non-branching and externally branching) was recorded for each sample, along with either the shell thickness (measured with the VGStudio Max 'calliper' tool) or shell volume (see individual experimental methods). Pores were classified as 'straight' if they showed no significant enlargement towards either the inside or outside of the shell, and 'internal branching' or 'externally branching' (Figure 2.3.1.) if they branched or showed a larger

(approximately more than three times the size the pores diameter) opening either internally or externally.

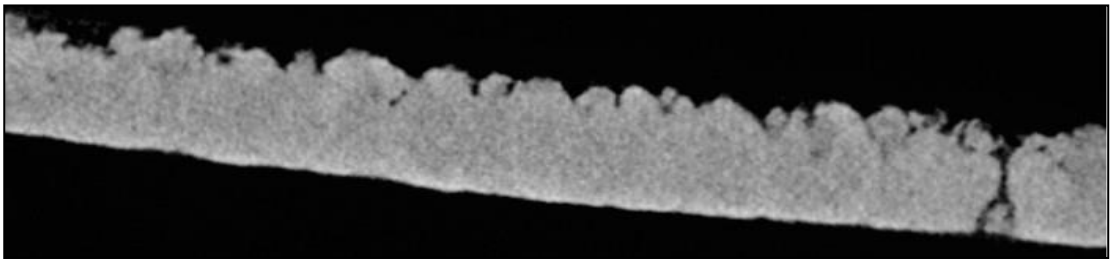


Figure 2.3.1. Two dimensional transverse cut typical of a CT scan output. The shell is oriented so the exterior is facing downwards. Note the externally branching pore on the right hand side of the image.

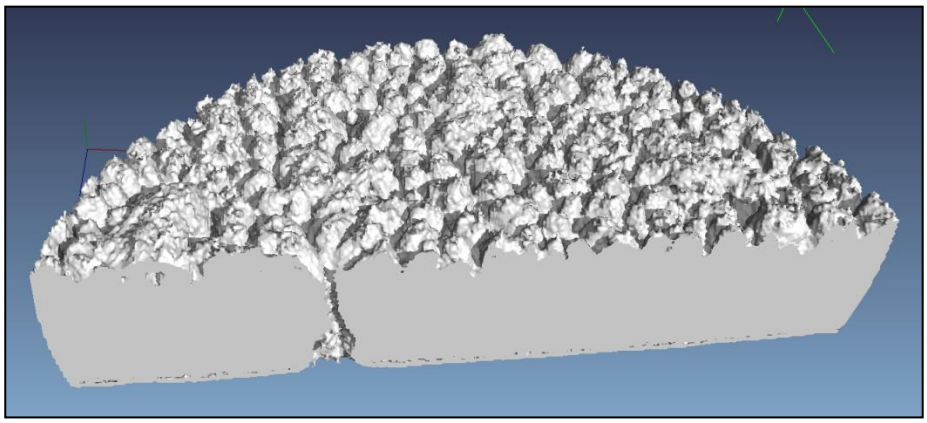


Figure 2.3.2. Three dimensional reconstruction of CT scan, showing the shells mammillary layer and externally branching pore structure through the shell (visible in 'cut' plane). These reconstructions provide little further information than the two dimensional transverse images.

2.4. Scanning Electron Microscopy (SEM)

The same shell samples were then prepared for SEM. The shell membrane was removed from the shell by soaking the shell sample in water for 5-30 minutes followed by peeling of the internal shell membrane and light physical rubbing to remove the inner region of the outer shell membrane. A Biorad 'PT7150' RF plasma barrel etcher was used to remove any remaining membrane at a relatively low temperature (150°C) without changing the inorganic components of the shell, this is achieved by producing a high oxygen atmosphere and passing through radio waves inducing plasma. Shell samples were then mounted onto aluminium stubs using a small amount of silver conductive paint. Samples were then gold sputter coated using a JEOL (MP-19020NCTR) Neocoater for 5 minutes (3 min + 2 min runs); this coats the sample in a thin layer of gold to improve electron conduction in the sample.

Figure 2.4.1. Biorad (PT7150) RF plasma barrel etcher. This utilises a high oxygen atmosphere and a radio waves to produce plasma which etches the organic material off the shell at a relatively low temperature (~200°C).

Figure 2.4.2. JEOL Neocoater (MP-19020NCTR) gold sputter coater. This coats samples in a thin layer of gold, increasing sample conduction and image quality in scanning electron microscopy.

Figure 2.4.3. Jeol Neoscope, bench top Scanning Electron Microscope (JM-5000)

A Jeol Neoscope bench top Scanning Electron Microscope (SEM) (JM-5000) was used to observe the structure of the shells and to take images at 100x and 200x magnification at an accelerating voltage of 10kV. Images were taken from the centre of the sample where there was no shell membrane.

2.5. SEM Image Analysis

Images from the JEOL Neoscope SEM were saved as JPEG files and scored for mammillary body size (-3 to 3), fusion (-3 to 3), the incidence of type A mammillary bodies (0 to 3), type B mammillary bodies (0 to 3) and cubic calcite formations (0 to 3) by visual inspection. The entire image was scored. Sample 'normal' shell mammillary layer scanning electron micrographs are shown in figures 2.5.1. and 2.5.2. Samples that were obscured by shell membrane such that the mammillary bodies were not visible were disregarded and not scored for any measures. Mammillary body size and mammillary layer fusion are both relative measures with a '0' score being considered 'normal'; therefore, scores range from -3 to +3 to characterise the size and fusion of the mammillary bodies. Type A and type B mammillary bodies as well as cubic formations are considered 'additional' formations that are not found in all shells and thus are scored on a 0 to 3 system.

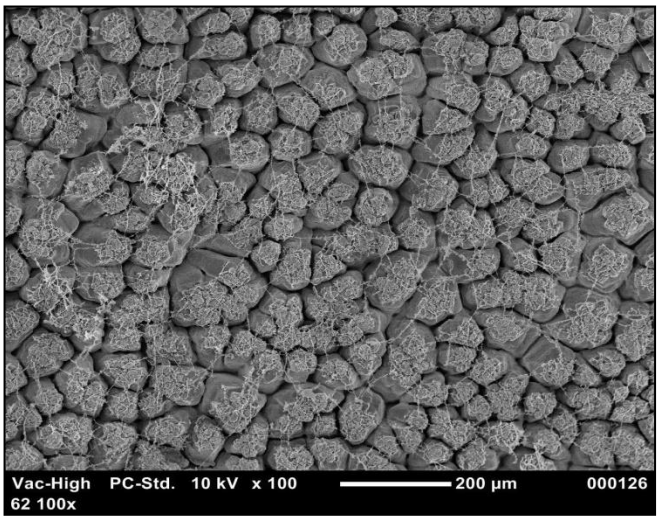


Figure 2.5.1. 100X magnification SEM image typical of a 'normal' table egg shell mammillary layer.

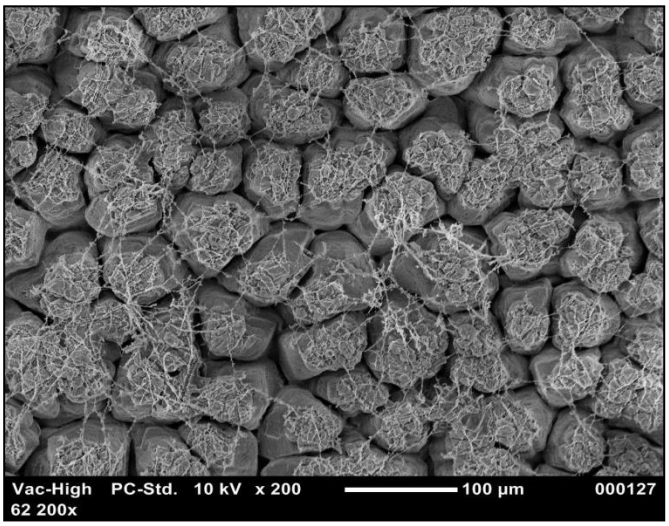


Figure 2.5.2. 200X magnification SEM image typical of a 'normal' table egg shell mammillary layer.

2.6. Statistical Analysis

Statistical analysis for all experiments was completed using JMP v.11 statistical software and Statview v.5.0.1., specific tests are described in each experimental chapter.

Chapter 3. Experiment 1. A pilot study, examining the imaging possibilities and statistical powers of imaging eggshells with Computed Tomography and Scanning Electron Microscopy.

3.1. Experiment 1 - Introduction

A pilot study was required to determine which shell structures could be identified and the potential power of the methods for the purpose of informing future experimental design.

In order to investigate the shell's internal structures, they must be visualised. X-ray CT produces a three dimensional digital (3D) representation of the shell which can be examined in two dimensional transverse cuts. CT scanning is a non destructive process, therefore allowing an individual sample to be scanned first with CT and then SEM. Eggshell CT scans can take somewhere between six and thirty minutes, and analysis of eggshell scans can take as little as two minutes or as long as thirty minutes if 3D renderings are required to visualise structures. Scanning electron microscopy allows for high resolution images to be produced of the shell's interior and exterior layers. The process of imaging shells with SEM can take up to five minutes per sample. Limiting the number of samples due to time and expense considerations may be achieved by reducing the number of samples from each group; however, if the number of shell samples is reduced to a point where critical structures are missed, the entire examination process may be futile.

Eggshell uniformity research has previously focused on comparing successive eggs from birds of the same flock or same species. Due to the process of egg formation in the hen (Chapter 1.3.1.), it is widely assumed that the shell around the midline is uniform; the poles of the egg may show altered structures due to their high relative concentration of calcification sites within the reproductive tract. It is expected that there is significant spatial variation in eggshell structures across the shell and, therefore, experiments will focus on the midline of the shell. Shell structures and abnormalities can be broadly divided into three classes; abnormalities of the shell's mammillary layer, abnormal structures extending through the shell (pores) and uneven distribution of the shell's cuticle.

Scanning Electron Microscopy (SEM) has been used to provide high magnification images of the shell's mammillary layer by many researchers (Board and Fuller, 1994; Burley and Vahedra, 1936; Chousalkar, et al. 2010). A number of mammillary layer defects and abnormalities have been observed using SEM. These include mammillary body abnormalities; type A mammillary bodies (altered mammillary bodies that do not contact the shell membrane), type B mammillary bodies (small, often spherical bodies that play little role in the strength of the shell) and cubic calcite formations (small cubic formations of calcite within the mammillary bodies). Mammillary layer abnormalities include: cuffing (the level of extra shell material around the mammillary bodies), early/late fusion of the mammillary bodies (the depth in which the mammillary bodies fuse to create a continuous layer), mammillary alignment (the borders of the mammillary bodies create an alignment of the edges of the mammillae) and mammillary erosions (erosions of the mammillary bodies).

Shell pores have been previously described by Board and Scott (1980) who described two pore structures, straight unbranching and externally branching. Ray and Roberts (2013) also described pores that branch internally towards the mammillary layer. Pores are generally described as simple structures whether straight unbranching or simple externally branching channels through the shell (Board and Scott 1980). The examination of the shells palisade layer with micro CT enables for imaging of the shells palisade layer to a more spatial observation rather than relying on a chance observation as previously achieved by SEM.

The aim of this experiment is three fold: a) to determine which shell structures can be identified using a combination of CT and SEM b) to make comparisons between the shell structures of low and high translucency score shells and c) to determine the statistical power of results and required sample sizes for future experiments.

3.2. Experiment 1 - Method

3.2.1. Sample Collection and Translucency Scoring.

Forty six eggs were selected from both stored and recently assembled collections of eggs. Fifteen of these egg shells were sourced from a previous research project, having been dried and stored at room temperature for between 12 and 48 months (Roberts, et al. 2013). Another 34 eggs were sourced from a commercial cage operation in Northern NSW, Australia. Eggs were selected on the basis of their translucency, 21 low translucency egg shells and 25 high translucency shells were hand broken to produce 3 replicate samples from the midline of each shell. Translucency for all eggs were scored on a 0 to 5 translucency scoring system as outlined in chapter 2.1. Scores between 4 to 5 were considered 'High' while scores of 0 or 1 were considered 'Low'.

3.2.2. CT Scanning.

Each sample was scanned using a GE Phoenix V-tomex CT Scanner, using the settings described in Appendix A.

3.2.3. CT Image Processing.

Computed tomography image processing was conducted according the methods described in Chapter 2 (2.1.). A standardised sub-sample of 4.17mm^3 (1 mm radius sphere) was isolated from the scan and the volume of voxels representing the volume of the shell was selected by isolating areas with similar greyscale values (an initial threshold of 2000 was used

and then adjusted for the best 'fit'). The volume of each sample representing shell was calculated from CT images using the VG studio 'volume calculator' tool.

3.2.4. Scanning Electron Microscopy.

The SEM procedure was completed as outlined in Chapter 2 (2.3.)

3.2.5. Statistical Analysis.

Statistical analysis was conducted with JMP v.5.0.1. High and low translucency scored shells were compared using students paired t tests for the direct count groups (CT measures) and Wilcoxon comparison tests were used to compare non-parametric (SEM) measures using JMP statistical software. Means and standard deviations were all calculated using JMP statistical software. Statistical powers were modelled using the highest standard deviation of the group means, and mean difference to detect was the difference between the group means.

3.3. Experiment 1 - Results

A number of structures were identified in CT images including three different pore types; straight unbranching, internally branching and externally branching shell pores. Internally branching pores were rare, with an average of 0.04 internal pores per 4.17mm^3. External pores were more common, appearing in most samples with an average of 1.40 pores per 4.17mm^{3}while straight pores were the most common of all pore forms, with an average of 2.35 straight pores per 4.17mm^3 (Table 3.3.1.). Straight and internally branching pores were commonly found to pass indirectly through the shell, occasionally proceeding laterally (figure 3.3.1.). The branching of pores towards the interior of the shell was difficult to distinguish from the morphology of the mammillary layer (Figure 3.3.2.). There was considerable variation observed in the observed degree of opening diameter in external pores. Figure 3.3.3. shows an externally opening pore with a very large external aperture. A typical example of externally branching pores is shown in Figure 3.3.4.

Scanning electron microscopy of the mammillary layer from three locations on the equator of the same shells found an average mammillary body size, and mammillary layer fusion of below zero. The range of mammillary body features can be seen in Figures 3.3.5. to 3.3.8. The average incidence of type A mammillary bodies was 0.65. The average incidence of type B mammillary bodies was 0.57. Cubic calcite formations were far less common than either of the altered mammillary bodes, with a total average of 0.07 cubic calcite formations(Table 3.3.2.)

Table 3.3.1. Abundance of egg shell features for all samples as observed by x-ray CT. Forty-six egg shell samples with three pseudoreplicates from each shell. Mean values are indicated with standard deviation in brackets.

	Straight pores (Count per sample)	**Internally Branching pores (Count per sample)**	**Externally Branching pores (Count per sample)**	**CT Determined Shell Volume (Count per sample)**
Average Score per Sample	2.30 (1.54)	0.03 (0.25)	1.42 (1.17)	1.04 (0.14)

Table 3.3.2. Average values for SEM measured shell features. Forty-six eggshell samples with three pseudoreplicates each. Standard deviation in brackets. (Average incidence per sample).

	Mammillary Body Size (-3 to 3)	**Mammillary Layer Fusion (-3 to 3)**	**Type A Mammillary Bodies (0 to 3)**	**Type B Mammillary Bodies (0 to 3)**	**Cubic Calcite Formations (0 to 3)**
Average Score per Sample	-0.66 (1.13)	-0.26 (1.47)	0.65 (0.68)	0.57 (0.86)	0.07 (0.28)

3.3.1. Shell Structures - Transverse CT Images

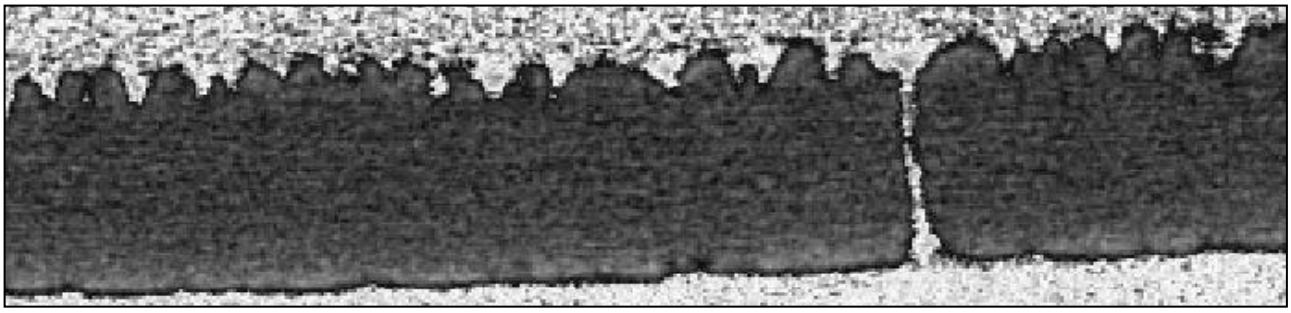


Figure 3.3.1. Transverse 'cut' through reconstructed micro CT volume of an eggshell. The shell is oriented so that the exterior is facing down and the mammillary layer is facing upwards. Demonstrating a straight, unbranching pore proceeding radially through the shell (~70x magnification).

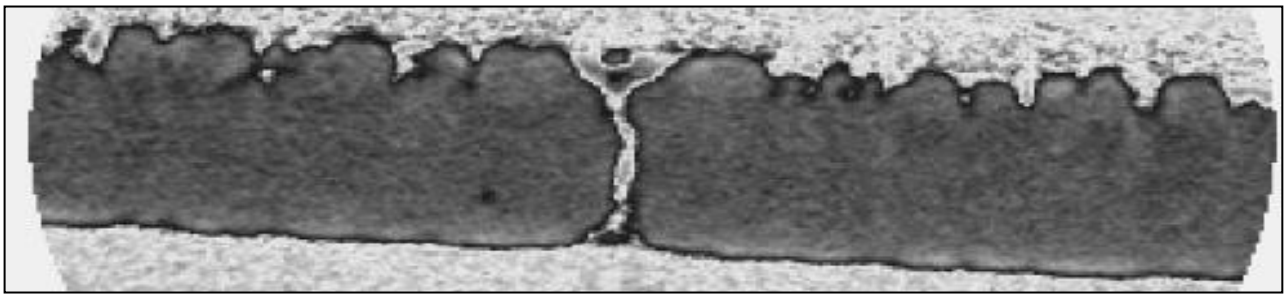


Figure 3.3.2. Transverse 'cut' through reconstructed micro CT volume of an eggshell. The shell is oriented so the exterior is facing down. Demonstrating an internally branching pore, showing the branching pore within the mammillary bodies (~70x magnification).

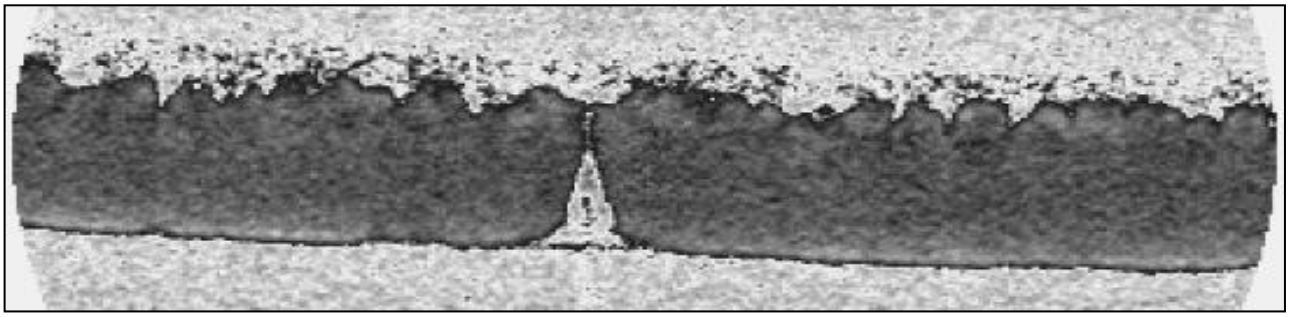


Figure 3.3.3. Transverse 'cut' through reconstructed micro CT volume of an eggshell. The shell is oriented so the exterior is facing down. Demonstrating an externally branching pore with a large external opening (~70x magnification).

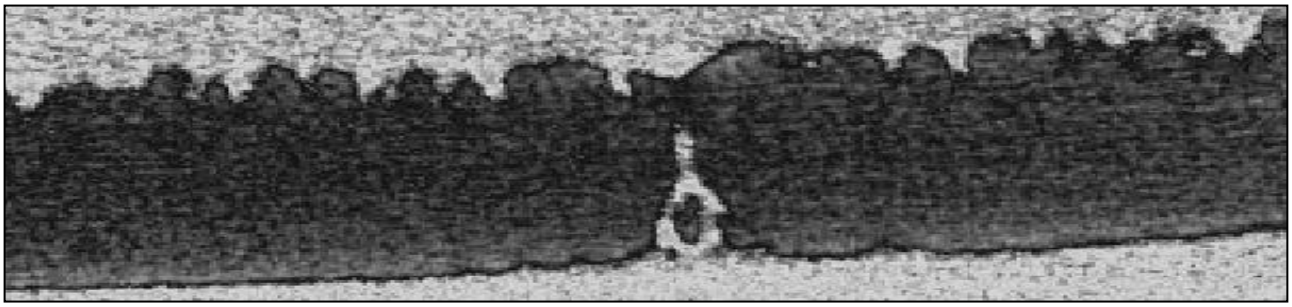
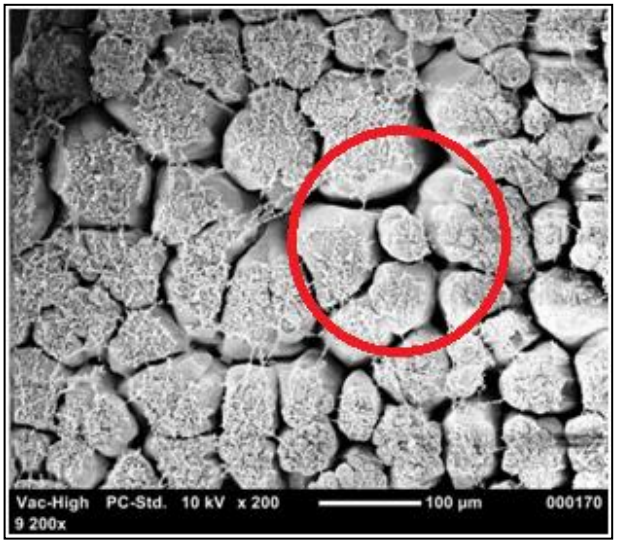


Figure 3.3.4. Transverse 'cut' through reconstructed micro CT volume of an eggshell. The shell is oriented so the exterior is facing down. Demonstrating a more common appearance of an externally branching pore (~70x magnification).

