Mechanical Properties of Avian Eggshells

Leah McManus, BSc.

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School of Mechanical and Manufacturing Engineering

Dublin City University

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Under the supervision of

Dr Owen Clarkin

&

Dr Harry Esmonde

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Engineering is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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List of Abbreviations

AFM - Atomic force microscopy

DNA - Deoxyribonucleic acid

EBSD - Electron backscatter diffraction

EOH - Eggs from older hens

EYH - Eggs from young hens

FEA - Finite element analysis

FWHM - Full width half maximum

GAGs - Glycosaminoglycans

Hmax - Maximum depth

HPLC - High performance liquid chromatography

LEFM - Linear elastic fracture mechanics

MS - Mass spectrometry

OC-17 - Ovocleidin 17

OTM - Organic trace mineral

Pmax - Maximum force

RA - Roughness average

SEM - Scanning electron microscopy

SEVNB - Single Edge V notch beam

TCD - Theory of critical distances

TEM - Transmission electron microscopy

VCL - Vertical crystal layer

XPS - X-ray photoelectron spectroscopy

XRD - X-ray diffraction

μCT - X-ray micro computed tomography

2D XRD - 2 dimensional X-ray diffraction

Abstract

Mechanical Properties of Avian Eggshells – Leah McManus

The following document is an in depth analysis of avian eggshell. Specifically, the assessment of mechanical properties of avian eggshell. Eggshell is a common food staple around the world and the quality of the shell is a major influence to consumers. Eggshell defects and flaws are an immense burden on farmers and suppliers with a large number of eggs discarded before reaching store shelves. The poultry industry is constantly assessing ways of decreasing flaws and fracture in eggshell in order to reduce waste and financial loss. The following documents discusses and analyses the mechanical impact which dietary bird organic trace mineral supplement could have on the eggshell. This analysis was conducted, mainly on hen eggshell, and provides a thorough analysis of dietary changes can make to the shell. In addition, this was assessing birds over time which allowed for an assessment of bird age on the quality of eggshell.

Additional validation studies and size analysis studies were completed to optimise methodology parameters and attempted to identify mechanical differences related to consumer eggs.

The project presented the findings that there was no significant change in mechanical properties of eggshell with varying supplement in the diet. In addition, the project presented evidence which indicated that fracture toughness as a material property, increased with bird age. Porosity was also seen to increase with the ageing birds. Thickness of eggshell remained relatively unchanging throughout the project which indicated that perhaps thickness was not directly associated with changing quality over time. Nanoindentation studies provided proof that along the cross section of the shell, mechanical properties were significantly different.

The practical results were also used in line with probability methods to identify if probability approaches could be used in eggshell quality assessment.

Chapter 1. Introduction

Avian eggs are a common staple in households around the world. The eggshell has evolved over millions of years to providing perfect conditions for chick development; it supplies the developing embryo with calcium and facilitates gas exchange, whilst also protecting the contents (Tullet, 1978). Eggshell is a perfect functional biomaterial, being strong enough to protect the contents from external forces yet brittle enough that the hatchling can fracture the shell. The latter part of this functionality has led to issues in the commercial egg industry. The delicate nature of the eggs resulting in significant economic losses for the poultry industry. Annually, 8-10% of eggs produced are discarded due to shell defects (Macleod, Bain and Hancock, 2006). Bird age is thought to impact the properties of the eggshell. Older birds produce larger eggs, which have greater commercial interest. However, what is not clear is if the change in characteristics over time is as a result of thickness, rather than a material property.

There has been a focus on researching avian eggshell in the hope that understanding the shell can lead to improvements in quality. Greater insight may influence developments in eggshell quality, with the objectives of reducing waste and financial loss. Research into improving egg quality has been ongoing for decades. Biological ceramics are of interest as they are formed at low temperatures, unlike their manufactured counterparts. Bioceramic materials such as nacre and dental enamel have piqued the interest of multiple research teams due to their structure and properties. Alternative methods of creating a similar product are of interest to commercial industries as they may be provided with cheaper and more sustainable options when looking at how natural ceramic materials are made. Essentially, the more information gathered relating to these biomimetic materials, the greater possibility of commercial benefit.