3.3.2. Shell Structures - SEM Images of the Shell's Mammillary Layer.



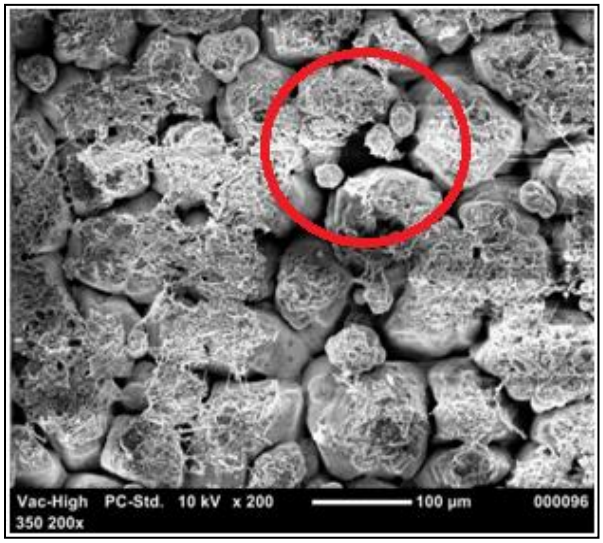
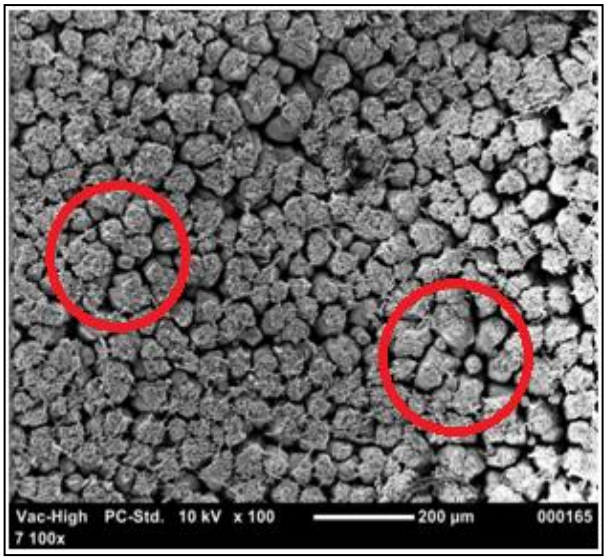


Figure 3.3.5. (left) 200x Scanning electron micrograph of an eggshell mammillary layer. Showing some large mammillary bodies and a prominent type A mammillary body.

Figure 3.3.6. (right) 200x Scanning electron micrograph of an eggshell mammillary layer. Showing some regions of normal and late mammillary layer fusion and a number of type B mammillary bodies.



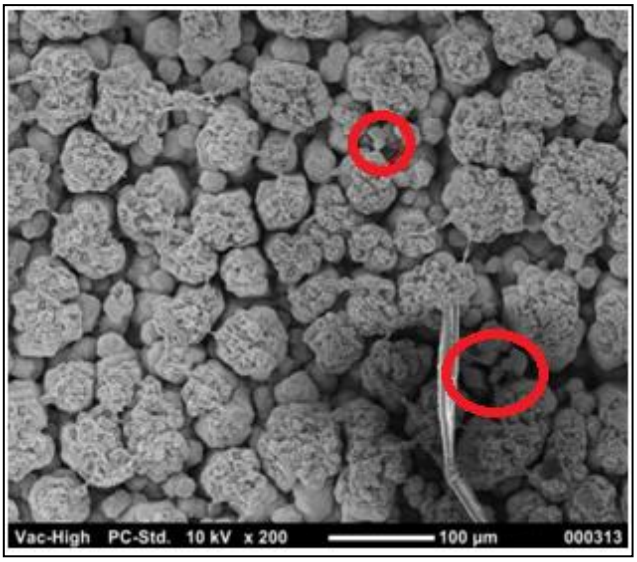


Figure 3.3.7. (left) 100x Scanning electron micrograph of an eggshell mammillary layer. Demonstrating small mammillary body size, late mammillary layer fusion and a large number of type B mammillary bodies.

Figure 3.3.8. (right) 200x Scanning electron micrograph of an eggshell mammillary layer. Showing another late mammillary layer fusion shell with 2 small cubic calcite formations (circled).

3.3.3. Comparison Between Low and High Translucency Scored Shells.

There were fewer straight pores ($p < 0.001$) and more externally branching pores ($p < 0.001$) in the high translucency score group than the low translucency score group (Table 3.3.3.). Egg shell volume as measured by CT was larger for the high translucency score group at 1.10 mm^3 compared to 0.99 mm^3 ($p < 0.001$). There were no differences in the number of internally branching pores with 0.06 and 0.04 for low and high translucency groups respectively. Statistical power to detect the differences observed was high for all CT measures, with all groups being above 0.98.

The high translucency score group had higher scores of both type A and type B abnormal mammillary body types. Mammillary body size and mammillary layer fusion scores were similar for both low and high translucency and all scores were negative. Observations of cubic calcite formations were rare, 0.02 and 0.11 (0-3 scale) (low and high respectively) and the difference was not significant. Statistical power was high (>0.8) for all significantly different measures, with the exception of CT measured Sample Volume where it was 0.12. Measures where the translucency group means were not significantly different all showed a lower statistical power (Table 3.3.4.).

Table 3.3.3. Averages of CT measures compared between low and high translucency scored shells. Standard deviation in brackets. P-Values are given for measures that are statistically different. Statistical power is given for each measure.

Translucency	Straight Pores (count)	Internally Branching Pores (count)	Externally Branching Pores (count)	CT Sample Volume (mm^3)
Low (63)	2.73[a] (1.54)	0.06 (0.20)	0.80[a] (0.89)	0.99[a] (0.10)
High (76)	1.95[b] (1.32)	0.04 (0.29)	1.97[b] (1.02)	1.10[b] (0.86)
P-Values	0.0017	NS	<0.0001	<0.0001
Statistical Power	0.84	0.07	1	0.12

Table 3.3.4. Averages of SEM measures compared between low and high translucency scored shells. Standard deviation in brackets. P-Values are given for measures that are statistically different. Statistical power is given for each measure.

Translucency	Mammillary Body Size (-3 to 3)	Mammillary Layer Fusion (-3 to 3)	Type A Mammillary Bodies (0 to 3)	Type B Mammillary Bodies (0 to 3)	Cubic Calcite Formations. (0 to 3)
Low (n= 63)	-0.59 (1.16)	-0.48 (1.15)	0.46[a] (0.62)	0.43[a] (0.62)	0.02 (0.13)
High (n= 76)	-0.74 (1.14)	-0.08 (1.73)	0.94[b] (0.66)	0.84[b] (1.04)	0.11 (0.36)
P-Values	NS	NS	<0.0001	0.0362	NS
Statistical Power	0.12	0.27	0.99	0.64	0.31

3.4. Experiment 1 - Discussion

This study provided pilot information on the shell structures underlying the appearance of eggshell translucency. Higher rates of type A and type B mammillary bodies were found in the high translucency score group, potentially indicating a greater mammillary layer disruption in shells exhibiting higher translucency. More straight unbranching pores were observed in the low translucency score group and more externally branching pores were found in the high translucency score group. The link between these pore formations and translucency is not clear; however, externally branching pores did show larger external openings than other pore structures. These larger openings were observed to continue into the shells palisade layer and may facilitate the retention of water within the shell, the retention of water within the shell has been previously related to the appearance of eggshell translucency (Solomon, 1991; Ray and Roberts, 2013; Tyler and Geake, 1964). Further work is required to properly identify all underlying structures and this will be completed in chapter 5 with larger sample sizes. The use of combined imaging technologies is a novel idea and as such has the potential to demonstrate the relationships between shell structures like never before.

This experiment was successful at demonstrating a number of previously unknown factors relating to the overall project. Pore formations were successfully imaged using micro CT and mammillary layer structures were visualized using SEM. The abundance of pores and mammillary structures were relatively rare, creating challenges for statistical comparison.

The presence of pore formations identified was infrequent, less than two pores were identified per sample. More straight pores were observed then externally branching pores and

there were very few internally branching pores. Using a shell sample size of 5mm by 5mm allows a high magnification but limits investigations to a small area. Increasing the physical size of the sample to increase the number of observed features would reduce the magnification of the CT images to the point of making the features unobservable. The time required for imaging a single sample through both CT and SEM was approximately 25 minutes with additional time for sample preparation of approximately 30 minutes. As CT and SEM require a large amount of time to image each sample, reducing the number of pseudoreplicates from each shell is important for allowing greater numbers of shells to be scanned. The usefulness of both CT and SEM have been demonstrated in this experiment. CT scanning allows for systematic imaging of the shells palisade layer and has provided the most accurate imaging of the palisade layer to date. Scanning Electron Microscopy is an established technique for imaging eggshell mammillary layer and produces high magnification and high contrast images, however imaging is isolated to the mammillary layer.

The sample numbers used in this experiment (139) provided statistical power in excess of 0.8 for all measure means that were different between low and high translucency with the exception of shell volume. It is likely that shell volume had a lower statistical power due to the small difference between the means and higher variance of the 'High' translucency score group. Increasing sample sizes in future experiments will further increase statistical power for all measures. The high statistical power for the difference in straight and externally branching pores (0.84 and 1 respectively) shows that for visual identification of pores sample sizes greater than 139 are suitable to produce accurate results. This technique offers an opportunity to explore the spatial distribution of shell structural features across the whole egg. Such investigation would indicate how many pseudoreplicates are required to actually describe a single egg shell.

The methods of identification and scoring process for candling, CT and SEM as used in this experiment are subjective processes with the potential for error and bias. Solomon (1986) comments that the visual scoring for translucency is a subjective process. The scoring of CT and SEM results in this project rely on visual scoring, and to maintain a reliable process all images were counted or scored by the same person (the candidate).

This pilot study was conducted in preparation for larger studies, the shell structures measured by SEM and CT were identified and pilot results demonstrated that there is a different abundance of structural features in shells of low and high translucency. The statistical power provided by this small study was sufficiently high to identify differences and future experiments should achieve the employ sample sizes (or greater) to identify statistical relationships.

Chapter 4. Experiment 2. Analysis of eggshell structures of consecutive eggs from individual hens.

4.1. Experiment 2 - Introduction

When comparing the external features or internal structures of eggs, there can be significant diversity among eggs laid by different species, within a single species, within a single flock and even among eggs laid by an individual hen (Panheleux, et al. 1990; Thompson, et al. 1983). In order to compare eggs with different externally visible features, such as translucency, it is important to compare successive eggs laid by the same hen to obtain an understanding of the diversity within each bird. The diversity of eggs laid by the same bird on successive days needs to be tested before experiments can be conducted assuming similarities.

While some workers have reported longitudinal measurements of eggs laid by the same hen these have not included shell details such as scoring for translucency, imaging the mammillary layer with Scanning Electron Microscopy (SEM) or examination of the palisade layer with Computed Tomography (CT). Leleu, et al. (2009) found that the vitelline membrane strength was not significantly different between eggs laid on successive days. Indicating some level of replicability, Thompson, et al. (1983) found that there was significant diversity among consecutive eggs in six measures including specific gravity, shell thickness, non destructive deformation and quasi-static compression force. The two measures with the highest correlations were egg weight and shell weight. Thompson, et al. (1983) concluded that egg values can be used as an indication of future egg results and that means from a group of eggs are more accurate

than a single sample. This work will examine and including, translucency, CT and SEM measures on successive eggs laid by individual hens.

There are a number of changes in the egg post-lay, many are associated with changes in the albumen properties. The two major changes in the albumen are an increase in pH (Aboonajmi, et al. 2010; Kim, et al. 2012; and Samli, et al. 2005) and a change in albumen viscosity (Aboonajmi, et al. 2010; Jones and Musgrove, 2005; Kemps, et al. 2010; and Silverside and Scott; 2001). These are likely to cause changes in the protein activity in the albumen (Kemps, et al. 2010), potentially reducing the anti-microbial activity of the albumen; however, this is partially offset by the increase in pH reducing bacterial growth (Messens, et al. 2004).

This experiment sought to examine the eggshell structures of eggs laid consecutively by the same hen, and to determine if successive eggs can be used as comparative samples. This knowledge is important to inform later experimental design, specifically to determine if an egg laid by an individual hen on one day could be used as a control for an egg laid on a subsequent day. Also this experiment examined the differences in eggs between no storage and after 1 week (7 days) unrefrigerated storage. It is known that unrefrigerated storage increases the incidence of bacterial penetration through shells (Brown, et al. 2006) and this study aimed to observe if there are any observable changes in the shell structure.

4.2. Experiment 2 - Methods

4.2.1. Sample Collection

Ninety eggs were collected from an experimental layer flock at the University of New England; eggs were collected from 6 hens on each of 15 days. Eggs were collected 5 days each week with a gap of two days in between. The term “day set” is used to describe a 5 consecutive day egg collection.

The first 60 eggs were collected in two five-day intervals with a two day interval in between (sampling days 1-5 and 8-12) and immediately weighed (Sartorius 'Quintix 513-1S' 3 decimal place balance). Eggs were candled for translucency immediately post-collection on an egg candling light box. Eggs were hand cracked, the contents were removed and the inside of the shell washed with tap water. For the third 5 day collection, 30 eggs were collected and placed in unrefrigerated storage at room temperature (mean of 18°C) for 7-8 days before being weighed (a second time), scored for translucency, cracked, emptied and washed.

Shells were allowed to dry thoroughly before being weighed again. From each shell, a small sample (10 mm by 5 mm) was taken from the equator of the shell. To remove the shell membrane from the sample, the shell sample was submerged in water for 30 to 60 minutes and the membrane was then removed from the shell by light physical rubbing.

4.2.2. CT Scanning.

Each shell sample was scanned with a GE Phoenix V-tomex CT Scanner, using the settings described in the Appendix (Chapter 9.3)

4.2.3. CT Image Processing.

CT Image processing was conducted as described in Chapter 2.

4.2.4. Scanning Electron Microscopy.

The SEM procedure was completed as described in Chapter 2.

4.2.5. Statistical Analysis.

The immediately analysed eggs and eggs stored for 7 days were compared using students paired t tests for the direct count groups (CT measures) and Wilcoxon (2 group comparisons) or Kruskal-Wallis (2+ group comparisons) tests were used to compare non-parametric (SEM measures) measures using JMP statistical software. The detectable difference in means was calculated using a power analysis tool within JMP; an alpha value of 0.05 and a statistical power of 0.8 were used. The sample number differed slightly between investigation periods and the relevant sample number was used to calculate power. The effect of egg weight on shell features, CT measures and SEM measures was examined by regression plots using JMP statistical software. To test the fit of the regression line, p values and R^2 values were used from the regression plot.

4.3. Experiment 2 - Results.

4.3.1. Consecutive Eggs

Egg Measures

The average egg weight for each investigation period ranged from 58.39g to 60.37g, with an average standard deviation of 4.74g. Given this level of variance and assuming a power of 0.8, the smallest difference in means detectable was 14.56g. There was very little difference between the average shell weights and standard deviation was similar between investigation periods (standard deviation 0.56g-0.60g). Eggshell translucency varied substantially between inspection periods and the smallest detectable difference in means was large. With the level of variance observed, treatment differences in means less than 2.3 score units (out of 5) remain undetectable (Table 4.3.1.1.).

Individual birds display considerable visually observed variability in consecutively laid eggs (Figure 4.3.1.2). Individual birds demonstrate the variability seen in members of the flock (figures 4.3.1.2. and 4.3.1.4.). Over days 8-12 and 15-19, compensatory effects can be observed between egg weights on consecutive days. A low egg weight is often followed by an egg that is heavier although the same relationship was less marked over days 1-5 (Figure 4.3.1.2).

Table 4.3.1.1. Average values of eggs from six hens, taken over 19 days in three five day periods. Standard deviations (in brackets).

Day Group	Egg Weight (g)	Shell Weight (g)	Translucency (0-5)
Day 1 - Day 5	60.30 (5.40)	5.66 (0.60)	1.70 (0.65)
Day 8 - Day 12	60.37 (4.64)	5.60 (0.57)	1.93 (0.84)
Day 15 - Day 19	58.39 (3.94)	5.65 (0.56)	1.38 (0.68)
Total Day 1 - Day 19	59.69 (4.74)	5.64 (0.56)	1.67 (0.75)
Detectable difference in means	14.56	1.72	2.3

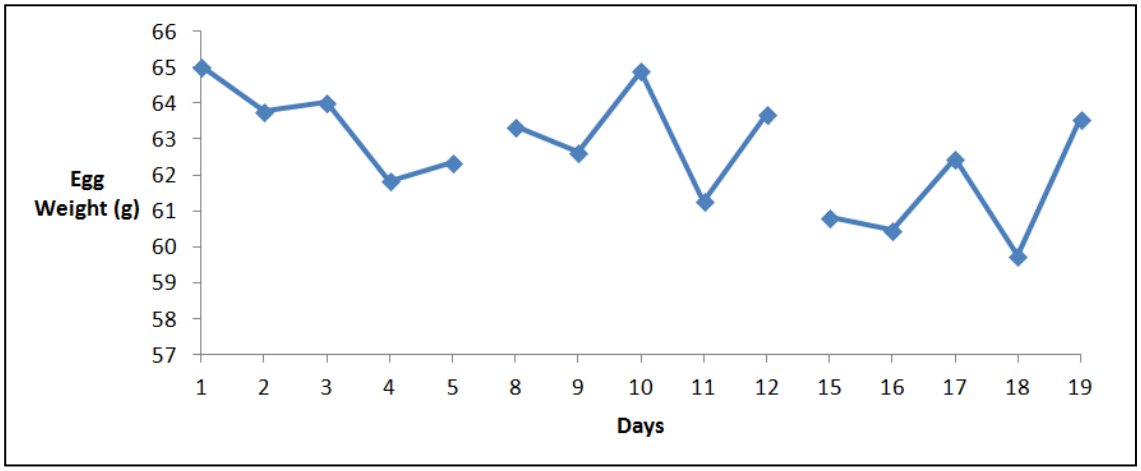


Figure 4.3.1.2. Consecutive egg weights from a single hen, three periods of five days over 19 days inclusive. Points depict individual observations and indicate a high degree of variance.

CT Measures

The degree of variance was so high amongst the CT measured shell features and the power to detect differences in means was therefore, low. In fact, for most measures the smallest shift in means detectable was substantially larger than the average number of features detected (table 4.3.1.2.). For example, to detect a treatment effect in the number of straight pore counts, a treatment would have to have on average 4.6 more pores per sample area (4.13 mm^2) than the control (table 4.3.1.2.). For shell thickness, which had more consistent means between investigation periods, the detectable difference was still 0.09. The largest difference between investigation period averages was found in externally branching pores ranging from 1.72 to 2.5 between the five day investigation periods (days 15-19 and 8-12 respectively). Standard deviations were high for all measures except shell thickness. For all pore counts the standard deviations were greater than half of the calculated average indicating significant variation around the mean.

There is no apparent relationship in the occurrence of externally branching and straight pores in consecutively laid eggs from a single hen (Figure 4.3.1.4.). There does appear to be some level of compensation between the two occurrences.

Table 4.3.1.2. Average CT measure values of eggs from six hens, taken over 19 days in three five day periods. Investigation periods were separated by two days each. Standard deviations (in brackets).

Day Group	Straight Pores (count)	Internally Branching Pores (count)	Externally Branching Pores (count)	CT Measured Shell Thickness (mm)
Day 1 - Day 5	2.57 (1.38)	0.07 (0.22)	1.85 (1.16)	0.37 (0.03)
Day 8 - Day 12	2.21 (1.21)	0.02 (0.09)	2.50 (1.49)	0.36 (0.03)
Day 15 - Day 19	2.45 (1.86)	0.05 (0.20)	1.72 (1.58)	0.37 (0.03)
Total Day 1 - Day 19	2.41 (1.50)	0.05 (0.18)	2.02 (1.44)	0.37 (0.03)
Detectable difference in means	4.61	0.55	4.42	0.09

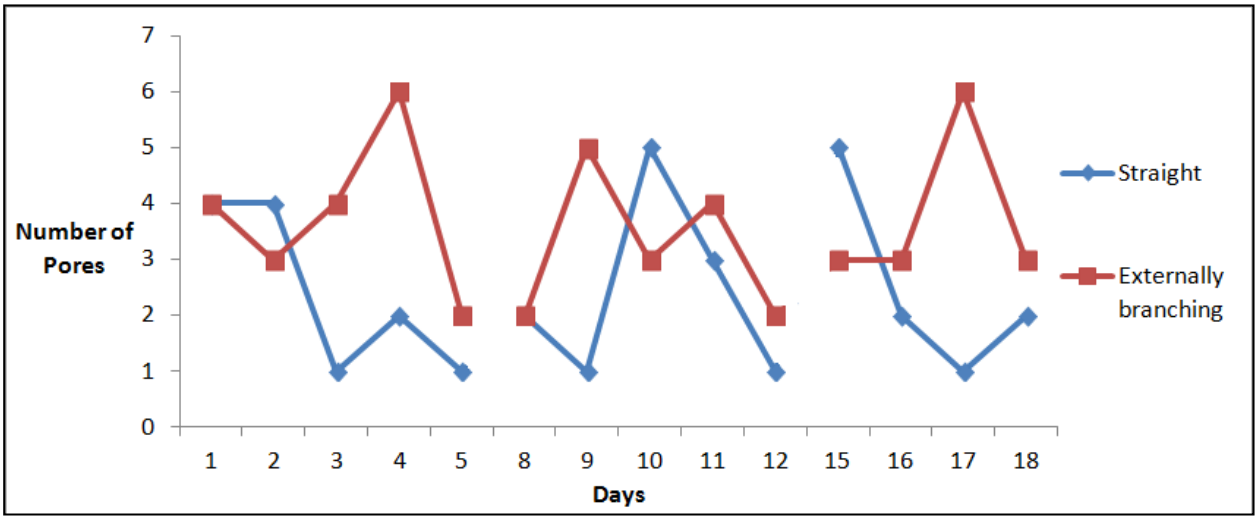


Figure 4.3.1.4. Consecutive pore counts from a single hen, both straight and externally branching pores over three periods of five days over 19 days inclusive. Points indicate observations.

SEM Measures

There were larger differences between investigation period means in SEM measures than physical egg or CT measures. Mammillary body size and mammillary layer fusion both increased with time, the incidence of type A and type B mammillary bodies showed no linear relationship and there was still considerable variance. Standard deviation for all measures was high relative to the range of possible scores. The smallest standard deviation observed was in the first investigation period of type B mammillary bodies was 0.82 with a scoring range of 0-3.

Due to the high degree of variance in all egg measures, the capacity to detect small shifts in means was low (Table 4.3.1.3.). For the abundance of type A and type B mammillary bodies the minimum detectable difference in means was 2.79 and 2.86 respectively. These represent more than 50% of the scoring range. For mammillary body size, the smallest difference in means detectable was 3.22 which represents 46% of the scoring range. Likewise for mammillary layer fusion under the current experimental conditions, treatment effects greater than 5.16 would have been detectable which represents 73.7% of the scoring range.

Table 4.3.1.3. Average SEM measure values of eggs from six hens, taken over 19 days in three five day periods. Standard deviations (in brackets).

Day Group	Mammillary Body Size (-3 - 3)	Mammillary Layer Fusion (-3 - 3)	Rate of Type A Mammillary Bodies (0-3)	Rate of Type B Mammillary Bodies (0-3)
Day 1 - Day 5	-1.50 (0.86)	-1.60 (2.75)	0.97 (0.89)	0.47 (0.82)
Day 8 - Day 12	-0.70 (1.03)	-0.70 (1.03)	0.96 (0.94)	0.74 (0.90)
Day 15 - Day 19	-0.23 (1.27)	-0.23 (1.27)	1.50 (0.91)	0.73 (1.08)
Day 1 - Day 19	-0.84 (1.17)	-0.95 (2.09)	1.13 (0.93)	0.64 (0.93)
Detectable difference in means	3.22	5.16	2.79	2.86

4.3.2. Storage Time

After eggs were stored at room temperature for 0 days and 7 days, there were no significant differences in egg weight, shell weight and percentage shell between the 0 and 7 days storage times. On average, shell translucency was 0.44 scoring units lower for eggs stored for 7 days (p=0.0103) than those measured immediately.