The research on eggshell has proven beneficial, yet is not fully conclusive in addressing improvements in the quality of the eggshell itself. Many of the research overlaps but does not offer a complete comprehensive understanding of the eggshell. Due to the nature of shell being a biological product, variation is inevitable and differences amongst research groups is evident and will be discussed. Nevertheless, for such a ubiquitous product, definitive knowledge should be sought. The overall perception of the structural attributes appears to be somewhat understood. However, research on factors which could well influence the mechanical properties of the shell, such as bird diet, bird age, shell porosity and shell crystallinity are not so definitive. Therefore, it is proposed that further investigation into these potential influences be analysed with the intention of attempting to answer some of the unknown questions about avian eggshell.

1.1 Project Aims

The objective of this project is to perform an in-depth analysis of the structural and mechanical properties of avian eggshell, with a focus primarily on domestic hens (*Gallus domesticus*). Samples of breeder duck eggs (*Anas domesticus*) are also briefly examined. By reviewing the research literature on eggshells in detail, a greater understanding of the factors which influence eggshell mechanical properties was obtained. This provides a foundation for assessing what can be controlled to understand eggshell properties.

Diet and age were the primary factors which were investigated in this project. The specific dietary analysis was observing any influence supplement trace minerals, which were bound to an organic peptide or 'organic trace minerals' (OTM), have on eggshell properties. Eggs from supplier farms were used. The birds on these farms was given different diets; one group was given an OTM diet supplement while another group remain on the generic feed given on the farm. Eggs were taken from these birds over a period of time and tested.

The mechanical properties which were assessed were strength, fracture toughness, stiffness and hardness.

In addition to this, structural analysis on the eggs was assessed. This was performed by crystallographic and porosity analysis. These tests were performed in order to identify any changes which could occur as a result of the change in mineral supplement diet, Structural tests were performed after the mechanical tests, and specifically performed on groups based on their mechanical results.

Other studies on store purchased hen eggs was completed. Differences in mechanical properties of eggs from different sized groups was investigated *i.e.,* medium, large and very large eggs. The mechanical properties which were assessed was strength and fracture toughness. The main function of store purchased eggs was to optimise procedures and gather primary data. The aim was to better understand the material, obtaining shell from various sources was beneficial as it allowed for a more robust study.

1.2 Project limitations

It is of importance to note limitations which were encountered during this project. Covid-19 significantly inhibited access to college campus and laboratories. The unprecedented nature of the closures resulted in laboratories being inaccessible for several months.

Supply of eggs from the diet and age study was also impacted by this. In addition, an avian influenza struck Irish farms which impacted egg supply. Several studies were terminated due to lack of supply. Essentially there were multiple studies that ended before conclusive results could be drawn. As a result, alternative studies occurred due to this disruption such as the testing the mechanical properties of store purchased eggs which compared egg size.

Chapter 2. Literature review

2.1 Introduction

Approximately 315 Million years ago organisms moved out of water and began existing on land. The amniotic egg ensured reproductive life survived this transition. It evolved a special set of membranes and a hard-shell exterior which functioned to facilitate new life (Stein *et al.*, 2019). Avian eggshell is a prime example of a secure biologically capsule. Composed of calcium carbonate embedded into an organic matrix, it provides physical protection, gas exchange and calcium for the developing embryo. It has a main structural role of protection yet is weak enough to allow the hatchling break free in a process called pipping.

The shell is designed to allow for gaseous exchange, water loss and act as a calcium store for the developing bird. The water and gas exchange is facilitated by a vast network of pores branching through the shell (Tullet, 1978) . The dissolution of the inner section of the egg occurs to supply the developing embryo with calcium. This is promoted by the presence of acid provided by the chorioallantoic membrane (Leeson and Leeson, 1963).

The features of eggshell ensure the perfect conditions for bird preservation. However, it is not naturally produced to withstand commercial handling. Due to the fragile property of the shell, it is prone to damage during handling. With 70 million tonnes of eggs produced per annum, approximately 10% are damaged and discarded (Macleod, Bain and Hancock, 2006). Improving eggshell quality may benefit agriculture by means of decreasing product waste. Layer hen lifespan is tied together with the quality of their eggs. As the hen ages, eggshell quality deteriorates. The result of this being that the hen can no longer produce good quality products and ultimately leads to the end of its life. Therefore, it is understood that eggshell quality is linked to the commercial layer hen's lifespan. Commercial hens laying lifecycle spans around 72 weeks, after which point, they are considered 'spent' (RSPCA, UK), (Stuttgen, 2020).