Table 4.3.2.1. Storage time related to egg quality. Superscripts indicate significant differences and standard deviation in brackets. NS is not statistically significant.

Storage Time	Egg Weight (g)	Shell Weight (g)	Percentage Shell (%)	Translucency (0-5)
0 Days (n=60)	60.33 (5.00)	5.63 (0.58)	9.0 (0.001)	1.81^{a} (0.75)
7 Days (n=30)	58.39 (3.94)	5.65 (0.56)	10.0 (0.002)	1.35^{b} (0.63)
p Value	NS	NS	NS	0.0103

Despite the differences in translucency, there was no difference found in any of the CT measures. Straight pores and externally branching pores were more common then internally branching pores. Shell thickness was the same for both zero and seven days storage groups (0.37 mm).

Table 4.3.2.2. Storage time related to CT identified shell structures. Standard deviation in brackets. NS is not statistically significant.

Storage Time	Straight Pores (count)	Internal Pores (count)	External Pores (count)	Shell Thickness (mm)
0 Days (n=10)	2.39 (1.30)	0.04 (0.17)	2.17 (1.36)	0.37 (0.03)
7 Days (n=5)	2.73 (1.75)	0.06 (0.22)	1.92 (1.55)	0.37 (0.03)
p Value	NS	NS	NS	NS

The 7 days storage group had a higher mammillary body size (p =0.001) and incidence of type A mammillary bodies (p =0.0146). All other measures tended to be higher in the 7 days storage eggs (table 4.3.2.3.).

Table 4.3.2.3. Storage time related to SEM identified shell structures. Superscripts indicate significant differences and standard deviation in brackets. NS is not statistically significant.

Storage Time	Mammillary Body Size (-3 to 3)	Mammillary Layer Fusion (-3 to 3)	Type A Mammillary Bodies (0-3)	Type B Mammillary Bodies (0-3)	Cubic Calcite Formations (0-3)
0 Days (n=10)	-1.12^{a} (1.02)	-1.18 (2.32)	0.96^{a} (0.91)	0.60 (0.86)	0.02 (0.13)
7 Days (n=5)	-0.23^{b} (1.27)	-0.46 (1.39)	1.50^{b} (0.91)	0.73 (1.08)	0.07 (0.39)
p Value	0.0010	NS	0.0146	NS	NS

4.3.3. Relationship with Egg Weight

The relationship between egg weight and other egg and shell features was determined by regression analysis (Figures 4.3.3.1. to 4.3.3.4.). As egg weight increases, the shell weight as a proportion decreases (Fig 4.3.3.1.). While this is statistically significant (p = 0.0051), the coefficient of determination is low (R^2=0.09). Translucency scores decrease as egg weight increases (Fig 4.3.3.2.), however, the correlation is poor (R^2=0.07). Both the incidence of internally branching pores (Figure 4.3.3.3.), and mammillary body size (Figure 4.3.3.4.) decreased with increasing egg weight. The R^2 values for each plot were all less than 0.2 indicating large variation around the trend and very poor relationships.

Figure 4.3.3.1.

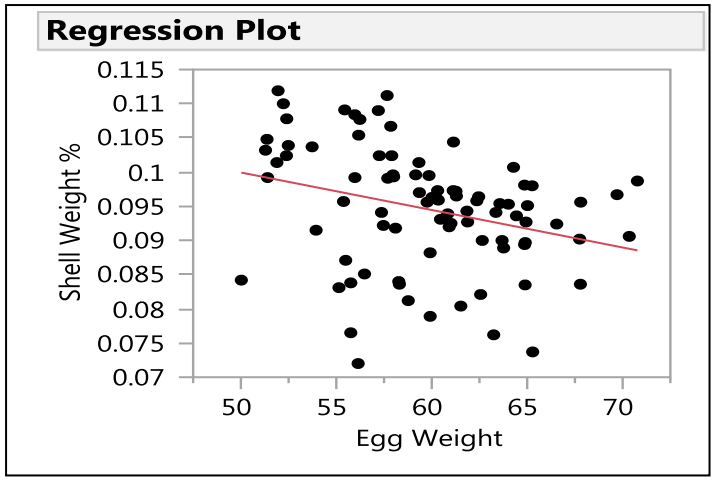


Figure 4.3.3.2.

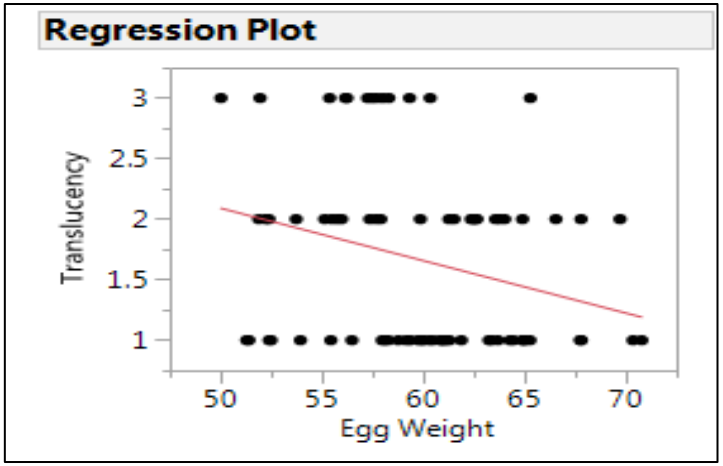


Figure 4.3.3.1. Regression plot, egg weight compared to shell weight as a percentage of egg weight.
P Value = 0.0051
R^2 = 0.09

Figure 4.3.3.2. Regression plot, egg weight compared to egg translucency.
P Value = 0.0102
R^2 = 0.07

Figure 4.3.3.3.

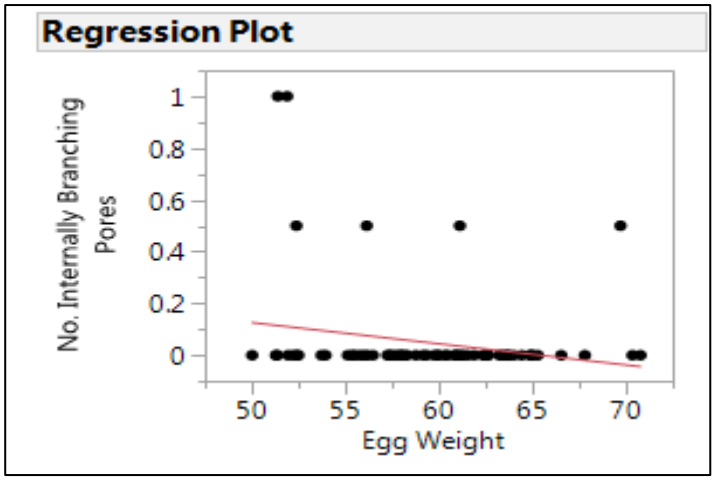


Figure 4.3.3.4.

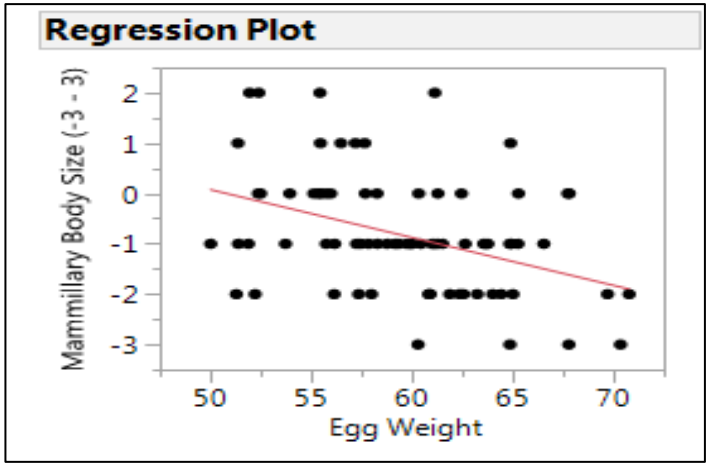


Figure 4.3.3.3. Regression plot, egg weight compared to internally branching pores.
P Value = 0.0478
R^2 = 0.05

Figure 4.3.3.4. Regression plot, egg weight compared to mammillary body size.
P Value = 0.0003
R^2 =0.15

4.4. Experiment 2 - Discussion

There is a large amount of variation between eggs laid on successive days. The variation is shown by the high standard deviations within investigation periods, and in the overall time period. Some birds exhibited cyclic results, with a smaller egg laid before/after a larger egg. This may be a survival mechanism to ensure there are resources to produce a larger eggs within a clutch. These results are consistent with those of Thompson, et al. (1983) who comments that while one egg can be used as an indication of what to expect the next day, this is not always reliable and a sample group is more accurate than any individual egg. A high detectable difference in means indicates that even substantial treatment effect could not be identified. Some factors such as internally branching pores had such low counts that the results are zero inflated and unreliable.

The eggs that were stored for seven days prior to analysis had larger mammillary body size and higher occurrence of type A mammillary bodies. This is not the result of the storage but of the different eggs used in each group. An increase in eggshell translucency was expected (Ray, unpublished thesis, 2013), potentially due to the transfer of moisture from the albumen of the egg into the structures of the shell. There was a lower translucency score in the 7 days storage group compared to the immediately analysed group, which is inconsistent with previous work that has reported an increase in translucency during storage (Ray, unpublished thesis, 2013). As the eggs showing lower translucency were different eggs, it is possible that these eggs had a lower translucency score at lay. It remains unclear what effect the timing of an egg within a clutch has on the translucency and shell structures and this is an avenue of research that should be considered. As candling and translucency scoring is a non-destructive process, comparisons of

translucency scores could be conducted on the same rather than successive eggs in future experimentation. The increase in mammillary body size and the abundance of type A mammillary bodies may be related to the higher eggshell translucency.

Significant relationships were identified between egg weight and the following features, mammillary body size, translucency and the number of internally branching pores. However, only weak correlations existed between these features and egg weight and were subject to influential points. Therefore, no categorical conclusions can be made about the relationship between egg weight and any shell structural features. Any future work designed to identify these relationships should include larger sample sizes and ensure a more complete 'spread' of egg weights.

Future studies in this area should strive to analyse a continuous sequence of eggs, rather than the 5 days a week completed here. This would remove the requirement of having to use day sets and the eggs could be compared as a single group. Longer periods of analysis may facilitate the identification of changes in the egg shell structure over time and the cyclic nature of egg shell structures. The subjective visual scoring of CT and SEM images used in this experiment has its limitations; however, it does provide a convenient mechanism that ensures accurate counts especially for CT identified shell pores.

The examination of shells was essential to determine if consecutive shells can be compared as part of the larger study on translucency and shell penetration rates by bacteria. This experiment has confirmed that there is considerable variation between consecutive eggs laid by the same bird and that future trials comparing shells should be completed on the same egg, or include sample sizes large enough to compensate for the variation.

Chapter 5. Experiment 3. Identifying the Relationship Between Eggshell Translucency and Shell Structures With Computed Tomography And Scanning Electron Microscopy.

5.1. Experiment 3 - Introduction

The appearance of eggshell translucency has been shown to be due to the presence of water within the shell structures; however, these underlying structures remain uncharacterised. There is considerable variation in many aspects of egg and eggshell morphology, and strong variation has been found to occur between eggs laid on consecutive days, as outlined in Chapter 4. Further adding to the uncertainty surrounding translucency, there is considerable variation in the amount and type of translucency in the shell and there may also be significant variation in the underlying shell structures that are thought to cause translucency.

Translucency has been discussed by many researchers, generally related to other shell factors that it may influence. Translucency changes in its extent and appearance over time post lay but much of the literature appears to overlook the effect of storage. Previous work has confirmed that, in more than 90% of eggs, translucency has fully developed by day 21 (Ray, unpublished thesis, 2013). The extent of shell translucency refers to the area of the shell that could be considered translucent and appearance relates to the degree of translucency. The lack of this critical information means that most previous work is difficult to interpret; this experiment will include both initial translucency (day 0), final translucency (day 21) and any change in translucency for all basic, CT or SEM measures.

Ray and Roberts, (2013) confirmed that translucency is related to the presence of moisture within the shell, and is independent of shell contents. When the shell is dried, the translucency disappears and when the shell is submerged in water the translucency returns, originally to the same locations and eventually through the entire shell. Having determined that the appearance of translucency is dependent on the presence of moisture within the shell, the question was which structures within the shell are acting to 'contain' the water.

The theories suggested in Table 5.1.1. have all been disproved. Moisture was first implicated as the cause of translucency by Talbot and Tyler (1973b). Solomon, (1986) added that the shell must possess structures that allow the moisture to build up, and that pores are a potential cause.

Table 5.1.1. Previously suggested structural basis of eggshell Translucency that have now been disproved.

Previously Suggested Structural Basis of Translucency	**Reference**
Shells deficient in manganese were more likely to have translucent regions	Leach and Gross, (1983).
Damage to the freshly laid shell results in shell damage causing translucency.	Talbot and Tyler, (1973).
In larger eggs, there is greater distance between the nucleation sites, resulting in a less 'complete' shell.	Solomon, (1986).
The uneven distribution of fat or oil through the shell, which changes the transmission of light causing translucency.	Holst, et al. (1931).
Thinner regions of shell transmit light resulting in translucency.	Holst, et al. (1931).
Variation in crystal calcium formations resulting in air spots in the shell.	Holst, et al. (1931).
Remaining unsure of the exact cause, Solomon (1986) determined that the morphology of the mammillary layer was responsible for translucency.	Solomon, (1986).

In addition to the body of Scanning Electron Microscope (SEM) studies examining the shell's mammillary layer (Board and Fuller, 1994; Burley and Vahedra, 1936; Chousalkar, et al. 2010) this study includes micro Computed Tomography (CT) scans on shell sections. The examination of the shell's palisade layer may indicate abnormalities or porous structures that provide space for moisture to be retained in the shell. Eggshell pores are a potential cause of eggshell translucency due to their nature as a channel for air and moisture from the inside to the outside of the shell. Different pore structures including externally branching pores as described by Board (1979) may result in increased areas within the shell for moisture to occupy. The shell's mammillary layer as the source of nucleation sites for the true shell is also a likely source of abnormalities that may disrupt the palisade layer. By using CT to observe the palisade layer

abnormalities, and SEM to observe mammillary layer abnormalities this can produce a comprehensive picture of the shell's structures.

The aim of this experiment is to identify which shell structures cause the appearance of translucency. Also under examination is any change in the appearance of translucency over time post lay, during both refrigerated and non-refrigerated storage of eggs.

5.2. Experiment 3 - Method

5.2.1. Sample collection and translucency scoring.

Over 5000 eggs were sourced from commercial egg operations in Northern NSW and Southern Queensland, Australia. Eggs were collected from the grading floor or directly from the cage fronts on the day of lay (Day 0). Eggs were collected from hens of different ages (flock ages between 14 and 60 wks). Each egg was scored for translucency using a zero to five translucency scoring system as soon as practical (between 2 and 10 hours) using a University of New England egg candler (UNE workshops). Eggs were then divided into two groups and stored in either a cool dry room or in a commercial grade refrigerated walk-in cool room (~4°C) and removed at days 7, 15 and 21 post lay for further scoring. Further scoring was done ‘blind’ without viewing previous scores to avoid scoring bias.

At day 21, all eggs exhibiting very low (0) and very high (4-5) translucency were selected, 157 from the 'low' translucency score group and 243 from the 'high' translucency score group. A single sample was selected from the midline of each egg and hand broken to produce a sample of approximately 10mm by 5mm in size. These samples were then allowed to dry in airtight containers containing silica gel desiccant.

5.2.2. CT scanning.

Each sample was scanned with a GE Phoenix V-tomex CT Scanner, using the settings described in the appendix (Chapter 9.3.)

5.2.3. CT Image Processing.

CT Image processing was conducted as described in Chapter 2 (2.1.).

5.2.4. Scanning Electron Microscopy.

The SEM procedure was completed as described in Chapter 2 (2.3.).

5.2.5. Statistical Analysis.

Change in translucency was calculated by subtracting the initial translucency from the final translucency. Initial and final translucency were compared for all variables (CT and SEM) using JMP v.11. Means, standard deviation and ANOVA tables were produced for all comparisons. Students paired t tests were used to compare direct count groups (CT) and Wilcoxon (two group comparisons)/ Kruskal-Wallis (more than two group comparisons) comparison tests were used to compare non-parametric (SEM) measures. Refrigerated and non refrigerated treatments were compared for translucency using a paired t test. Regression analysis was used to identify relationships between flock age and shell structures. Regression analysis was also used to identify relationships between the change in translucency (over 21 days) and shell structures. To test the fit of the regression line, p values and R^2 values were recorded from the regression analysis.

For the purpose of statistical analysis the following groups were defined.

Initial Translucency

Low - 0 and 1, moderate - 2 and 3 and high - 4 and 5.

Final Translucency

Low - 0 and 1 and high - 4.5 and 5.

5.3. Experiment 3 - Results

5.3.1. Storage Conditions.

Refrigerated eggs had a significantly lower final translucency than the non-refrigerated eggs (Table 5.3.1). Refrigerated eggs also showed a significantly smaller change in translucency when compared to non-refrigerated eggs (0.29 and 0.36 respectively).

Table 5.3.1. The effect of refrigeration and non-refrigeration storage on the final translucency and change in translucency scores. Standard deviation in brackets and superscripts indicate significant differences.

Status	Final Translucency (21 Days) (0-5 Score)	Change in Translucency
Refrigerated (n=980)	1.82[a] (0.86)	0.29[a] (0.72)
Non-Refrigerated (n=2752)	2.37[b] (0.94)	0.36[b] (0.94)
P Value	<0.0001	0.0484

5.3.2. Flock Age.

The effect of flock age was compared for a number of shell structures as measured by CT and SEM using regression plots; only significant relationships are displayed (Figures 5.3.1. 5.3.2. and 5.3.3.). There was a significant but small increase in the incidence of type B mammillary bodies with increasing flock age (figure 5.3.3.). However, all of these regression plots show a low correlation of fit (R^2= 0.03 for all three groups).

Flock Age.

Figure 5.3.1.

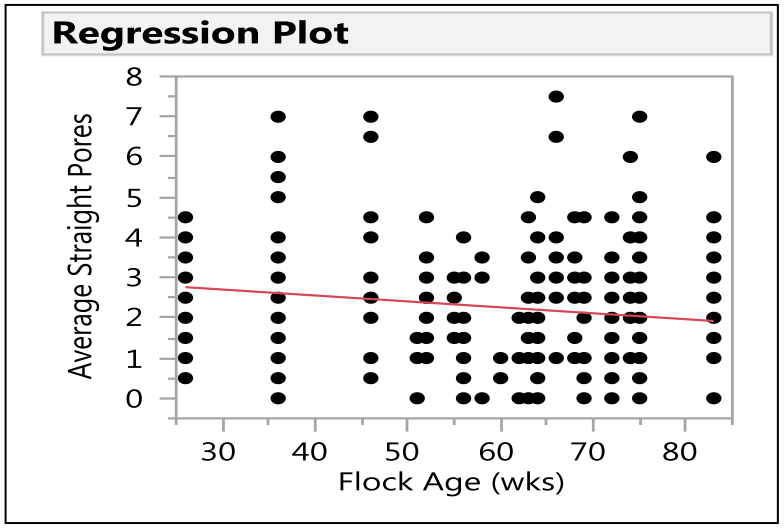


Figure 5.3.2.

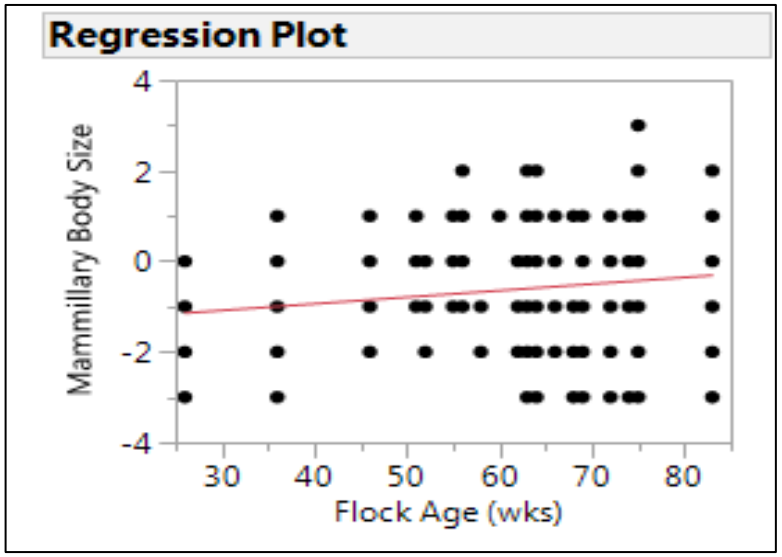


Figure 5.3.3.

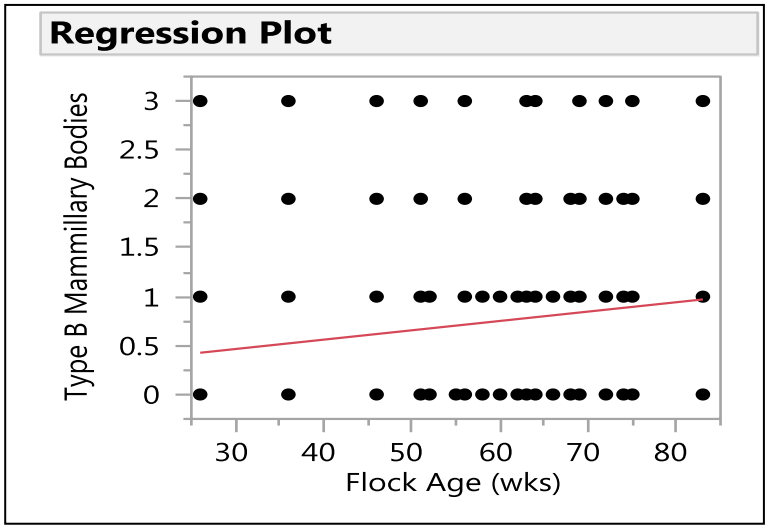


Figure 5.3.1. Flock age compared to the average number of straight pores.
P value = 0.0012
R^2 = 0.03

Figure 5.3.2. Flock age compared to the mammillary body size.
P value = 0.0011
R^2 = 0.03

Figure 5.3.3. Flock age compared to the rate of type B mammillary bodies.
P value = 0.0015
R^2 = 0.03

5.3.3. Egg Initial Translucency.

Eggs with initial high translucency were found to have a significantly different shell thickness between the low and moderate/high groups with no difference between the moderate and high groups (Table 5.3.2.). The low initial translucency group had a lower number of externally branching pores (low=0.71, moderate=1.54, high=1.52) and a higher number of straight unbranching pores when compared to the moderate and high groups (2.69, 1.92 and 2.04, low, moderate and high respectively). There were no significant differences in the average number of internally branching pores between initial translucency groups with scores ranging from 0.05 (moderate) to 0.11 (high).

Table 5.3.2. Initial (Day 0 post lay) Translucency compared by CT measures. Standard deviation in brackets and superscripts indicate significant differences.

Initial Translucency	Average Straight Pores (count)	Average Internally Branching Pores (count)	Average Externally Branching Pores (count)	CT Determined Shell Thickness (mm)
Low (n=161)	2.69[a] (1.51)	0.07 (0.24)	0.71[a] (0.82)	0.37[a] (0.04)
Moderate (n=87)	1.92[b] (1.31)	0.05 (0.16)	1.54[b] (0.90)	0.39[b] (0.06)
High (n=104)	2.04[b] (1.49)	0.11 (0.39)	1.52[b] (1.15)	0.39[b] (0.06)
P Value	<0.0001	NS	<0.0001	0.0128

The low initial translucency group was different from the high translucency group in all measures except mammillary body size, and significantly different from the moderate group in all measures except mammillary layer fusion. The incidence of type A and type B mammillary bodies, as well as the incidence of cubic calcite formations, were all significantly lower for the low initial translucency score groups than for the moderate and high initial translucency score groups (Table 5.3.3.). The incidence of cubic calcite formations was low (0.02 to 0.11) in all groups with over 92.5% of shells not showing any cubic formations.

Table 5.3.3. Initial (Day 0 post lay) Translucency compared by SEM measures. Standard deviation in brackets and superscripts indicate significant differences.

Initial Translucency	Mammillary Body Size (-3 to 3)	Mammillary Layer Fusion (-3 to 3)	Type A Mammillary Bodies (0 to 3)	Type B Mammillary Bodies (0 to 3)	Cubic Calcite Formations (0 to 3)
Low (n=161)	-0.73 (1.17)	-0.66[a] (1.36)	0.62[a] (0.78)	0.52[a] (0.73)	0.02[a] (0.14)
Moderate (n=87)	-0.55 (1.35)	-0.54[a] (1.64)	1.13[b] (0.92)	0.90[b] (1.07)	0.11[b] (0.36)
High (n=104)	-0.36 (1.23)	-1.10[b] (1.39)	1.11[b] (0.91)	0.90[b] (1.08)	0.09[b] (0.33)
P Value	NS	0.0184	<0.0001	0.0012	0.0160

5.3.4. Egg Final Translucency.

The average number of straight pores per sample was significantly higher in low translucency shells as compared with high translucency (Table 5.3.4.). The number of externally branching pores was statistically significantly higher in the high translucency shells as compared with the low translucency shells (Table 5.3.4.). Shell thickness and the number of internally branching pores were not significantly different between the high and low translucency groups.

Table 5.3.4. Final (21 Day post lay) Translucency compared by CT measures. Standard deviation in brackets and superscripts indicate significant differences.

Final Translucency	Average Straight Pores (count)	Average Internally Branching Pores (count)	Average Externally Branching Pores (count)	CT Determined Shell Thickness (mm)
Low (n=155)	2.70[a] (1.51)	0.06 (0.21)	0.68[a] (0.79)	0.37 (0.05)
High (n=246)	1.98[b] (1.39)	0.08 (0.33)	1.65[b] (1.05)	0.39 (0.05)
P Value	<0.0001	NS	<0.0001	NS

Mammillary body size and fusion were not significant different between the low and high final translucency groups (Table 5.3.5.). All other measures including; type A mammillary bodies, type B mammillary bodies and cubic calcite formations were significantly more common in the high final translucency score group.

Table 5.3.5. Final (21 days post lay) Translucency compared by SEM measures. Standard deviation in brackets and superscripts indicate significant differences.

Final Translucency	Mammillary Body Size (-3 to 3)	Mammillary Body Fusion (-3 to 3)	Type A Mammillary Bodies (0 to 3)	Type B Mammillary Bodies (0 to 3)	Cubic Calcite Formations (0 to 3)
Low (n=155)	-0.74 (0.10)	-0.72 (1.32)	0.58^{a} (0.77)	0.53^{a} (0.73)	0.02^{a} (0.14)
High (n=246)	-0.53 (0.08)	-0.69 (1.54)	1.08^{b} (0.88)	0.84^{b} (1.04)	0.09^{b} (0.34)
P Value	NS	NS	<0.0001	0.0018	0.0136

5.3.5. Change in Egg Translucency.

The relative change in translucency over a 21 day period ranged from -1 (indicating a lower final translucency then initial) to 4, (indicating a much higher final translucency than initial). As the change in translucency increased there were increased changes in a range of eggshell structures. The abundance of externally branching pores, the incidence of type A mammillary bodies and the extent of mammillary layer fusion all increased with increasing translucency change (Figures 5.3.4. to 5.3.6.). All three significant regressions indicated limited predictive power with R^2 values from 0.01 to 0.03.

Change in Translucency.

Figure 5.3.4.

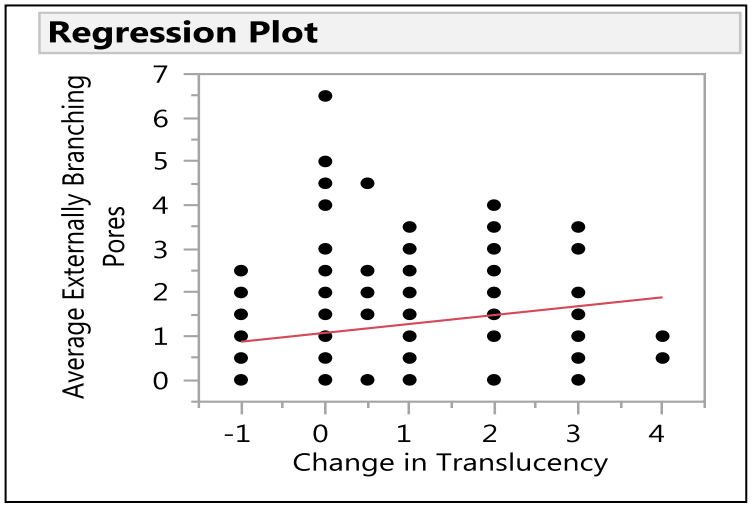


Figure 5.3.5.

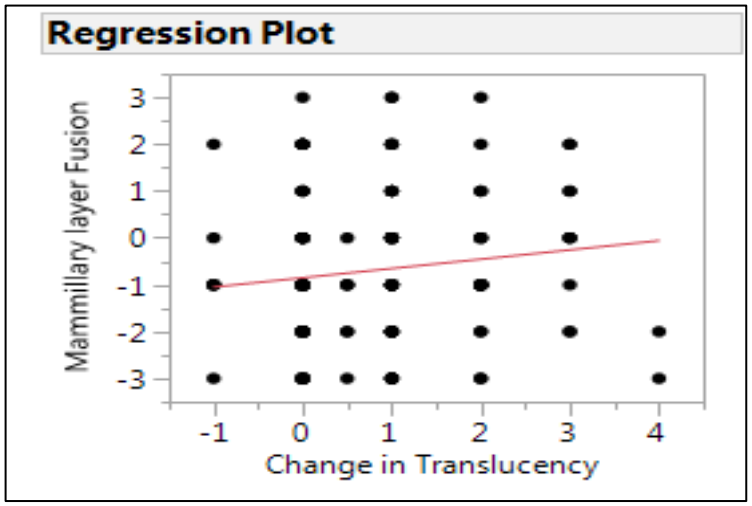


Figure 5.3.6.

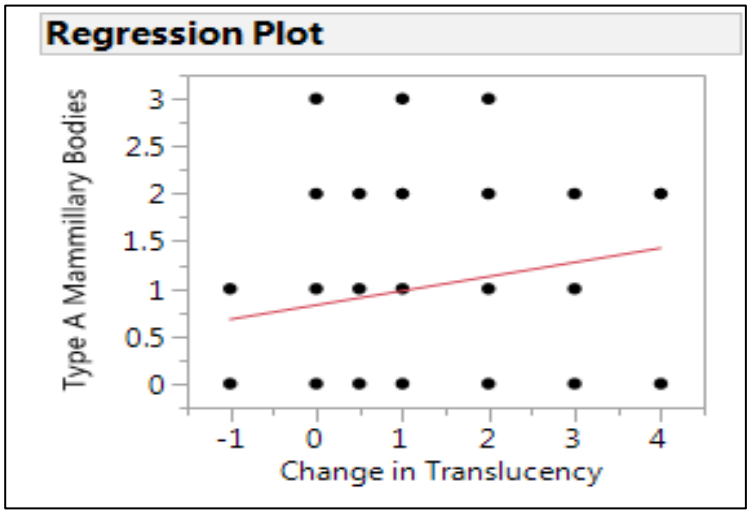


Figure 5.3.4. Change in translucency compared with average externally branching pores.
P value = 0.0017
R^2 = 0.03

Figure 5.3.5. Change in translucency compared to the mammillary layer fusion.
P value = 0.0325
R^2 = 0.01

Figure 5.3.6. Change in translucency compared to the rate of type A mammillary bodies.
P value = 0.0092
R^2 = 0.02

5.4. Experiment 3 - Discussion

The purpose of this experiment was to identify which shell structures are associated with the appearance of eggshell translucency. A number of eggshell structures observed using CT and SEM were significantly different between the low and high translucency score groups. Other components of this experiment included comparing the effects of refrigerated and non-refrigerated storage on the development of translucency. The final component was to examine how flock age affected the eggshell structures.

In order to properly examine the structural basis of translucency both the initial (0 days) and final translucencies (21 days) of shell samples were examined. A lower number of straight pores and higher number of externally branching pores in the shells with a higher level of translucency. For the SEM measures, higher translucency shells showed an elevated incidence of type A and type B mammillary bodies and cubic calcite formations. While there is no single factor that causes eggshell translucency or the change of translucency, it can be seen that, as translucency increases, so do a number of shell defects. There is no conclusive link between these identified structures and the presence of water in the shell. However, externally branching pores may provide space within the exterior region of the eggshell for water to occupy. The other abnormalities identified as being related to translucency are all in the shell's mammillary layer, which is in agreement with Solomon (1986). Type A mammillary bodies are those bodies that have no attachment to the shell membrane; however, they provide bulk to the shells palisade layer. Type B mammillary bodies are small spherical or nearly spherical bodies that may have attachment to the membrane but provide no 'bulk' to the true shell. Cubic calcite and type B

bodies potentially disrupt the formation of the palisade columns that form the palisade layer. It remains unclear how mammillary layer abnormalities affect the 'bulk' or build-up of the shell, it is possible that the disruption of the mammillary layer results in gaps in the shell's palisade layer and reduces the strength of the contact between the shell membranes and the mammillary cones. A strong consistent mammillary layers is the basis for a consistent palisade layer, therefore, any disruption to the mammillary layer can result in an increased incidence of shell pores.

It is likely that translucency is not caused by a single shell structure but, rather, that there are a number of shell structures that can disrupt the mammillary and palisade layers of the shell. Variation in these structures may explain the variation in the appearance of translucency. While there have been a number of suggestions about potential causes of eggshell translucency no previous work has made certain findings. Solomon, (1986) suggested that, in larger eggs, there is more room between the nucleation sites resulting in fewer calcite columns and therefore translucency. However, the results of the present study show no difference in mammillary body size between translucency groups for either the initial or final translucency measures. Holst, et al. (1931) made two predictions; the first, that thinner regions of the shell were the cause of translucency, is disproved by the current study finding no significant difference in thickness between low and high translucency shells the second prediction by Holst, et al. (1931) is that translucency is caused by variations in the crystal column formations resulting in air spots. This is probably accurate, with the qualifier that the spaces in the shell are occupied by the water from the egg rather than air. Talbot and Tyler, (1973) indicated that translucency is caused by damage

to the freshly laid egg immediately post lay, and while this is possible it does not explain the variation or extent of translucency commonly observed.

The changing appearance of translucency during storage is likely caused by the movement of water through the shell. It is known that translucency is caused by the presence of water within the shell structures (Ray, unpublished thesis, 2013; Ray and Roberts, 2013). The loss of water by the egg post lay (Ar and Rahn, 1985) is likely to affect the amount of water in the shell and therefore, the appearance of translucency. Eggs (3732) were examined and those stored in commercial refrigeration had both reduced final translucency and a reduced change in translucency. It is likely that the colder temperature reduced the water loss from the egg, and therefore, reduced the progression of water through the shell. Water loss from the egg is dependent on the difference in vapour pressure between the inside and outside of the shell, while refrigerated there is a lower water potential gradient. Change in translucency over the 21 day storage period was examined with reference to shell structure in this experiment, although there were few significant results. Linear regression analysis showed some relationships between change of translucency and the extent of mammillary layer fusion, the incidence of type A mammillary bodies and the number of externally branching pores. It seems likely that externally branching pores and high incidence of type A mammillary bodies increases the change in translucency by influencing the rate and extent of movement of water through the shell.

It has been previously reported that translucency increases as flocks age (Solomon, 1986) however, the present experiment found no such evidence. This may be due to the fact that the older laying flocks had been subjected to an induced moult at approximately 65 wks. Moulting

has been shown to increase productivity although it does not necessarily influence shell weight or thickness (Ahmed, et al. 2005). It is likely that since moulting has an effect on some egg quality features and may also affect translucency score.

5.4.1. Sources of Error and Improvements in Method

Visual scoring is a well-known error source with translucency scoring being labelled as subjective (Solomon, 1986). In order to reduce errors translucency scoring was conducted 'blind', (where the previous scores were not viewed when scoring), and standard score images were available and referred to. When identifying pore structures (especially internally opening pores) some samples were viewed in the three dimensional reconstruction of the pore, which clearly showed if a pore branched. Future work could focus on flock age and investigate the influence that induced moulting has on the abundance of eggshell structures.

Micro CT technology represents a major breakthrough in the examination of eggshells and when combined with the already established SEM of the mammillary layer it allows us to develop a more complete picture of eggshell structure. The visual identification and scoring of pores, and measurement of the shell thickness is a subjective and elementary use of technology that is by nature quantitative. Future work should focus on using CT density volumes to examine the shells for subtle changes in density through the shell. Computed tomography is able to measure the thickness of the mammillary layer over large regions of the shell and measure the abundance and profile of shell pores quantitatively rather than qualitatively, this work is hopefully just the beginning in micro CT analysis of commercial eggs and their shells. Further

avenues of research may also include analysing the makeup of eggshells using mass spectroscopy.

This work has focused on the list of potential structural defects that cause translucency and these can now be related to the rates of bacterial penetration in experiment 4 (chapter 6). Identifying potential shell structures by non-destructive candling may allow for a number of future opportunities. Selecting specific low translucency eggs for immunocompromised populations or transferring high translucency eggs to pasteurization and Liquid Egg Processing (LEP) may be a means of improving industry food safety if a confirmed relationship between translucency and egg susceptibility to horizontal infection can be confirmed.

Chapter 6. Experiment 4. Agar Egg Penetration of Shells by Salmonella Typhimurium Phage Type 9 and the Relationship between Penetration and Shell Translucency and Other Shell Structures.

6.1. Experiment 4 – Introduction

The egg is a vital source of protein and nutrients for developed and developing countries alike and, for this reason, ensuring its food safety is vital. The eggshell is an often overlooked structure providing both protection and a convenient container for the egg contents (Jones, et al. 2004). There are two ways that eggs can become infected with pathogenic bacteria, vertical or pre lay infection and horizontal or post lay infection (De Reu, et al. 2006; Dolman and Board, 1991; Gantois, et al. 2008; Miyamoto, et al. 1996). Horizontal infection is of serious concern due to both the rate of infection and the number of points in the handling chain that may facilitate the infection process.

Eggshell penetration by *Salmonella enterica* serovars is a serious concern to both industry and the consumer. If eggs that represent a potential bacterial risk (due to a shell providing poor protection), can be identified on farm by a non-destructive method, these could be re-directed to commercial pasteurised egg products. This would produce a safe product for consumers yet offset the cost to producers by facilitating income from the potentially unsafe eggs.

There a lack of published information comparing the relative risk of horizontally infected (post lay infection) eggs to vertically infected eggs (pre-lay infection) for the development of

human illness. In Australia, there is no evidence that *Salmonella* Typhimurium enters the egg contents via vertical transmission and *Salmonella* Enteritidis is not endemic to Australian layer flocks.

Salmonella is responsible for between 696,000 and 3,840,000 cases of human *Salmonella* food poisoning in the US each year (Lin, et al. 1997) and 11,992 notifications for *Salmonella* food poisoning in Australia (OzFoodNet Working Group, 2010); and is therefore, a considerable problem. The Australian cases of Salmonella food poisoning were made up by *S*. Typhimurium (5,241 cases – 43%), *S*. Enteritidis (836 cases – 7%), *S*. Virchow (571 cases – 5%), *S*. Saintpaul (422 cases – 4%) and *S*. Virchow (323 cases – 3%) (OzFoodNet Working Group, 2010).

Considerable work has been conducted comparing shells penetrated with bacteria to unpenetrated shells and penetration rates under a range of conditions. Several researchers have compared various infection conditions to infection rates. However, with one exception, no one has discussed the storage conditions pre-infection, and no one has discussed in detail the significance of egg shell translucency (Bain, et al. 2011; Berrang, et al. 1998; De Reu, et al. 2006; Dolman and Board, 1991; Schoeni, et al. 1995). Dolman and Board (1991) standardised their experiments by using eggs that were 2 days old in their agar penetration experiments.

6.1.1. Salmonella Typhimurium

Salmonella is one of the major food borne pathogen causing gastrointestinal food poisoning in humans. Normally the disease progresses as a gastrointestinal illness characterised by pain, fever, diarrhoea and vomiting in humans (Lin, et al. 1997). *Salmonella* Typhimurium also infects a range of domestic livestock and fowl resulting in a spectrum of outcomes ranging from

severe disease to asymptomatic carriage. Bacteria readily spread between these reservoirs and the human population (Ibarra and Steele-Mortimer, 2009, Moragn, et al. 2004).

In Australia, serovar *S.* Typhimurium is considered the most significant serovar causing human salmonellosis. *Salmonella enterica* is a group of over 2,500 serovars that readily contaminate the internal contents of the egg following often asymptomatic infection of the hen (Guard-Bouldin and Burh, 2006). Barrow (1989) determined that, internationally, *S*. Enteritidis and *S*. Typhimurium were the most important serovars, and Ibarra and Steele-Mortimer (2009) comments that *S.* Typhimurium is one of the most common causes of food borne gastroenteritis in humans.

Salmonella Typhimurium, along with other *Salmonella* serovars, is of significant risk to eggs due to the nature of the infection of the hen. Morgan, et al. (2004) found that many *Salmonella* serovars colonise the gastrointestinal tract which is often associated with faecal shedding. Faecal shedding is a likely source of horizontal infection of eggs by bacteria as eggs often come into prolonged contact with faeces. *Salmonella* Typhimurium was further implicated by Bauer-Garland, et al. (2006), who reported that the *S.* Typhimurium serovar appears to be more capable of developing resistance to multiple antimicrobial agents. Salmonella Typhimurium was also found to shed more into the environment than other serovars, and that this may be responsible for increased infection rates among birds.

6.1.2. Disinfection Agents used in laboratory for egg penetration experiments

In order to conduct any microbiological experiments on eggs it is necessary to disinfect the shells to prevent experimental cross-contamination. There has been significant diversity in

the disinfection agents used in research to study bacterial penetration of eggs. Several examples are listed in Table 6.1.1. Disinfection methods that involve harsh chemicals such as flaming following ethanol immersion or repeated immersion in ethanol and either mercuric chloride or hydrogen peroxide are potentially sources of cuticle or shell damage. De Reu, et al. (2008a) commented that burning ethanol off the shell was not suitable for eliminating spore forming bacteria and McLaren, et al. (2010) confirmed that the effective use of disinfectants in an on-farm environment is fundamental for the control of zoonotic infections. Understanding what effect (if any) these disinfection procedures have on the shells cuticle, may influence the choice of agent for experimental procedure and assist with interpretation of results.

Table 6.1.1. Previously used methods to disinfect eggshells.

Description of Disinfection Method.	**Reference.**
Immersion in 70% ethanol for 30 seconds	Schoeni, et al.(1995)
Immersion in 70% ethanol for an unspecified time period	Clay and Board (1991) & Dolmon and Board, (1991)
Immersion in 70% ethanol for 5 minutes and flaming over a burner	Miyamoto, et al. (1997)
Immersion in Hydrogen peroxide for 10 seconds, followed by sprinkling with 75% ethanol and burning off	De Reu, et al. (2008a)
Dipping eggs in 1.5% lugol's solution of iodine 5 times for an unspecified time period	Guard-Bouldin and Burh, (2006)
Washing in a sanitising detergent (no other information given) followed by immersion in 1% mercuric chloride for 1 minute and immersion in 70% ethanol for 2 minutes	Lifshitz, et al. (2011)

6.1.3. The Agar Egg, Penetration Experiment

The agar egg method is a technique for examining the effect of the eggshell on preventing contamination of the egg contents. The agar egg method was first used by Board and Board (1967) and has since been widely used to infect eggs through the shell and measure rates of shell penetration under various conditions (Bain, et al. 2011, Berrang, et al. 1998, Chousalkar, et al. 2010, De Reu, et al. 2006, Messens, et al. 2005_b, Schoeni, et al. 1995). The method involves removing the contents of the egg with a syringe and refilling the egg with molten agar; the egg can then be exposed to bacteria, either as a solution, spray or dry matter. In an intact egg the chemical and physical defences of the albumen would result in lower rates of infection. The benefit of this method is that it allows only the contribution of the shells physical defence to be identified.

This experiment aims to identify which eggshell features including translucency (initial, final and any change in translucency) and eggshell internal structures correlate with increased incidence of bacterial penetration by *Salmonella* Typhimurium phage type 9. In order to examine the shells of penetrated eggs, an artificially high rate of penetrated shells is required, therefore, a high bacterial load will be utilised. It also aims to examine how pre-infection storage and post-infection incubation affect shell penetration rates. Comparisons were made between samples of shell from control eggs and inoculated eggs as part of a population study. In addition, comparisons were made within individual shells which had been inoculated, between penetrated and non-penetrated adjacent areas of shell.

6.2. Experiment 4 – Method

6.2.1. Disinfection Agents and Their Effects on the Shell and Cuticle

As a preliminary measure we sought to determine what effects (if any) various disinfection agents have on the shell and cuticle coverage.

Twenty eggs, sourced from a research control flock (no treatments) at the University of New England, were randomly divided into 4 groups of 5. An eggshell sample of approximately 10 x 20 mm in size was taken from the equator of each egg.

Disinfection agents included iodine (1% w/v iodine Betadine™), hydrogen peroxide (3%w/v Faulding Remedies™) 100% ethanol and 70% ethanol. A line was drawn down the centre of the sample. One half was submerged for 60 seconds in one of the disinfection agents. Following exposure, shells were allowed to air dry before being prepared for Scanning Electron Microscopy (SEM) as described in Chapter 2 in (2.3.). Cuticle coverage was compared visually from SEM images.

6.2.2. Sample Collection and Translucency Scoring

Eggs (number= 360) were collected from a commercial layer operation in South Australia, on the day of lay or day 1 post lay. All eggs with cracked shells or obvious faecal contamination were discarded. Initially, all eggs were scored for translucency using a CE portable egg candler (Model, ID 05-2010, 3549, a 0 to 5 translucency scoring system) and weighed (Mettler Toledo PB3002-3, 2 decimal place balance). Eggs were divided into six groups, of 40, 30 experimental eggs and 10 control eggs. The remaining eggs were used as spares. The six groups were stored in

a cool room (~4°C) to prevent growth of any contaminating microorganisms until their use of experiment. Eggs were removed for experimentation after 0, 2, 4, 7, 15 and 21 days of storage for groups 1-6 respectively (Figure 6.2.1.). Eggs were again scored for translucency and weighed on the day of experimental infection.