Concentrating on the aging and nutritional influence, the following review will analyse what affect diet and age has on shell structure and size. To develop commercial egg products, the ultrastructure and the physical properties of the eggshell must be thoroughly understood. The egg of the domestic hen (*Gallus domesticus*) is more frequently analysed than that of other bird species. There is a greater structural understanding of the composition of the eggshell of the hen. Throughout this review, an analysis of a number of species will be conducted. However, there will be greater focus on the domestic hen due to the minimal research that has been completed on various other species.

Bird nutrition is an essential part of egg quality, for both the content and the shell. The natural degradation process which occurs as the bird ages results in increased egg mass and decrease in shell quality (Molnar *et al.*, 2016)(Shafey, 1996). The increase in egg size also correlates with decreasing strength and shell thickness (Roberts, Chousalkar, and Samiullah, 2013) (Rodriguez Navarro *et al.*, 2002). Larger eggs are commercially sought after, but their production is limited as the bird's health deteriorates along with the eggshell itself becoming too thin to survive handling and storage. The poultry industry encourages innovative research so that better quality eggs are produced from older layer birds. There is an obvious interest in increasing the lifespan of layer birds. Longer living birds that can still produce good quality larger eggs is a favourable and sustainable objective which aims to improve animal health and financially benefiting the industry.

Composed mainly of the calcium carbonate polymorph, calcite, eggshell is an example of a naturally occurring bioceramic. However, the eggshell behaves slightly differently to other bioceramics found in nature. With a high Young's modulus and low fracture toughness, it singles itself out from other natural bioceramics. Bones, teeth and shells are all tough biomineral calcium composites which function to withstand impact. The organic material is thought to have an influence on the properties of these ceramics. Nacre, a component of mollusc shell, is composed of 95% calcium carbonate in the form of aragonite. However, it is three times tougher than pure aragonite (Rabiei, Dastjerdi and Barthelat, 2012). This suggests that the structural organisation and the organic components distributed within it could play a role in this increased overall toughness. Bone, another example of a bioceramic material, has a network of intricate collagen scaffolds where hydroxyapatite crystals develop (Ball, 2001). These biomaterials, although almost exclusively made from inorganic components, are influenced by organic materials. As a result, it can be proposed that eggshell is similar to these bioceramic materials in that the organic influence may play a role in eggshell's physical characteristics. Protein and glycosaminoglycan make up the organic components of the eggshell and is referred to as the organic matrix of the eggshell. The calcite crystalline structure is integrated and laid down in a controlled manner alongside the matrix. Eggshell ultrastructural analysis gives an insight into the biomineralisation process and the impacts this process has on the overall properties of the shell. Along with the organic influence, mineral uptake in the diet has been found to impact shell strength and ultrastructure. Inorganic dietary additives such as zinc, manganese, copper, iron and selenium have been suggested to improve egg quality *i.e.*, breaking strength and thickness (M. Ketta and Tůmová, 2016). However, the uptake of these mineral elements into the bird upon ingestion can vary and has incited research interest.

The change in egg size as the bird ages is of interest as it is linked to a decrease in eggshell quality. This will also be discussed in the following review. As the eggshell is formed in an acellular environment, the mechanisms of mineralization have been a challenge to understand. It is important to have a clear interpretation as by doing so, alterations to the bird's life may be made to enhance egg quality.