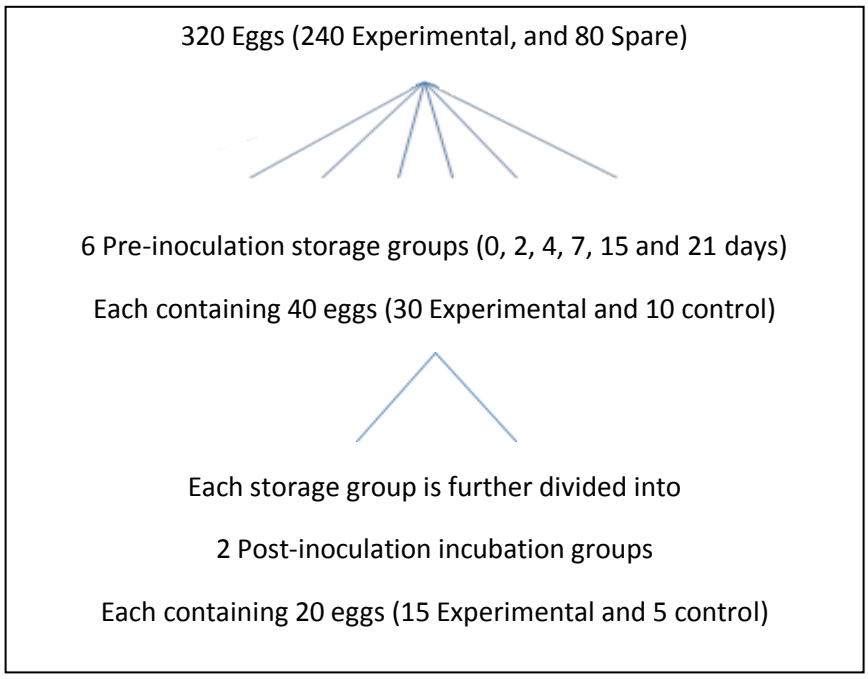


Figure 6.2.1: Experimental Design Chart.

6.2.3. Preparation of XLD Agar

XLD agar (Oxoid CMO46g X.L.D. Medium) was prepared as per product instructions and poured into disposable Petri dishes. Ampicillin was added at 10µL/L to the agar (Sigma Aldrich – A0166-59 lot. BCBK2970V).

6.2.4. Preparation of *Salmonella* for Infection Trial

Salmonella Typhimurium type 9 (MLVA type 03-24-11-11-523) was originally isolated from a commercial layer farm dust sample; this serovar was used as it is resistant to the antibiotic Ampicillin. The *S*. Typhimurium type 9 was stored at -80°C and plated onto XLD plates in order to isolate colonies. A bacterial suspension was prepared using 0.5 McFarland standards, 10 serial dilutions were plated and counted and, using these counts, a final bacterial concentration of 10^6 CFU/mL was prepared. All microbiology work was conducted under sterile conditions inside a laminar flow cupboard to prevent contamination of the samples.

6.2.5. Infection Procedure

Whole eggs were disinfected by immersion in 70% ethanol for 30 seconds and allowed to air dry. The contents of each egg were removed with a 10 mL syringe and an 18 gauge needle; during this operation every effort was made to maintain the hole as small as possible. The inside of each shell was washed with 10 mL of Phosphate Buffered Saline pH 7.0-7.4 (PBS) to remove any remaining contents without effecting pH. And the exterior of the shell was wiped with 70% ethanol. The shells were then allowed to dry fully before being filled using a 10mL syringe and 18ga needle with 30 mL to 40 mL of XLD containing ampicillin. The eggs were left for the agar to cool and solidify in a biosafety cabinet.

The bacterial solution (140 mL) was divided between two containers. Each agar filled shell was partially submerged (large end of egg) in 70 mL of bacterial solution for 90 seconds and allowed to dry in a biosafety cabinet. Eggs were further divided into two groups for incubation;

one half (15 experimental eggs + 5 control eggs) were stored for three days and the other half for six days. Both incubated at 32°C (S.E.M. (SA) Pty. Ltd.).

After incubation the shells were removed from the incubator into a biosafety cabinet. The top pole of the egg was removed by physical breaking and the agar contents were examined for bacterial contamination. *Salmonella* contamination is conspicuous with the agar changing colour from red to black, radiating out from the infection sites.

Shells were allowed to dry from the agar in the biosafety cabinets and the internal surface of the shell was lightly sprayed with 70% ethanol. The shells were then exposed to UV light overnight to ensure any remaining bacteria were killed. Following disinfection, sites where penetration was apparent (black or brown markings) were labelled with permanent marker so they could be selected later.

6.2.6. Fixed sample preparation for Scanning Electron Microscopy

Following infection and incubation, 38 egg shell samples were taken before exposure to ethanol or UV. The samples were selected from 37 penetrated shells and one non-penetrated control shell. In penetrated shells they were selected from regions where bacterial penetration was evident. The samples were less them 10 mm square and included attached and detached membrane. Following the breaking the samples were stored in a glutaraldehyde fixative solution, to ‘fix’ (preserve) any bacteria and prevent their degradation. Two methods were used to critical point dry the samples. The first 12 were dried in a Tousimis 931 Series critical point dryer (Adelaide Microscopy Unit) and the remaining samples were dried by washing in an organic

solvent. The first wash was 50% hexamethyldisilazane in ethanol, and the second was 100% hexamethyldisilazane each for 10 minutes, and then allowed to dry in air.

Shell samples were mounted onto aluminium stubs using a small amount of silver conductive paint. And gold sputter coated using a JEOL Neocoater for 5 minutes (3 min and 2 min runs) which coats the sample in a thin layer of gold to improve electron conduction in the sample. A JEOL JSM-6010LA Analytical and Scanning Electron Microscope was used to examine samples at 3,000x to 20,000x to visualise any colonising bacteria.

6.2.7. Remaining Sample Preparation for Computed Tomography

A single eggshell sample was taken from all incubated shells (including the control shells that were not inoculated) and an additional shell sample was taken from the un-infected region of the equator of infected shells. As a result, all samples were not necessarily from the midline of the shell as the sample was taken from a known infected area often around the broad pole of the eggs. In penetrated eggs with no apparent point of bacterial penetration, non-penetrated and control eggs, the samples was taken from the lower third of the egg closest to the broad pole. These samples were hand broken into approximately 10 mm by 5 mm sizes and allowed to dry in a container containing silica gel desiccant before imaging.

6.2.8. CT scanning and Processing

Each sample was scanned with a GE Phoenix V-tomex Computed Tomography (CT) scanner, using the settings described in the appendix (Chapter 9.4.)

Computed tomography image processing was conducted as described in Chapter 2 (2.1.).

6.2.9. Scanning Electron Microscopy

Scanning electron microscopy procedure was completed as described in Chapter 2 (2.3.).

6.2.10. Statistical Analysis

All treatments were compared with JMP v.11. Means, standard deviations and ANOVA tables were produced for all comparisons. Students paired t tests were used to compare direct count groups (CT) and Wilcoxon (two group comparisons)/ Kruskal-Wallis (more than two group comparisons) comparison tests were used to compare non-parametric (SEM) measures. Shell bacterial penetration rates for the pre-inoculation and incubation storage time groups were all compared using an ANOVA table and students t test.

6.3. Experiment 4 – Results.

6.3.1. Study of the Effect of Disinfection Agents on the Eggshell Cuticle.

There was no observable difference between the total cuticle coverage of eggshells treated with the different disinfection agents, 70% ethanol, 100% ethanol and hydrogen peroxide (Figures 6.3.1. 6.3.3. and 6.3.4). The iodine solution did not appear to remove any cuticle although cracks or abnormalities in the cuticle layer became more pronounced after immersion and drying (Figure 6.3.2). The iodine solution left the shell with a darker appearance due to the deposition iodine on the surface of the shell. The minor colour shade differences between images in the figures are due to brightness adjustments during image acquisition.

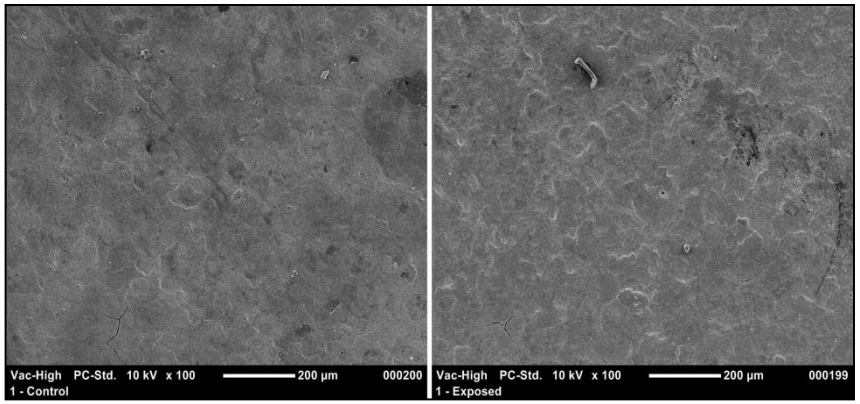


Figure 6.3.1. Examination of eggshell exterior for cuticle damage, hydrogen peroxide exposed and control from the same shell. (100x scanning electron micrograph).

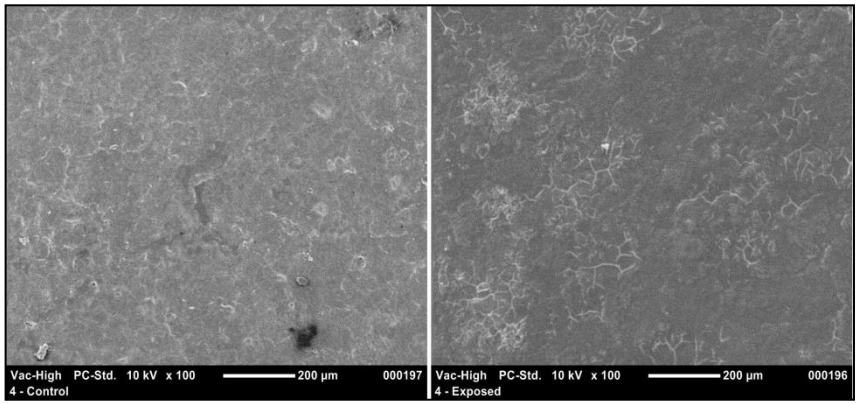


Figure 6.3.2. Examination of eggshell exterior for cuticle damage, Iodine solution exposed and control from the same shell. (100x scanning electron micrograph).

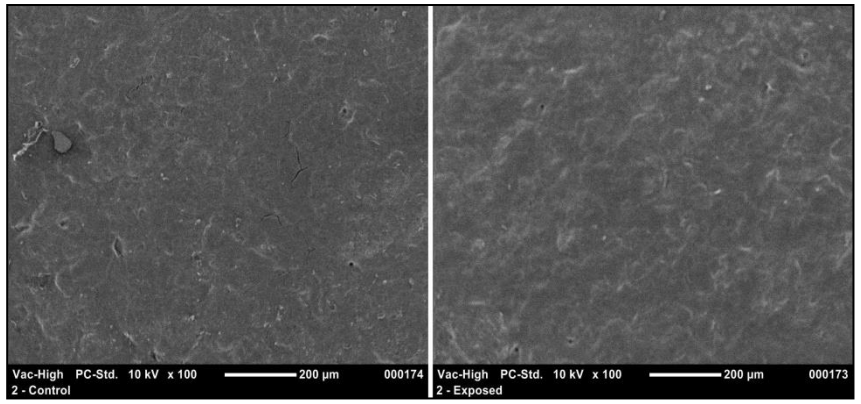


Figure 6.3.3. Examination of eggshell exterior for cuticle damage, 70% Ethanol solution exposed and control from the same shell. (100x scanning electron micrograph).

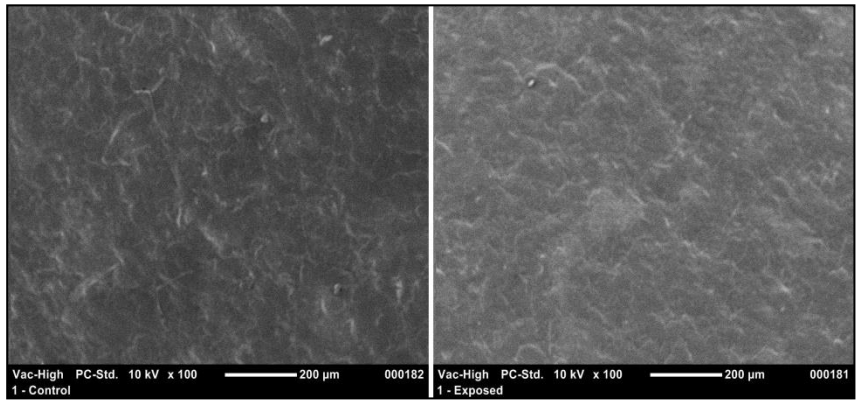


Figure 6.3.4. Examination of eggshell exterior for cuticle damage, 100% Ethanol solution exposed and control from the same shell. (100x scanning electron micrograph).

6.3.2. Observations of 'Fixed' Shell Samples.

Viewing the 'fixed' (preserved) shell samples of penetrated eggs, there were a number of rod shaped bodies that were potentially bacteria. These bacteria-like structures were found only on the inside of the inner shell membrane (Figures, 6.3.5. and 6.3.6.). No such structures were identified on the exterior of the shell, interior of the shell (under the membrane) or on the exterior side of the membrane. Bacteria-like structures were commonly found clustered in groups of 10-100. Individual bacteria may have been present, but may have been hard to identify against the fibres of the membrane.

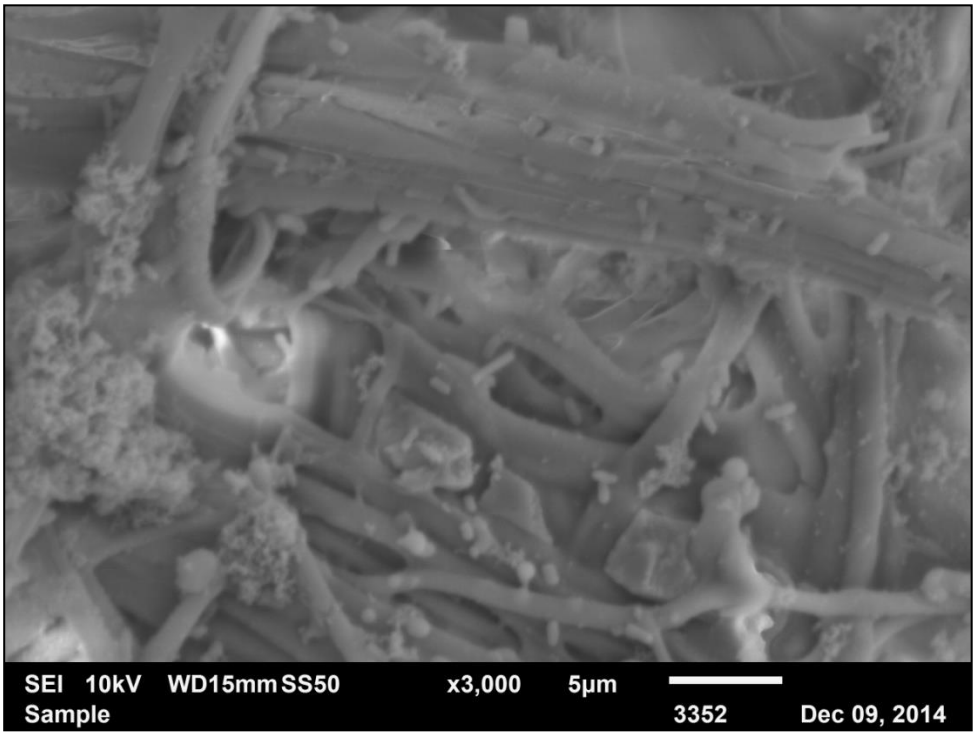


Figure 6.3.5. 3,000x Magnification scanning electron micrograph showing the interior side of the shell membrane (previously in contact with XLD agar), the small rod shaped objects are suspected *Salmonella* Typhimurium.

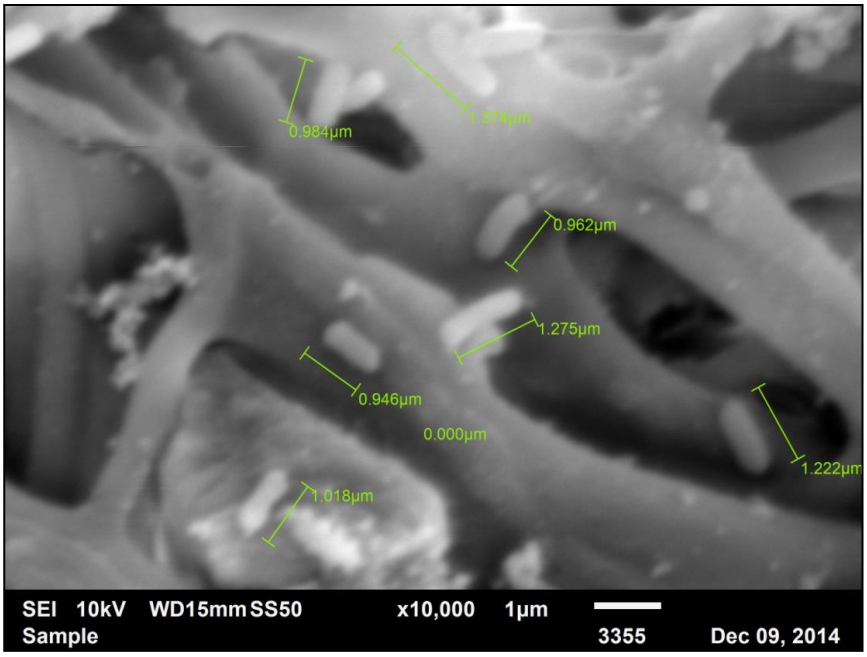


Figure 6.3.6. 10,000x Magnification scanning electron micrographs showing the interior side of the shell membranes (previously in contact with XLD agar), small cylindrical objects are suspected *Salmonella* Typhimurium bacteria. Ruler measurements have been added on all suspected bacteria.

6.3.3. Effect of Incubation and Pre-Incubation Storage times on Eggshell Penetration by *Salmonella* Typhimurium

There was considerable variation in the rate of penetration in both the storage days (pre inoculation) and incubation days (post incubation), with results ranging from 50% to 100% of shells penetrated by *Salmonella* Typhimurium (Figure 6.3.7.). Only the day 0 storage group was significantly different from the 3 days incubation (50%) and 6 days incubation groups (85.71%). Penetration rates tended to be highest for days 2-4 of pre-incubation storage of the experiment but were also high after 15 days of pre-incubation storage. However, both incubation groups for the 7 days storage were below previous results (73.33% and 70.59% respectively). Both incubation periods in the 7 day pre inoculation storage group were significantly different from adjacent storage times (4 and 15 days).

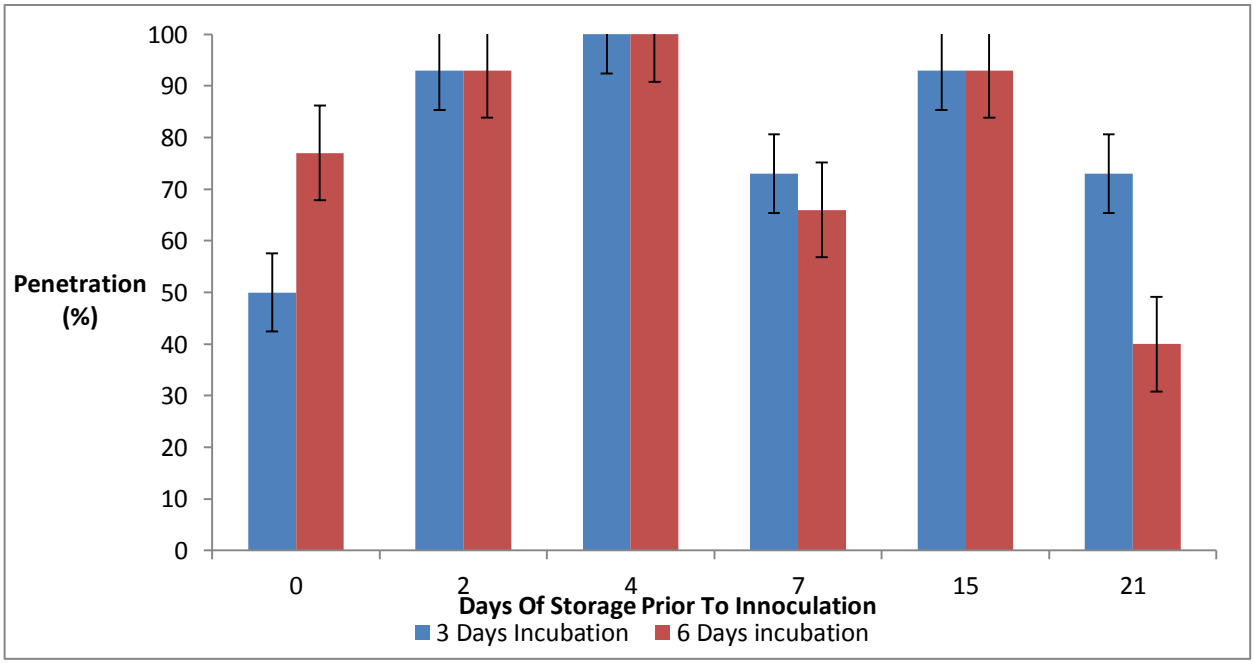


Figure 6.3.7. Penetration rates of *Salmonella* Typhimurium into egg shells with up to 21 days of pre-inoculation storage. Blue bars refer to treatment groups that were incubated 3 days following inoculation, while red bars indicate treatment groups incubated for 6 days following inoculation. Incubation was at 37°C. Error bars depict standard error (n=15).

6.3.4. Eggshell Features and Bacterial Penetration

There were no significant differences identified when the presence or absence of bacterial penetration was compared with whole egg factors (Table 6.3.1.). Penetrated eggs tended to lose more weight per day (-0.015g and -0.012g), and penetrated eggs also had a slightly higher initial and final translucency, although the change in translucency was lower the in unpenetrated eggs (0.11 and 0.21).

Table 6.3.1. Penetrated and non-penetrated eggshell groups examined by egg measures. Initial and final measurements for both translucency and egg weight refer to changes over the pre-inoculation storage time. Standard deviation in brackets.

Group	Initial Translucency (0-5)	Final Translucency (0-5)	Change in Translucency (Final – Initial)	Egg Weight Initial (g)	Egg Weight Final (g)	Egg Weight (Change / Day) (g/day)
Penetrated (n=136)	2.36 (0.69)	2.47 (0.73)	0.11 (0.45)	62.68 (2.17)	62.51 (2.18)	-0.015 (0.02)
Non-Penetrated (n=29)	2.21 (0.77)	2.41 (0.97)	0.21 (0.58)	62.71 (2.48)	62.52 (2.49)	-0.012 (0.01)
P - Value	NS	NS	NS	NS	NS	NS

There were no significant treatment effects in any of the CT measures (table 6.3.2.). There were trends towards an increasing incidence of straight pores (2.28 and 2.10 respectively) and a decreased incidence of externally branching pores (1.26 and 1.33) in shells penetrated by *Salmonella* Typhimurium. The number of internally branching pores was lowest in the non-penetrated shells. There were no significant differences between the total number of pores in each group, with the mean pores of penetrated and non-penetrated groups differing by only 0.02. CT measured shell thickness was very similar at 0.39 mm in the control and non-penetrated groups and 0.40 mm in the penetrated sample group.

Table 6.3.2. Control, penetrated and non-penetrated shell group means examined by CT measures. Standard deviation in brackets.

Group	**Mean Straight Pores**	**Mean Internal Pores**	**Mean Straight Pores**	**Mean External Pores**	**Mean Total Pores**	**CT Measured Shell Thickness (mm)**
Control (n=59)	2.06 (1.22)	0.05 (0.18)	2.06 (1.22)	1.37 (1.02)	3.51 (1.53)	0.39 (0.04)
Penetrated (n=200)	2.28 (1.53)	0.06 (0.28)	2.28 (1.53)	1.26 (1.06)	3.92 (1.69)	0.40 (0.06)
Non-Penetrated (n=30)	2.10 (1.33	0.03 (0.13)	2.10 (1.33	1.33 (1.06)	3.90 (1.57)	0.39 (0.05)
P – Value	NS	NS	NS	NS	NS	NS

Shells showing bacterial penetration had significantly increased mammillary body size from the controls. While not significant the non-penetrated group was moderate (Table 6.3.3.). The extent of mammillary layer fusion was not different among the groups but tended to be lower in the control group. The penetrated group showed significantly fewer type A mammillary bodies than the non-penetrated group (1.01 to 1.46) but neither was significantly different from the control group. There was a trend towards reduced incidence of type B mammillary bodies (0.49 and 0.75) and cubic calcite formations (0.05 and 0.13) in the penetrated shell group, although these differences were not statistically significant.

Table 6.3.3. Control, penetrated and non-penetrated shell groups examined by SEM measures. Standard deviation in brackets and different superscripts indicate significant differences.

Group	**Mammillary Body Size (-3 - 3)**	**Mammillary Body Fusion (-3 - 3)**	**Type A Mammillary Bodies (0 - 3)**	**Type B Mammillary Bodies (0 - 3)**	**Cubic Calcite Formations (0 - 3)**
Control (n=59)	-0.58^{a} (1.22)	-0.04 (1.38)	1.29^{ab} (0.99)	0.71 (1.10)	0.04 (0.21)
Penetrated (n=200)	0.01^{b} (1.23)	-0.25 (1.47)	1.01^{a} (0.89)	0.49 (0.84)	0.05 (0.26)
Non-Penetrated (n=30)	-0.29^{ab} (1.46)	-0.21 (1.28)	1.46^{b} (1.06)	0.75 (1.15)	0.13 (0.61)
P - Value	0.0218	NS	0.0393	NS	NS

6.3.5. Eggshell Characteristics and Bacterial Penetration

When comparing a penetrated region of the shell to an adjacent non-penetrated sample from the same shell, the penetrated group had significantly more straight pores (2.70 as compared with 2.04) and a significantly thinner shell (0.38 mm as compared with 0.44 mm). There was also a trend of more internally and externally branching pores in the penetrated group although these results were not statistically significant (Table 6.3.4).

Table 6.3.4. Penetrated and non-penetrated adjacent shell regions of the same egg examined by CT measures. Standard deviation in brackets and different superscripts indicate significant differences between regions.

	Average Straight Pores	**Average Internal Pores**	**Average External Pores**	**Average Total Pores**	**CT Measured Shell Thickness (mm)**
Penetrated (n=63)	2.70^{b} (1.65)	0.09 (0.42)	1.27 (0.12)	3.21 (1.48)	0.38^{b} (0.03)
Adjacent (n=62)	2.04^{a} (1.39)	0.02 (0.19)	1.10 (0.13)	3.09 (1.67)	0.44^{a} (0.06)
P - Value	0.0163	NS	NS	NS	<0.0001

The penetrated regions of the shells showed significantly more cubic calcite formations than the non-penetrated adjacent regions, with no cubic calcite formations being recorded in the adjacent samples. There were trends of lower mammillary body size and mammillary layer fusion in penetrated regions of the shell, as compared with the adjacent non-penetrated regions. The incidence of type A and type B mammillary bodies was not significantly different between the two regions of the shell (Table 6.3.5.).

Table 6.3.5. Penetrated and non-penetrated adjacent shell regions of the same egg examined by SEM measures. Standard deviation in brackets and different superscripts indicate significant differences between regions.

	Mammillary Body Size (-3 - 3)	**Mammillary Body Fusion (-3 - 3)**	**Type A Mammillary Bodies (0 - 3)**	**Type B Mammillary Bodies (0 - 3)**	**Cubic Calcite Formations (0 - 3)**
Penetrated (n=63)	-0.21 (1.36)	-0.64 (1.52)	1.15 (0.94)	0.64 (0.99)	0.12^{b} (0.42)
Adjacent (n=62)	0.04 (1.06)	-0.36 (1.32)	0.85 (0.86)	0.62 (0.86)	0.00^{a} (0.00)
P - Value	NS	NS	NS	NS	0.0358

6.4. Experiment 4 – Discussion.

Eggshell structures associated with bacterial entry have been identified; however, there were some differences between the analysis of penetrated and non-penetrated shell samples taken from the same egg and the comparison of treated and control shells from different eggs. The secondary examination of how pre and post inoculation storage times affect bacterial penetration rates was not conclusive, with no real trends observed. The 'fixing' of bacteria on the inside of the eggshell to preserve them for SEM visualization was successful, with potential bacteria identified on the inside of the shell membranes of multiple shell samples.

6.4.1. Disinfection Agents and the Eggshell Cuticle

The most commonly used disinfection agents; 70% and 100% ethanol, iodine and hydrogen peroxide did not damage the eggshell cuticle or produce any observable change to the exterior of the shell. Iodine was eliminated as an option as it left a residual film on the shell, which may have also occupied the pores and structures within the shell. Alcohol (70%) was used for experiments as it is previously used (Clay and Board, 1991, Dolmon and Board, 1991 & Schoeni, et al. 1995), readily available and is effective in killing bacteria.

6.4.2. Visual Observations of 'Fixed' Shell Samples

The visual identification of *Salmonella* Typhimurium Phage Type 9 (PT9) showed potential *Salmonella* bacteria by SEM. Identification of bacteria was difficult against the background of the shell membrane. Removal of the membrane, as was completed in other parts of this experiment, was not an option as the plasma asher would have removed the bacteria also. Several clusters of

suspected *Salmonella* bacteria were observed on the inside of the shell membrane (in contact with the albumen); however, the exact identity of these structures could not be confirmed. This is consistent with the findings of Nascimento, et al. (1992) who identified *Salmonella* Enteritidis bacteria on the inner membrane surface of infected shells. Further immunoelectron microscopy studies could be conducted in future to further confirm the location of bacteria. In other regions of the shell, identification of the bacteria was uncertain. Bacteria sized objects could be identified individually but could not be confirmed as bacteria. The lack of bacterial clusters on the other shell components tested (shell exterior, shell interior, and membrane exterior) may indicate that, after three days incubation (post inoculation), the bacteria have all proceeded though the shell. The agar egg model requires the contents of the egg to be replaced with agar, and XLD agar is a more attractive base for bacteria then the hostile environment of the egg albumen. Because of these artificial changes, our results may not accurately represent the location of bacteria in an infected 'whole egg' model.

6.4.3. Effect of Storage and Incubation Time on Eggshell Penetration by *Salmonella* Typhimurium

Our experimental aim to compare shell penetration rates over time was secondary to our aim of producing a series of penetrated and non-penetrated samples for the purpose of examining shell structures associated with increased ease of bacterial penetration. Therefore, a high overall rate of penetration was produced by selecting a high inoculation concentration of 10^6CFU/mL. Gole, et al. (2014_a; and 2014_b) used two bacterial solution concentrations of 10^3 CFU/mL and 10^5 CFU/mL in order to replicate different levels of environmental contamination

and Schoeni, et al. (1995) used 10^4 CFU/mL. Messens, et al. (2005_b) made bacterial solutions up to $5.2x10^6$ CFU/mL, which was consistent with the current experiment. Penetration rates reached 100% for 4 subgroups and were above 50% for all 6 groups and 12 incubation subgroups. Due to these high penetration rates it is hard to identify trends in the penetration rates. Days seven and 21 both showed lower penetration rates than the previous days. It is possible that this is due to small differences in the concentration of the inoculums, as calculating inoculums requires counting multiple plates of bacteria small mistakes are possible. Any future work should be completed with the same inoculum solution, reducing minor differences in bacterial load. Post inoculation incubation time was compared with three and six days being examined. Four pre-incubation storage days (0, 2, 4 and 15) showed higher infection rates after six days. The remaining two pre-incubation storage days (7 and 21) showed reduced penetration rates after six days incubation. These two groups were also the groups showing decreased overall infection, possibly indicating a problem with the inoculum concentration.

We identified that penetration was more common on the blunt pole of the shell than the midline and this is in agreement with Messens, et al. (2005_b) who found 72.9% of penetration occurred in the bottom third of the shell. Riley, et al. (2014) identified that pores are more common on the lower third of the shell than the midline or the sharp end; they also showed that the average pore size was smaller in the sharp pole. Messens, et al. (2005_b) also found that key to shell penetration was the growth of bacteria on the outside of the shell with 43% of shells with external growth showing penetration and only 3% of non-penetrated shells showing any external growth. This demonstrates two possibilities; first, that external growth has induced the internal

contamination, and secondly, that after internal penetration has occurred the external contamination dies, potentially due to the lack of nutrients on the shells exterior.

6.4.4. Bacterial Penetration and Shell Properties

The shell structures associated with bacterial penetration of egg shells have previously been examined by several researchers, using a similar 'agar egg' microbiology infection model (Bain, et al. 2011, Berrang, et al. 1998, Chousalkar, et al. 2010, De Reu, et al. 2006 and Schoeni, et al. 1995). However, none have examined the eggshell structures using the different scanning techniques over the period of time as reported in this study. Examination of pores using CT indicates that there was a trend towards higher total pore numbers in shells and regions that were penetrated. Shell pores have been discussed by a number of researchers as possible entry points for bacteria; and there are no conclusive results implicating or absolving shell pores as a causative factor.

There were no shell structures that were identified as being associated with bacterial penetration in either the penetrated or non-penetrated egg populations; likewise the comparison between penetrated and adjacent regions of the same shell. However, some generalisations can be made. There was a significantly lower incidence of type A mammillary bodies in the penetrated group at the population scale, whereas the opposite trend was found within eggs, it is not clear if this relationship is causal. Although type A mammillary bodies do not contact the shell membrane they may play a role in stabilizing the mammillary layer. This would potentially add to the volume of the palisade layer, increased volume in the mammillary layer may produce a more resilient shell. There were increased rates of cubic calcite in the

penetrated locations of the shell, with no cubic calcite being found in non-penetrated 'adjacent' locations. The presence of cubic calcite is unlikely to be directly related to shell penetration, but may be an indication of regions of the shell with localized abnormalities. Cubic calcite is rarely found in shells with normal sized, consistent mammillary bodies, and more often found in regions with small mammillary bodies and inconsistent sizing and spacing. Cubic calcite may also be related to a higher incidence of abnormalities, particularly type B mammillary bodies, although current evidence is lacking. Penetrated shells were more likely to have smaller mammillary bodies and lower mammillary fusion. These mammillary differences may result in a less 'secure' shell, due to the lower mammillary fusion. Smaller mammillary bodies result in a greater number of mammillary columns and this may reduce the number of pores. Decreased rates of mammillary fusion have also been previously implicated in shell penetration with Chousalkar, et al. (2010) commenting that earlier fusion results in a stronger more complete shell.

Comparison of the control, penetrated and non-penetrated egg shells at the population level showed no differences and only a small trend of increasing penetration with increasing total pore numbers. There was an increase in the numbers of all pore types in penetrated shells; however, only the number of straight pores was significantly higher. This comparison of the same shells also found a significantly lower shell thickness in the penetrated samples. This is relevant as the population study found only a very small non-significant difference in shell thickness (0.40, and 0.39). This indicates that there may be localized differences in shell thickness of different regions of the shell, and that thinner regions are more likely to be penetrated by bacteria.

The two studies that form this experiment, the population comparison and the same shell comparison, have found mutually exclusive results. The population comparison is there to determine which eggshells are more likely to be penetrated out of the population while the same shell comparison shows which underlying shell structures are responsible for causing shell penetration. A factor that may influence the differing results here maybe due to the shell region being examined. Riley, et al. (2014) using CT identified mammillary body density, pore frequency and average pore size as being significantly different between the poles of the shell.

We have considered *S*. Typhimurium to be the largest food safety threat to Australian eggs, however, it needs to be determined if the different serovars of *Salmonella* have different proficiencies at penetrating the eggshell. Gole, et al. (2014_b) compared a number of *Salmonella* serovars including *S.* Adelaide, *S.* Singapore, *S.* Worthington and *S.* Livingstone and found that all survived on the eggshell and penetrated the agar egg to a similar extent. Stokes, et al. (1956) showed that *S.* Oranienburg, *S.* Typhimurium, *S.* Montevideo, *S.* Pullorum and *S.* Gallinarum all showed the ability to penetrate the eggshell which is notable as *S.* Pullorum and *S.* Gallinarum are non-motile. Schoeni, et al. (1995), found that *S*. Enteritidis, *S.* Typhimurium and *S.* Heidelberg were able to penetrate the shell and Gantois, et al. (2008) examined the ability of *S.* Enteritidis (2 serovars), *S.* Typhimurium, *S.* Heidelberg, *S*. Virchow and *S.* Hadar to survive in the albumen, with *S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg being able to survive in the albumen for over 24 hours. It can be concluded from these studies that there are a wide range of *Salmonella* serotypes that can colonise eggs and, while some are of greater food safety concern than others, all must be considered as potential hazards to the egg industry.

Further work needs to be completed to examination shell thickness and penetration rates. Shell thickness should be taken at many points around the eggshell and these related to the places in infection and the structures underlying them. The determination of how the storage time of eggs affects shell penetration rates was not conclusive. This could be repeated with a single inoculum to avoid any differences in the concentration to which the eggs were exposed. It could also include a comparison of different storage conditions, including refrigerated and non-refrigerated environments.

6.4.5. Conclusion

This experiment has successfully determined which shell features are more likely to result in horizontal infection of eggshells. The presence of increased incidence of straight pores and cubic calcite formations as well, as decreased rates of type A mammillary bodies and increased mammillary body size, were all significantly associated with shell penetration by *Salmonella* Typhimurium. The individual variation in eggs has resulted in a number of insignificant trends rather than significant determinations so future work should include a greater number of samples to help reduce the chance of individual egg variation affecting results. This work has not answered all the questions asked; it has proven the effectiveness of the microbiology method and CT and SEM imaging methods and confirmed some previous work through the comparisons of disinfection agents. This experiment did not identify any relationship between shell translucency and shell penetration; however this may be due to individual egg variation. It is possible that increased shell susceptibility to bacteria and shell translucency are not related but instead, both could be independently related to malformations or disruptions of the shells

mammillary layer. There are no direct associations between high translucency eggs and increased risk of egg contamination by *Salmonella* Typhimurium. The key findings of this experiment combined with the findings of experiment three may allow for a more powerful comparison of the structures relating to both translucency and bacterial eggshell penetration.

Chapter 7. General Discussion

The aim of this project was to identify the structural abnormalities and observable features of eggshells that are associated with increased risk of bacterial penetration into the egg contents and, to a large extent, this project has successfully met its aims. As well as the preliminary work, it has determined what eggshell structures underlie the phenomenon of eggshell translucency and examined the shell structures that increase the risk of eggshell penetration by *Salmonella* Typhimurium. A major component of this study was attempting to relate the appearance of translucency to rates of bacterial penetration. While a clear relationship was not identified, some common shell structures were identified. Imaging and identification of shell features was completed on a range of equipment from simple egg candlers, to Scanning Electron Microscopes (SEM) and a micro Computed Tomography (CT) scanner.

Experiment one was conducted as a preliminary examination to find how many samples would be required to achieve a sufficient statistical power and to determine how shell structures could be observed. Due to the scanning time and cost of imaging samples with CT and SEM, minimising the number of shell samples taken while at the same time obtaining the required information was essential. The results showed that there was sufficient statistical power from the sample group.

Experiment two examined consecutive eggs laid by the same birds. The aim of this experiment was to examine the eggshell structures of eggs laid consecutively by the same hen, and to determine if successive eggs can be used as comparative samples (one as a control and a successive egg as an experimental comparison). This experiment found that there was variation

among successive eggs laid by the same hen in all measures scored. A high, detectable difference was found, and in many cases this was in excess of 50% of the scoring range. Thus we determined that, due to the variation in eggshells, a large number of shells must be examined in order to determine relationships between shell features and the underlying structures, and that consecutive eggs may be significantly different with respect to their shell structure.

Experiment three was a major component of this project; it aimed to determine which underlying shell structures are related to the appearance of eggshell translucency. Translucency was examined initially after lay and then finally after 21 days storage. Higher initial translucency had a significant correlation with lower numbers of straight pores, higher numbers of external pores, slightly greater shell thickness (0.01 mm) as well as increased incidence of a series of mammillary layer defects; mammillary layer fusion, type A bodies, type B bodies and cubic calcite formations. It remains unclear if these defects directly cause structural space in the shell for moisture to accumulate or if they act to disrupt the structures of the shell resulting in a disrupted palisade shell layer.

The effect of refrigerated and non-refrigerated storage for 21 days on the appearance of translucency was examined and it was found that translucency increases to a greater extent in eggs stored at room temperature, possibly due to increased loss of water through the shell and the effect of storage at higher temperature on albumen viscosity. Albumen viscosity and available moisture levels likely play a role in the appearance of translucency by supplying the moisture that occupies the spaces within the shell structures. This experiment also investigated

the effect of flock age on the appearance of shell abnormalities and found broad correlations between flock age and some ultrastructural features of the shell's mammillary layer.

Experiment four was a key component of this project and aimed primarily to determine which egg features and shell structures are related to increased rates of eggshell penetration by *Salmonella* Typhimurium Phage Type 9 (PT9). There were secondary objectives including identifying how pre inoculation storage and post inoculation incubation times affected shell penetration rates; these remained unclear due to the high inoculation concentration. Initially there was a small study component to determine if commonly used experimental disinfection agents have any effect on the eggshell cuticle.

There were a number of eggshell structures which were found more commonly in penetrated eggshells; shell samples were also taken from penetrated and non-penetrated adjacent parts of the same shell. These two measures produced different results indicating that shell structure may not be uniform around the pole of the shell and that penetration may be more likely in certain areas. The population comparison, which compared penetrated and non penetrated shells, identified which shells are more likely to be penetrated, and the same shell study identified where these shells are most likely to be penetrated.

Salmonella Typhimurium has been shown in the present study to penetrate the shell of intact eggs with a wide variety of shell structures, which is in agreement with the findings of Gantois, et al. (2008) and Schoeni, et al. (1995; and Stokes, et al. (1956). The penetration rates of the current study were high, however, due to the high concentration of the inoculum. The SEM examination of certain shells for the presence of bacteria, which was completed following a

'fixing' protocol, identified that bacteria were found on the inside of the shell membrane in contact with of what would have been the egg albumen. However, as the hostile environment of the albumen was replaced with a nutrient medium of XLD agar, it is not surprising that the bacteria were able to replicate in the agar once they had penetrated the shell.

Experiment four found no direct relationship between translucency, (initial, final or total change) and rates of bacterial penetration. However, there were a number of eggshell structures that were identified by both experiment three as being associated with shell translucency and experiment four as being associated with an increased incidence of bacterial penetration. As with the relationship between shell structures and translucency, it remains unclear if these structures directly increase shell penetration or act to disrupt eggshell structures, facilitating bacterial entry.

Initial translucency is of particular interest, as it is most relevant to commercial operations where eggs are usually processed on the day of lay. In CT measures, all pore types were at a higher incidence in the *Salmonella* penetrated sample group. When examining initial translucency, both internally branching and externally branching pores were found to increase; however straight pores decreased. Scanning electron microscopy measures include a small increase in mammillary body size in both higher translucency and increased rates of *Salmonella* penetration. There was a significant decrease in mammillary layer fusion in the higher translucency scores, and a corresponding minor decrease in the fusion of the penetrated group. While these simple trends have been identified, significant relationships remain unidentified.

Larger mammillary bodies and lower mammillary layer fusion were found to be associated with penetration by *Salmonella* Typhimurium and higher eggshell translucency. The increased size of mammillary bodies may act to increase the structural space between them, allowing moisture or bacteria to occupy spaces within the shell. Increased mammillary layer fusion acts to increases the volume of shell within the same shell thickness. The increased volume of the shell (a measure proportional to shell thickness) likely assists in resisting bacteria and reducing the potential space or increasing the path length for the transmission of water and/or bacteria within the shell structures.. Other SEM measures did not show any correlation between initial translucency and increased rates of shell penetration.

Experiment three showed no real trends in relating shell structures to the age of the hen, potentially due to an induced moult by a commercial operator who supplied a large proportion of our sample eggs. Messens, et al. (2005_b) found differences in the rate of penetration at different age groups. A significant difference was observed between early and late lay (45.0% and 31.6% respectively), these results run contrary to our expectation that penetration increases in older flocks due to increased shell abnormalities. Messens, et al. (2005_b), however, examined shell porosity, shell thickness and cuticle coverage found that major variations for all shell characteristics were observed irrespective of hen age.

A range of mammillary abnormalities was found at difference incidences. There was significant variation in mammillary body size and mammillary layer fusion across the four experiments. There was also considerable variation in the overall incidence of altered mammillary bodies, including types A and B and cubic formations. The underlying cause of these

malformed bodies remains unknown and future work needs to be conducted as to what causes these abnormal structures and altered pore structures.

It has been identified that translucency is a changing concept, and it has been noted that the changes are more significant immediately post lay and over the first 24-48 hours. The initial translucency measurements in both experiments three and four were taken after eggs were collected and returned to the laboratory, this resulted in different wait times for the different egg sources. These wait times (pre scoring) were limited as much as possible although variation may have effected results. The longest wait may have been in excess of 10 hours, (time of lay unknown), while the shortest wait time was potentially less than 4 hours. In any future work the time of translucency measurement should be standardised as much as possible.

Ar, et al. (1974) comment that shell thickness is a useful measure of pore length, assuming the pore runs radially through the shell. However, we found that pores regularly run at angles through the shell or have significant bends in them. Therefore, while shell thickness may be a convenient measure for pore length, it may not be accurate. However, there is no clear non-imaging replacement for shell thickness as the pore length measurement.

Due to the nature of CT scanning, to measure all samples equally a region of the shell is digitally isolated. The procedure of breaking shells by hand to produce samples results in irregularly shaped samples. If samples could be methodically broken into the same shape, this could be optimized to produce a larger region for digital isolation, and therefore maximize the region being examined. Use of a Dremel cutting tool would provide more regularly shaped structures but the cut edge of the piece of shell is more likely to be damaged.