2.2 Eggshell formation

Hen eggshell is composed of calcium carbonate (95 %) crystals, proteins (3.5%) and the remainder being that of trace elements (Marie *et al.*, 2015). Calcite and organic materials are deposited around the egg membrane, creating the shell (Nys *et al.*, 2004). The eggshell development is a mineralisation process and occurs independent of cellular control. The uterine fluid contains all of the organic material and minerals needed for egg development. The egg is bathed in this uterine fluid containing 6-10 mM of calcium and 70 mM bicarbonate (Nys *et al.*, 1991). This supersaturated solution deposits the necessary compounds to construct the shell. The eggshell forms from the inside out. Eggshell membranes are located between the albumen and the eggshell fibres. Changes in concentration composition in the uterine fluid influence the different formation stages, particularly the calcite crystal growth at different layers in the uterus (Nys *et al.*, 2004). The avian reproductive system is composed of the ovary and the oviduct (see Figure 2.1). Many bird species have only one working ovary and oviduct system, usually located on the left side. The second one degenerates during embryo development. Two theories which proposed to understand why only one ovary and oviduct is necessary are: 1. That to produce eggs requires a large concentration of calcium from the hen. This process would require far too great a demand of calcium from the bird and so it is more favourable for the hen to produce one egg at any one time (Taylor, 1970); 2. The weight of two ovaries may have curtailed the bird's ability to fly efficiently (Zheng *et al.*, 2013).

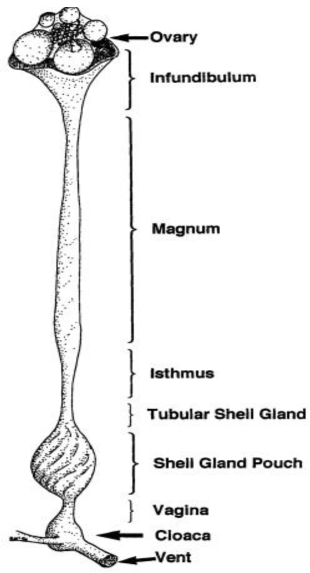


Figure 2.1 Schematic of egg development in the avian oviduct (Roberts, 2004).

The oviduct consists of 6 sections: the infundibulum, magnum, isthmus, uterus (with shell gland) vagina and cloaca. The process of laying an avian egg is termed *'oviposition'*. A follicle is released from the ovary and enters the oviduct. After ovulation, the egg is ready for its solid shell to be formed. It then enters the isthmus and uterus. It is in the isthmus where the egg establishes its characteristic shape (Smart, 1991). The two shell membranes are set here. The egg undergoes a process called plumping. Water and salts enter the egg and it begins to increase in size. The egg itself resides in uterine fluid containing calcium carbonate which is required for shell formation. Other ions such as S^{2-} and Co^{2+} are also present and involved in the shell formation (Park and Sohn, 2018). An immense amount of calcium is needed for egg production, where large quantities of the mineral are needed in the hen's daily diet for egg production. The bird mobilizes calcium from the bone which is transported to the oviduct (Graveland and Berends, 1997).

Eggshell formation occurs in the uterus and lasts 18-19 hours. The layers of eggshell are formed in a controlled process of calcium precipitation (Nys and Gautron, 2007) (Simmons and Hetzel, 1983). This process occurs between the membrane enveloped albumen and the uterine wall mucosa (Hincke *et al.*, 2012). The initial phase of the crystal growth consists of calcium carbonate spherulites nucleating on the shell membrane. The orientation of crystals forming at the site of nucleation in the mammillary cones initially occurs in all directions. Once available space has been occupied, the crystals are forced to grow away perpendicular to the membrane. The palisade layer forms by linear deposition of crystals. The average rate of calcium deposited is 0.33g/hr for roughly 10 hours in hens (Hincke *et al*., 2012). Hen eggshell thickness measures approximately 360 µm and duck eggshell thickness measures 342 µm (M Ketta and Tůmová, 2016) (Nys, Bain and Van Imerseel, 2011).

In hens, calcium concentration in the uterus begins to decline and proteins such as ovocalyxin-32 are seen to be present in the uterine fluid milieu the final stages of eggshell formation in hens. Phosphate anions inhibit calcium carbonate precipitation. Ovocalyxin-32 is a phosphoprotein and it had been proposed that this organic materials influence the termination of calcification (Gautron *et al.*, 2001). Finally, the cuticle forms on the outside of the shell. It is deposited 1.5-2 hours before laying by the shell gland in the uterus (Kusuda *et al.*, 2011) (Wilson *et al.*, 2017)

Since phosphate anions can inhibit calcium carbonate precipitation (36), the relative contribution of inorganic phosphate and phosphoproteins to termination of calcification remains to be determined. Ovocalyxin-32, a major phosphoprotein of the eggshell matrix, is concentrated in the outer eggshell and cuticle and therefore is a potential candidate as a proteinaceous crystal growth inhibitor (37)

2.3 Eggshell Structure

2.3.1 The membrane

Egg membrane is composed of two layers which separate the albumen from the mammillary layer. These two layers have significant roles in water exchange, support and microbial barriers. There is an internal and external membranous layer both on the internal layer of the shell (Ackerman, Dmi'el and Ar, 1985). The positioning of the membranes is depicted in **Figure 2.2** and **Figure 2.3**. The two membranous layers are almost indistinguishable from each other. Separation can be seen at the bottom of the egg, where an air sac is located between the two.