Experiment one compared the shell structures around the midline of the shell, however, experiment four examined regions that were either penetrated by bacteria or directly adjacent to those regions; these penetrated regions were far more likely to be found towards the blunt pole of the egg. The results in experiment four's comparison of penetrated and adjacent regions found significant differences in shell structures, indicating that there is greater variation in shell structures when you move away from the midline of the shell. The variation in findings between these penetrated and adjacent regions has also shown that shell thickness plays a role in shell penetration. Although logical, this possibility has been previously discounted by Messens, et al. (2005_b) who found no relationship between penetration and shell thickness, cuticle coverage, and number of pores. Messens, et al. (2005_a), however, may have made a similar assumption as we did that the shell thickness is relatively uniform, as their results are similar to our population study which compares different shells rather than shell samples from the same shell. Any further examination of penetration where the points of penetration can be isolated should examine shell thickness further with multiple pieces from the same shell.

Experiments one and three originally included a density measurement taken by the CT software. However, this measurement failed to account for minor differences in the grayscale histogram which would have caused alternations in the final results. Although these initial attempts were not productive, density mapping of shells is a potential future research avenue if standardisations can be made.

Micro CT as conducted in this experiment is a useful tool for examining shells. However, there are some limitations, including costs, scan times and sample requirements. Producing a

high magnification, high resolution scan is a time consuming process. For experiments two and four, scans of individual pieces of shell were completed in approximately 6 minutes while, in experiments one and three, scan times were in excess of 12 minutes. Scan times can be increased to increase scan quality. The SEM component of these experiments remained constant throughout the experiments while the CT scanning component evolved throughout the project. This was because significant work had already been conducted in the SEM processing and imaging of eggshells (Board and Fuller, 1994; Burley and Vahedra, 1936; Chousalkar, et al. 2010). The use of micro CT to examine the shell was novel and required developmental work to get the right balance of increased scan clarity and reduced scan times. One factor that affects final scan resolution is the size of the individual sample, the magnification of the scan is dependent on how close the sample is to the emitter and this is limited by the size of the sample. Therefore, some minor adjustments occurred between scans. While these minor adjustments most likely had no effect on the scans, there were some adjustments to the CT scan time, and number of scans taken from around the shell. Size of shell samples was kept small to maximize the magnification and resolution.

SEM has long been used to examine the shell's mammillary layer, high resolution images are produced quickly, with far less cost relative to imaging methods such as CT. There are however, limitations on SEM imaging which are mostly related to sample preparation. Prior to imaging with SEM, samples in all experiments had the shell membrane removed by submerging in water and physical rubbing. The remaining membrane was removed by plasma etching and samples are then mounted on an aluminium stub with silver paint and gold sputter coated. These

final two steps ensure that the sample is conductive producing better imaging results. Each step in this process alters the shell, and the entire process is destructive to the shell.

The eggs used in experiments one, two and three were sourced from two commercial cage operations in Eastern Australia; cage eggs were utilised as they remain the most commonly produced and consumed egg product in Australia. The two commercial operations chosen include a very large operation and a relatively small operation, representing a range of cage operations. Commercial cage egg farming makes up the majority of table eggs laid in Australia, therefore this research has focused on eggs produced this way.

7.1. Future Avenues of Research

There are several avenues of research that could be taken to continue this work. The analysis of pore structures from CT images by visual scoring is just the beginning of the potential analysis available through the CT and associated software. Comparing minor density differences in the exterior shell structures may further identify the areas of the shell being occupied by water. Metcher, (2009) comments that datasets produced from CT image series are becoming more versatile with the growing sophistication and availability of 3D image viewing, manipulation and analysis software. Examination of the shell's mammillary layer, including mammillary body counts, size of mammillary bodies (both depth and width) as well as other physical measurements may be conducted by CT in the future. CT may also provide more detailed information about the shell's mammillary layer than SEM as it has the potential for a number of analysis tools to be automated allowing more accurate and numerous measurements.

An examination should be conducted on regions of the same shell with different levels of translucency, similar to the same shell comparison in penetrated shells from experiment four. As translucency varies between regions of the shell in both extent and type, it is worthy of investigation to examine these regions and determine if they are different from normal 'adjacent' regions.

Further examination of the shells of penetrated and non-penetrated shells could include the use of mass spectroscopy or a similar method to confirm that there are no differences in chemical composition of a penetrated shell.

7.2. Conclusion

This project has successfully identified a number of eggshell structures that are related to both eggshell translucency and increased rates of bacterial penetration by *Salmonella* Typhimurium PT9. While there were no definitive structures that are found significantly more in both high translucency and high penetration shells there was a series of shell structure abnormalities that were present in both. It is likely that both the presence of water in the shell (translucency) and the increased rates of bacterial penetration are caused by a range of different disruptions to the shell's palisade layer. These structural abnormalities are likely tied to differences in the mammillary layer and the pore structures in the palisade layer; however there may be further shell structures that are yet to be identified. One of the original propositions of this project was the possibility of identifying egg susceptibility to horizontal bacterial infection by examining egg translucency on farm which would have allowed these high susceptibility eggshells

to be removed from the supply chain. The relationship between infection rates and translucency is not clear cut and there is still no non-destructive method to determine bacterial susceptibility.

The key to ensuring egg safety is to prevent the growth of any contaminating bacteria during all points in the supply chain and in personal households (Schoeni, et al. 1995). Refrigeration at all points in the supply chain combined with proper cooking practices will ensure the food safety of the table egg.

Chapter 8. References

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Appendices

Appendix A - Experiment One

CT Settings

Phoenix GE V|tome|x Nano tube 180 kV

X-ray source energy: 80 kV

X-ray source current: 180 mA

Focal spot size 4um

Power: 14.4V; 0.86 watts

Projections per Scan: 800

Exposure interval per projection: 800ms

Voxel size (resolution): 5.64 microns isotropic voxel side length (Some minor variation occurred due to sample size)

Magnification: 70.974 (Some minor variation occurred due to sample size)

Appendix B - Experiment Two

CT Settings

Phoenix GE V|tome|x Nano tube 180 kV

X-ray source energy: 80 kV

X-ray source current: 180 mA

Focal spot size 4um

Power: 14.4V; 0.86 watts

Images per scan: 400

Timing: 400ms

Voxel size (resolution): 4.95 microns.

Magnification: 40.40 - 40.50 (Minor variation occurred due to sample size)

Table B1. Six hens examined for egg weight over 15 days (g).

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	61.004	59.914	60.307	59.351	61.291	64.938	60.913	60.298	60.015	61.877	59.158	61.138	58.100	57.969	61.123
Hen 2	59.327	57.465	56.170	57.366	59.855	58.000	55.377	57.253	57.695	57.852	57.661	53.939	55.971	57.200	56.233
Hen 3	65.015	63.765	64.014	61.841	62.360	63.360	62.645	64.897	61.267	63.682	60.835	60.458	62.457	59.754	63.568
Hen 4	52.387	51.887	53.733	51.29	52.226		51.948	60.343	55.973	57.900	55.440	51.398	51.369	52.485	52.401
Hen 5	70.774	67.788	69.706	70.347	64.864	64.864	64.891	66.545	67.745	67.803	65.259	64.283	64.436	63.244	
Hen 6	59.917	61.523	55.138	62.561	55.757	55.757	56.142	55.49	50.023	65.285	55.468	58.271	58.768	58.304	56.477

Table B2. Six hens examined for shell weight over 15 days (g).

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	5.640	5.282	5.870	5.756	5.915	6.022	5.606	5.789	5.779	5.733	5.895	6.381	5.331	5.776	5.945
Hen 2	6.016	5.301	5.921	5.400	5.955	5.762	5.302	5.862	5.715	6.175	6.412	4.938	6.067	6.237	6.058
Hen 3	6.182	5.670	6.103	5.833	5.976	5.963	5.635	5.819	5.955	5.733	5.713	5.631	6.022	5.714	6.066
Hen 4	5.366	5.262	5.573	5.293	5.743		5.815	5.789	5.550	5.929	6.046	5.099	5.384	5.455	5.651
Hen 5	6.983	5.667	6.739	6.372	6.364	5.799	5.419	6.150	6.109	6.484	6.398	6.473	6.030	4.821	
Hen 6	4.725	4.945	4.583	5.137	4.263	4.674	4.042	4.834	4.211	4.809	4.829	4.895	4.774	4.876	4.809

Table B3. Six hens examined for translucency score over 15 days (0-5).

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hen 2	3	3	3	2	2	3	3	3	3	2	2	1	2	3	3
Hen 3	1	2	2	1	2	1	2	2	2	1	1	1	2	1	2
Hen 4	2	2	2	1	2		3	3	2	2	1	1	1	1	1
Hen 5	1	2	2	1	1	1	1	2	1	1	1	1	1	1	
Hen 6	1	2	2	2	2	2	3	2	3	3	2	1	1	3	1

Table B4. Six hens examined for straight pores over 15 days.

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	3.5	3	3	3.5	1	4.5	3	1	3	4.5	6	4	0	0	3
Hen 2	4	4	1	2.5	1	1.5	1	3	2.5	1.5	4.5	2	0.5	2	0
Hen 3	2	2.5	1.5	1.5	1.5	1	2	0.5	2	2.5	1	0.5	3.5	3	4.5
Hen 4	6	2.5	6	4	3.5		4	2	2.5	0	3.5	0	2	4	1
Hen 5	1	2	4	1.5	1	1	4	1.5	2.5	1	3	0	6	1.5	
Hen 6	2.5	1.5	2	1.5	2.5	3.5	1.5	3.5	2.5	1	2	1.5	4.5	2.5	5

Table B5. Six hens examined for internally branching pores over 15 days.

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5
Hen 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hen 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hen 4	0.5	1	0	0	0		0	0	0	0	0	1	0	0	0
Hen 5	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0	
Hen 6	0	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0

Table B6. Six hens examined for externally branching pores over 15 days.

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	1.5	2.5	1	0.5	0	1.5	1	0	0	2.5	1	0.5	0	2	1
Hen 2	3.5	3	4	5	2	2.5	5	4.5	4.5	1.5	3.5	3	6	3	0
Hen 3	1	2.5	2.5	1.5	1.5	1	3	3.5	2.5	2.5	0	2	2.5	1	0
Hen 4	1.5	3	0	1	1.5		0	3	0.5	2	0.5	0	5	2	4
Hen 5	1	1	0.5	1.5	3	3.5	2	3.5	4.5	4	3	0	1	2	
Hen 6	2.5	2.5	1	2	1.5	1.5	3	3	1.5	5	1.5	1.5	1.5	2.5	0

Table B7. Six hens examined for CT measured shell thickness over 15 days (mm).

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	0.34	0.32	0.37	0.37	0.39	0.36	0.34	0.37	0.37	0.4	0.4	0.4		0.39	0.37
Hen 2	0.38	0.36	0.41	0.36	0.38	0.38	0.36	0.37	0.4	0.39	0.37	0.34	0.42	0.4	
Hen 3	0.39	0.35	0.36	0.38	0.39	0.39	0.36	0.38	0.37	0.38	0.36	0.35	0.38	0.36	0.35
Hen 4	0.39	0.38	0.4	0.37	0.42	.	0.41	0.38	0.37	0.39	0.38	0.35	0.43	0.33	0.33
Hen 5	0.41	0.37	0.42	0.4	0.4	0.39	0.35	0.36	0.29	0.3	0.35		0.32	0.34	
Hen 6	0.32	0.32	0.33	0.34	0.29	0.33	0.3	0.34	0.35	0.29	0.33	0.41	0.38	0.39	0.38

Table B8. Six hens examined for mammillary body size over 15 days (-3 to 3).

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	-1	-1	-3	-1	0	-1	-2	-3	-1	-2	-1	2		-2	-1
Hen 2	-1	-1	-1	-2	-1	-2	0	-1	0	-1	1	0	0	1	
Hen 3	-2	-1	-2	-2	-2		-1	-1	-1	-1	-2	-1	0	-1	-1
Hen 4	0	-1	-1	-2	-2		2	0	0		2	-1	1	0	2
Hen 5	-2	-3	-2	-3	-3	-1	1	-1	0	0	-1		-2	-2	
Hen 6	-1	-1	0	-2	-1	0	-2	0	-1	0	1	0	-1	-1	1

Table B9. Six hens examined for mammillary layer fusion over 15 days (-3 to 3).

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	-1	-2	-3	-2	1	2	-1	-2	-1	-3	-1	1		1	2
Hen 2	-1	0	-2	-2	1	-3	-3	1	1	-1	1	-2	0	1	
Hen 3	-2	-1	-2	-14	-2		0	0	-2	-1	-1	0	-1	0	-1
Hen 4	-2	1	2	-2	-2		2	2	-1		1	-1	-1	1	-2
Hen 5	-1	-2	-3	-3	-2	-2	-2	-2	-2	1	-2		-3	-3	
Hen 6	0	-1	0	-3	2	0	-2	0	-2	2	1	1	-2	-1	-1

Table B10. Six hens examined for rate of type A mammillary bodies over 15 days (0-3).

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	0	0	2	2	0	0	1	1	1	1	1	2		1	0
Hen 2	1	0	1	0	0	0	0	0	1	2	0	1	2	0	
Hen 3	1	1	0	1	2		0	2	2	1	2	2	1	2	1
Hen 4	1	1	0	2	0		0	0	0		2	1	1	2	3
Hen 5	1	0	0	1	2	1	1	0	3	0	2	.	3	3	
Hen 6	2	1	2	2	3	2	3	1	1	2	2	0	2	2	1

Table B11. Six hens examined for rate of type B mammillary bodies over 15 days (0-3).

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	1	0	2	0	0	0	1	0	0	2	1	0		1	0
Hen 2	0	0	0	0	0	2	0	0	1	0	1	0	1	0	
Hen 3	1	0	0	0	0		0	1	0	2	1	0	0	0	0
Hen 4	0	0	0	1	0		0	0	2		0	0	0	3	3
Hen 5	1	0	0	0	2	0	1	1	3	0	0		3	3	
Hen 6	0	0	2	1	3	1	2	1	0	0	0	0	1	1	0

Table B12. Six hens examined for rate of cubic calcite formations over 15 days (0-3).

Hen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Hen 1	0	0	0	0	0	0	0	0	0	0	0	0		0	0
Hen 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Hen 3	0	0	0	0	0		0	0	0	0	0	0	0	0	0
Hen 4	0	0	0	0	0		0	0	0		0	0	0	0	0
Hen 5	0	0	0	0	0	0	0	0	0	0	0		2	0	
Hen 6	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0

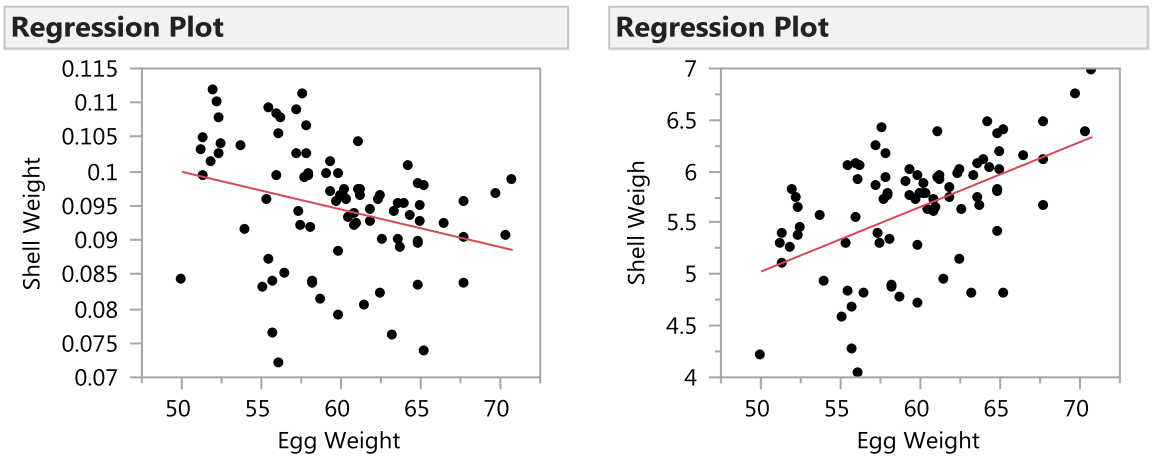


Figure. B13 Regression plot, egg weight compared to shell weight as a percentage of egg weight.
P Value = 0.0051
R^2 = 0.09
Figure. B14 Regression plot, egg weight compared to shell weight (g).
P Value = <0.0001
R^2 = 0.27

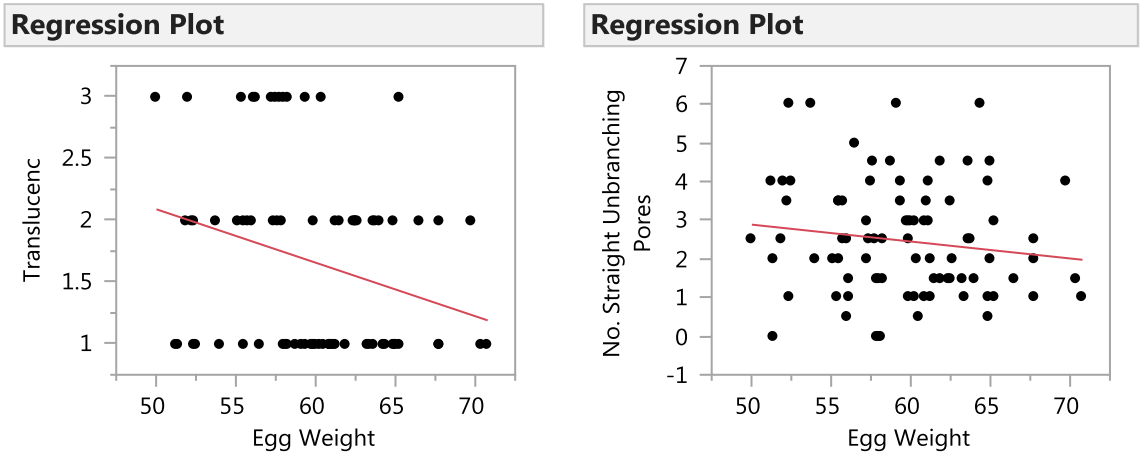
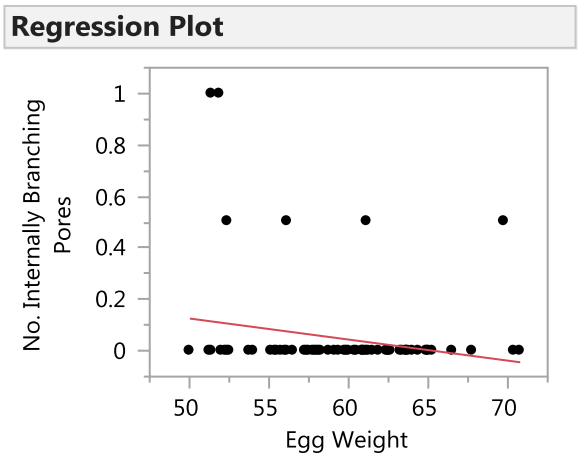


Figure. B15. Regression plot, egg weight compared to shell translucency.
P Value = 0.0102
R^2 = 0.07
Figure. B16. Regression plot, egg weight compared to the number of straight unbranching pores.
P Value = 0.1913
R^2 = 0.02



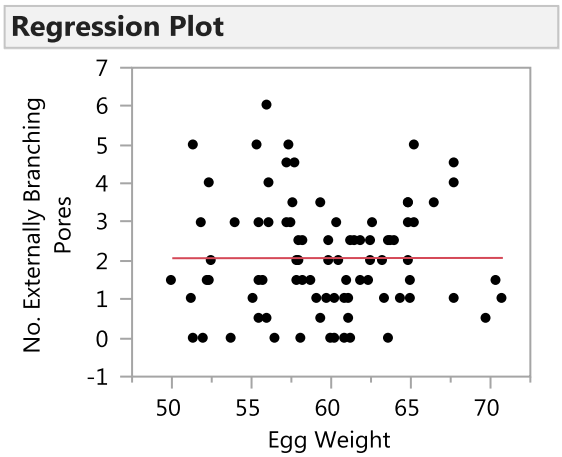
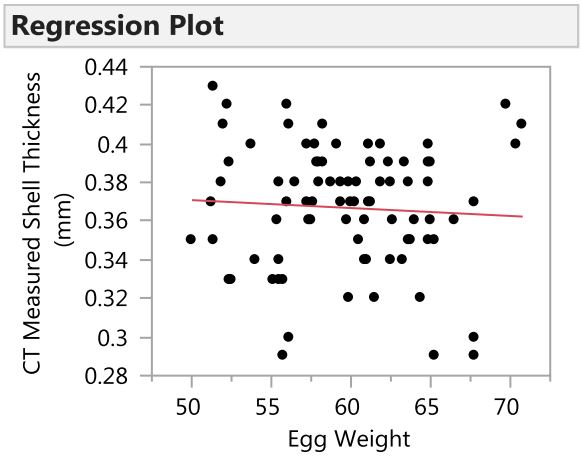


Figure. B17 Regression plot, egg weight compared to the number of internally branching pores.
P Value = 0.0478
R^2 = 0.05
Figure. B18. Regression plot, egg weight compared to the number of externally branching pores.
P Value = 0.9908
R^2 = 0.00



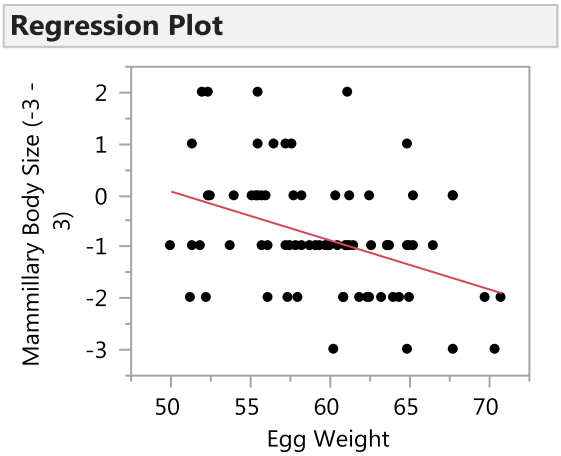
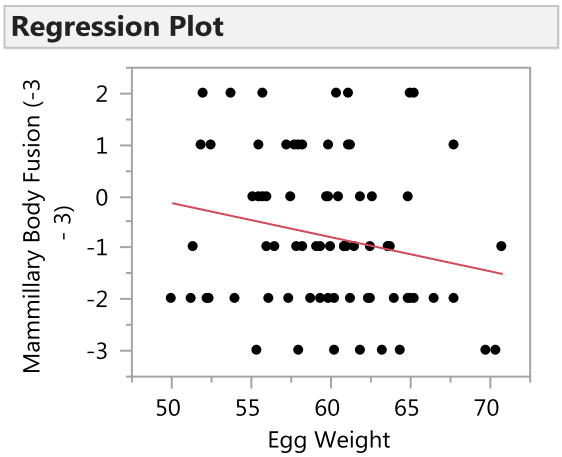


Figure. B19. Regression plot, egg weight compared to the CT measured shell thickness (mm).
P Value = 0.5753
R^2 = 0.00
Figure. B20. Regression plot, egg weight compared to mammillary body size.
P Value = 0.0003
R^2 = 0.15



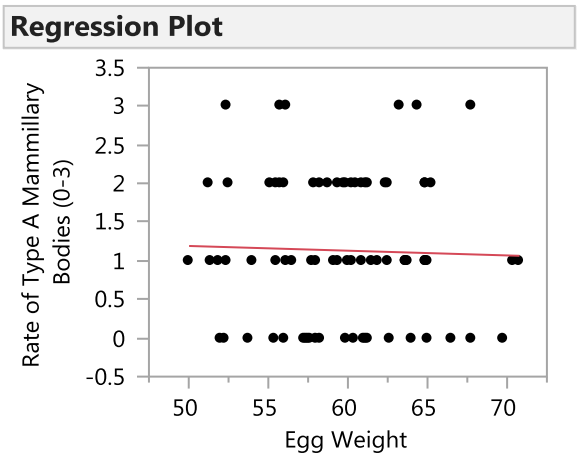
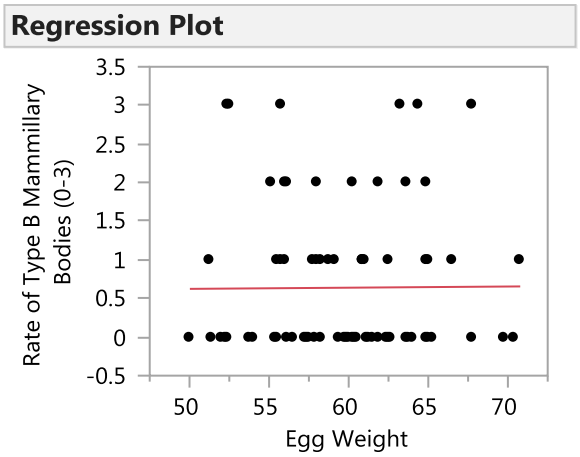


Figure. B21. Regression plot, egg weight compared to the extent of mammillary layer fusion.
P Value = 0.0519
R^2 = 0.05
Figure. B22. Regression plot, egg weight compared to the rate of type A mammillary bodies.
P Value = 0.7763
R^2 = 0.00



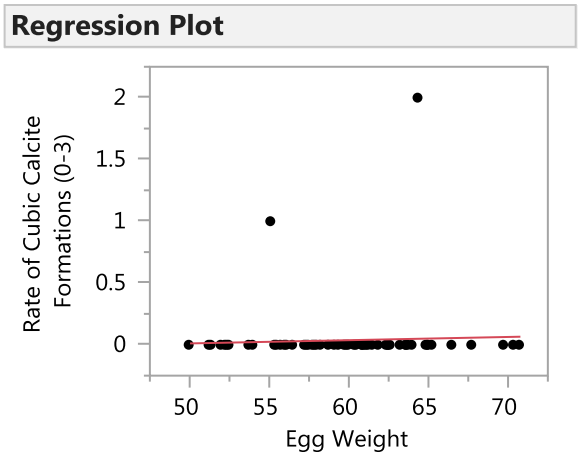


Figure. B23. Regression plot, egg weight compared to the rate of type B mammillary bodies.
P Value = 0.9436
R^2 = 0.00
Figure. B24. Regression plot, egg weight compared to the rate of cubic calcite formations.
P Value = 0.6429
R^2 = 0.00

Appendix C - Experiment Three

CT Settings

Phoenix GE V|tome|x Nano tube 180 kV

X-ray source energy: 80 kV

X-ray source current: 180 mA

Focal spot size 4um

Power: 14.4V; 0.86 watts

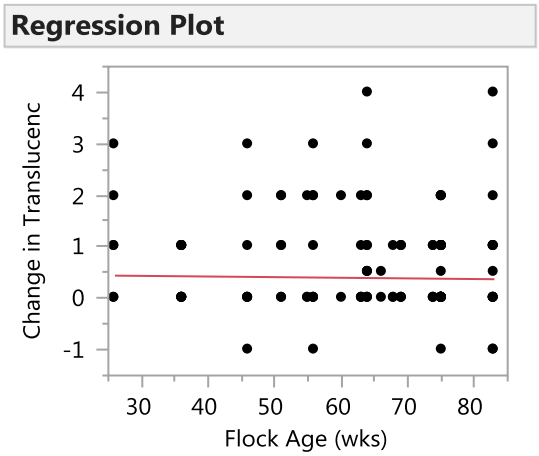
Images per scan: 400

Timing: 400ms

Voxel size (resolution): 5.64 microns (Minor variation occurred due to sample size)

Magnification: 70.974 (Minor variation occurred due to sample size)

Flock age Regression Plots.



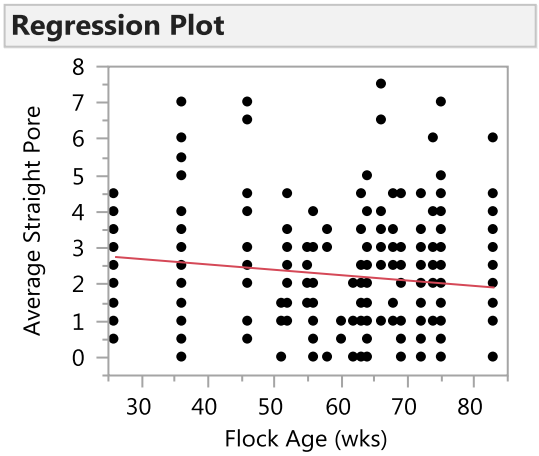


Figure. C1. Regression plot, flock age compared to change in translucency.
P Value = 0.6302
R^2 = 0.00
Figure. C2. Regression plot, flock age compared to average straight pores.
P Value = 0.0012
R^2 = 0.03

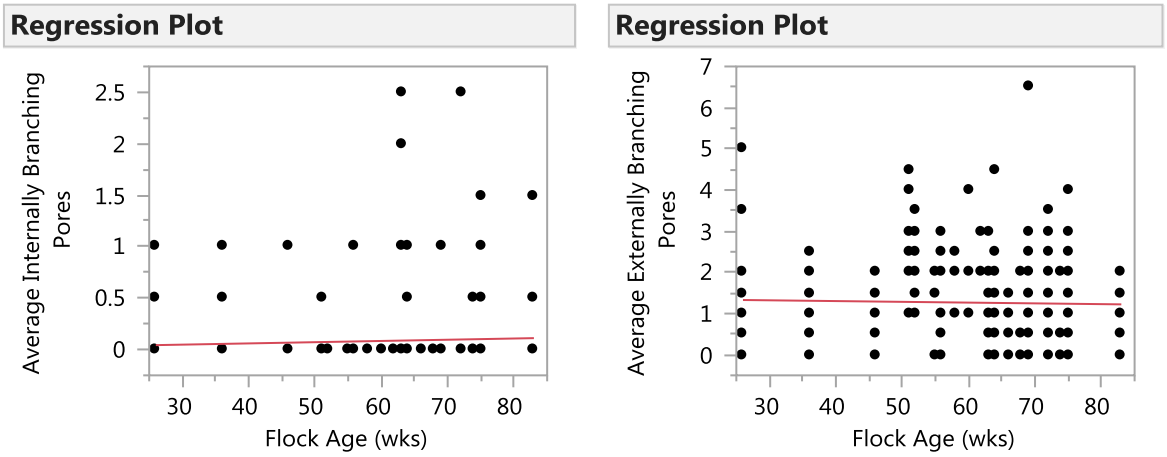


Figure. C3. Regression plot, flock age compared to average internally branching pores.
P Value = 0.215
R^2 = 0.00
Figure. C4. Regression plot, flock age compared to average externally branching pores.
P Value = 0.5682
R^2 = 0.00

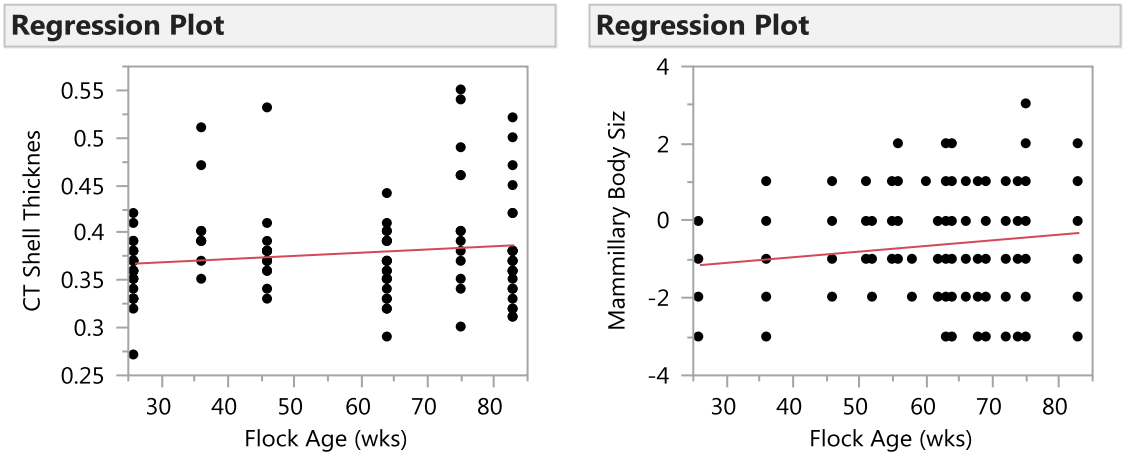


Figure. C5. Regression plot, flock age compared to CT measured shell thickness.
P Value = 0.1109
R^2 = 0.02
Figure. C6. Regression plot, flock age compared to mammillary body size.
P Value = 0.0011
R^2 = 0.03

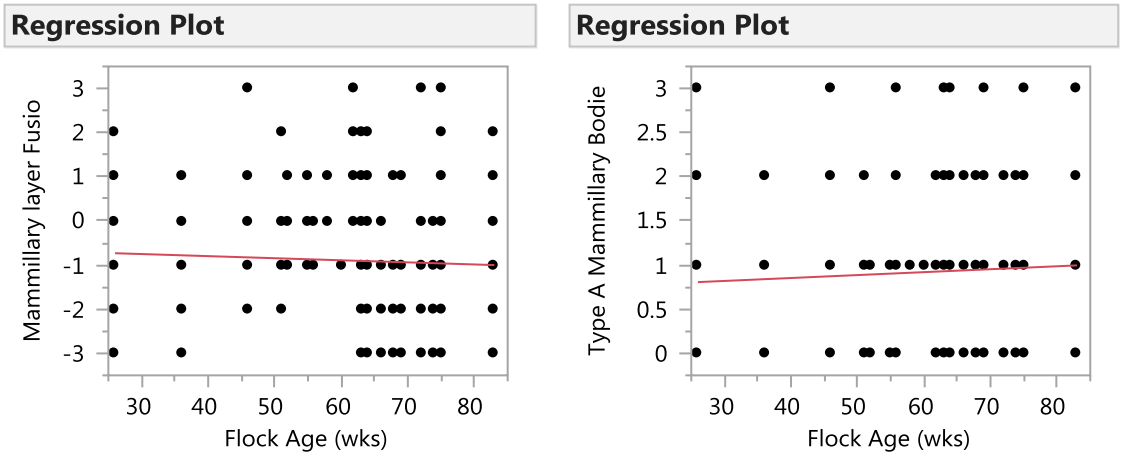


Figure. C7. Regression plot, flock age compared to mammillary layer fusion.
P Value = 0.2797
R^2 = 0.00
Figure. C8. Regression plot, flock age compared to the rate of type A mammillary bodies.
P Value = 0.2373
R^2 = 0.00

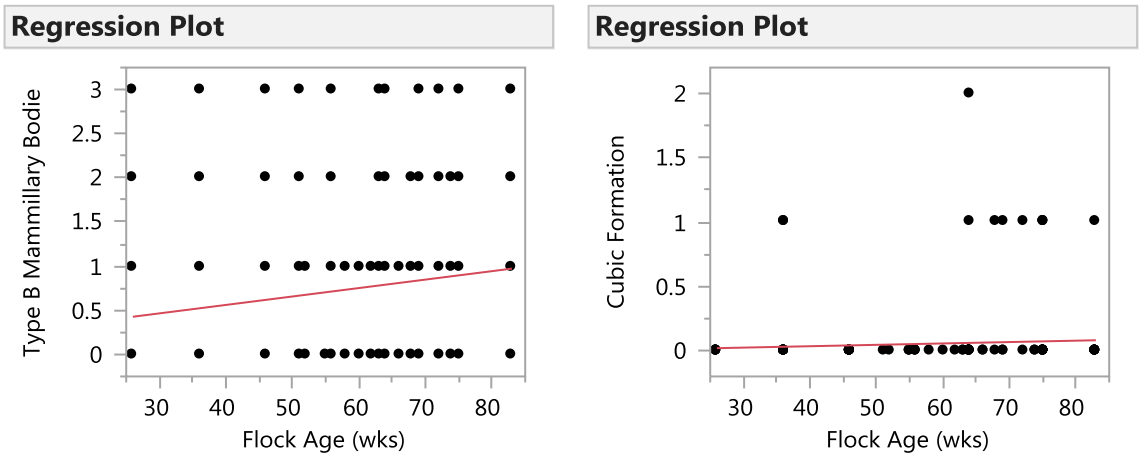


Figure. C9. Regression plot, flock age compared to the rate of type B mammillary bodies.
P Value = 0.0015
R^2 = 0.03
Figure. C10. Regression plot, flock age compared to the rate of cubic calcite formations.
P Value = 0.1597
R^2 = 0.01

Change in Translucency Regression Plots.

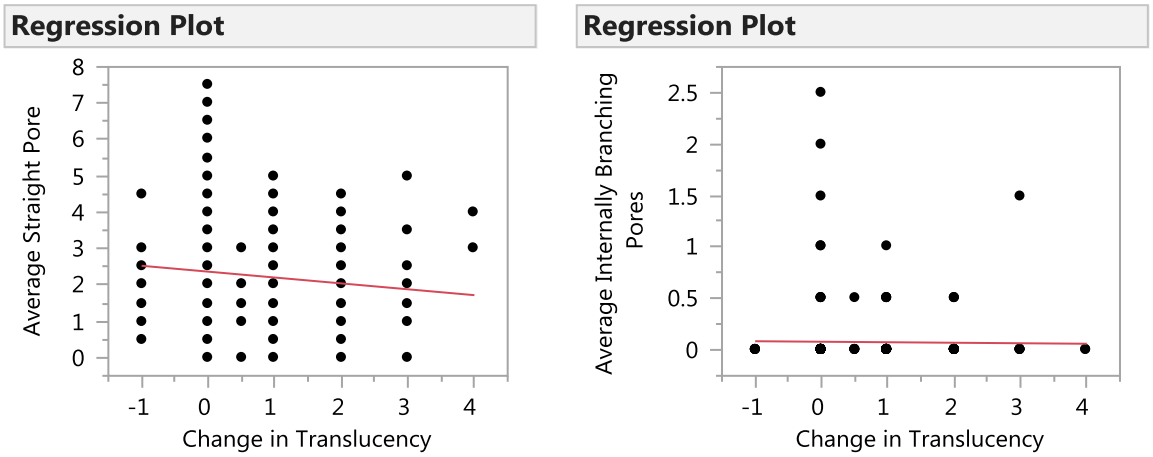


Figure. C11. Regression plot, change in translucency compared to the average straight pores.
P Value = 0.0855
R^2 = 0.01
Figure. C12. Regression plot, change in translucency compared to average internally branching pores.
P Value = 0.7834
R^2 = 0.00

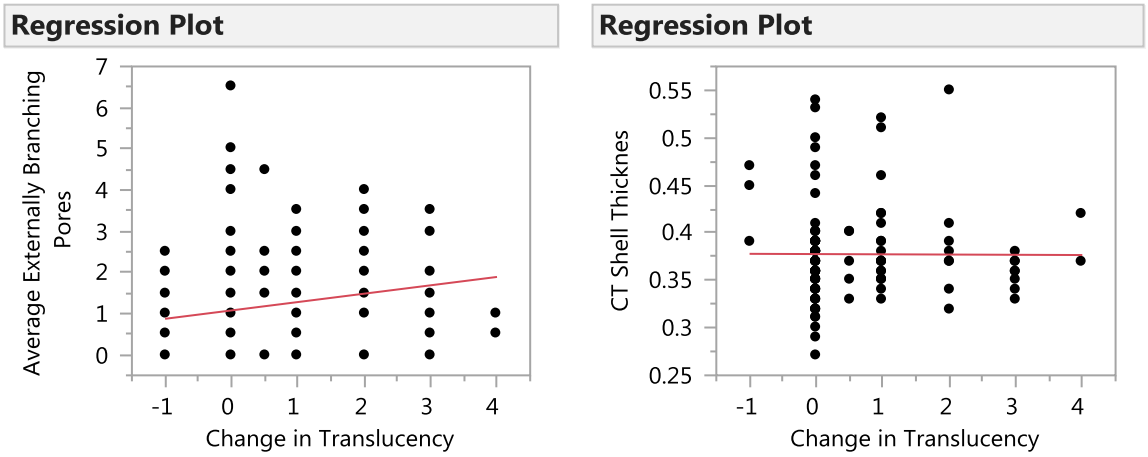


Figure. C13. Regression plot, change in translucency compared to average externally branching pores.
P Value = 0.0017
R^2 = 0.03
Figure. C14. Regression plot, change in translucency compared to CT measured shell thickness.
P Value = 0.9558
R^2 = 0.00

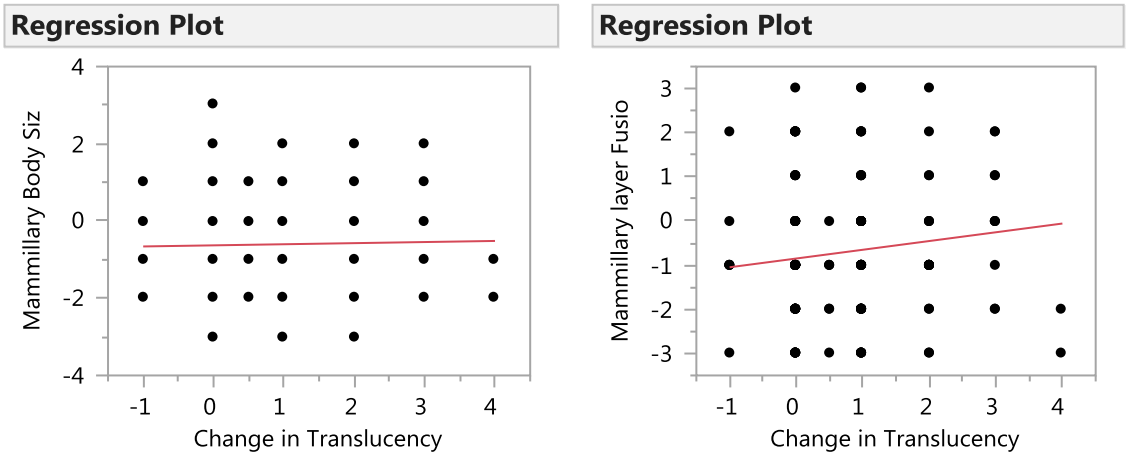


Figure. C15. Regression plot, change in translucency compared to mammillary body size.
P Value = 0.7579
R^2 = 0.00
Figure. C16. Regression plot, change in translucency compared to the extent of mammillary layer fusion.
P Value = 0.0325
R^2 = 0.01

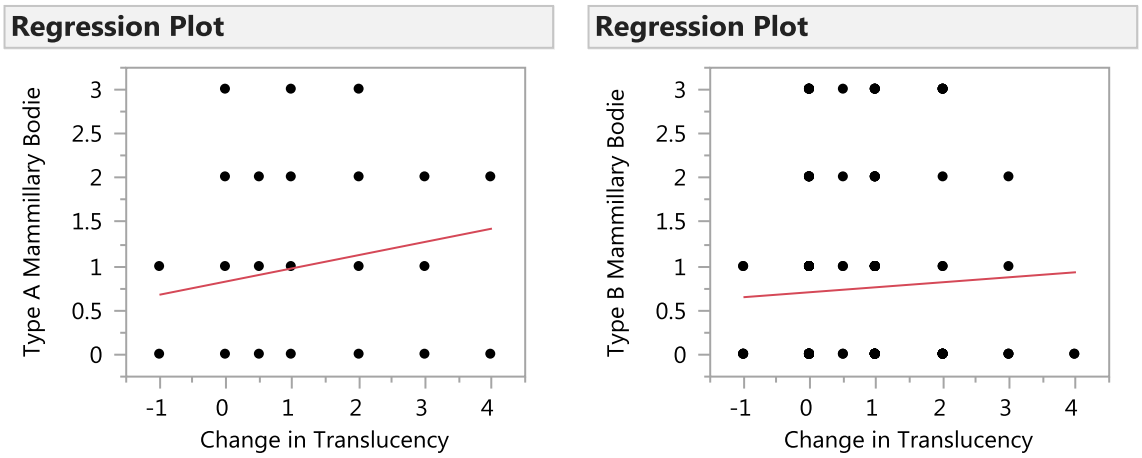


Figure. C17. Regression plot, change in translucency compared to the rate of type A mammillary bodies.
P Value = 0.0092
R^2 = 0.02
Figure. C18. Regression plot, change in translucency compared to the rate of type B mammillary bodies.
P Value = 0.3581
R^2 = 0.00

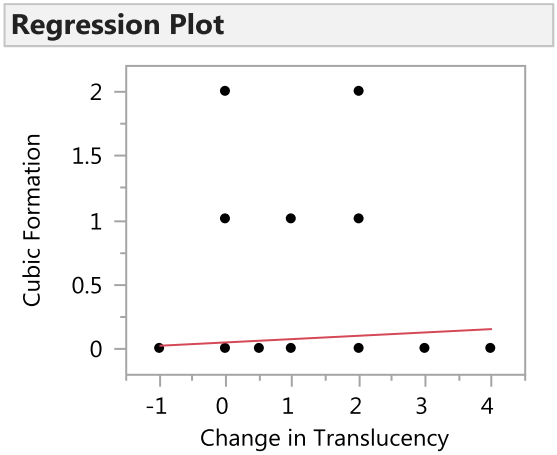


Figure. C19. Regression plot, change in translucency compared to the rate of cubic calcite formations.
P Value = 0.1311
R^2 = 0.01

Appendix D - Experiment Four

CT Settings

Phoenix GE V|tome|x Nano tube 180 kV

X-ray source energy: 80 kV

X-ray source current: 180 mA

Focal spot size 4um

Power: 14.4V; 0.86 watts

Images per scan: 400

Timing: 1000ms

Voxel size (resolution): 5.69 microns (Minor variation occurred due to sample size)

Magnification: 70.974 (Minor variation occurred due to sample size)

Table D1. Penetration rates of eggs by *Salmonella* Typhimurium phage type 9, over 21 days storage and 3 and 6 days incubation, superscripts indicate significant differences.

Storage Time Pre-Inoculation (Days)	**0**		**2**		**4**		**7**		**14**		**21**	
Incubation Time Post-Inoculation (Days)	**3** (n=10)	**6** (n=21)	**3** (n=28)	**6** (n=25)	**3** (n=16)	**6** (n=18)	**3** (n=15)	**6** (n=17)	**3** (n=22)	**6** (n=22)	**3** (n=18)	**6** (n=18)
Penetration Rate (%)	50^{e}	85.71^{abcd}	92.86^{abc}	100^{a}	100^{a}	100^{a}	73.33^{cde}	70.59^{de}	95.45^{ab}	100^{a}	77.78^{bcd}	66.67^{de}