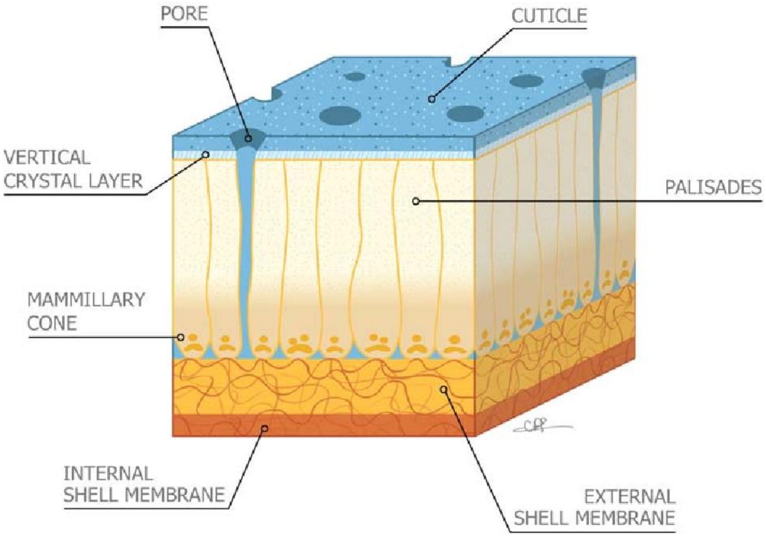


Figure 2.2 Digital drawing of cross-sectional view of avian eggshell. All layers of the shell are visible here including the membrane (Hincke *et al.*, 2012).

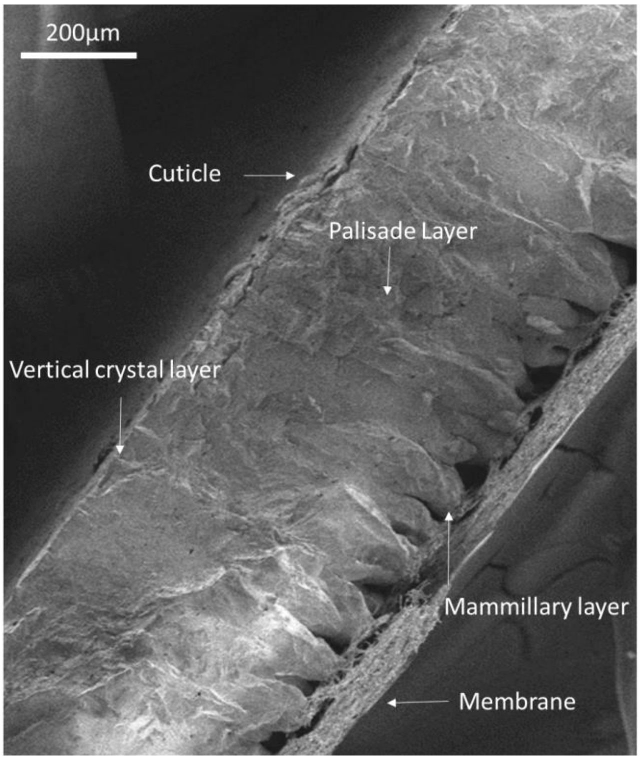


Figure 2.3 SEM of hen eggshell (project image).

Egg membrane is composed of interwoven fibres which can be seen in **Figure 2.4**. The fibres, which make up these layers, are measured at 1-3 µm in width. There are pores which allow gas and water to dissipate across the shell. These pores in the membrane, seen by scanning electron microscopy (SEM), range from 5-10 µm (Wang *et al.,* 2009). The outer portion of the membrane is the initiation site where crystallization occurs, where the nucleation sites forming mammillary cones is located (see **Figure 2.5**.)

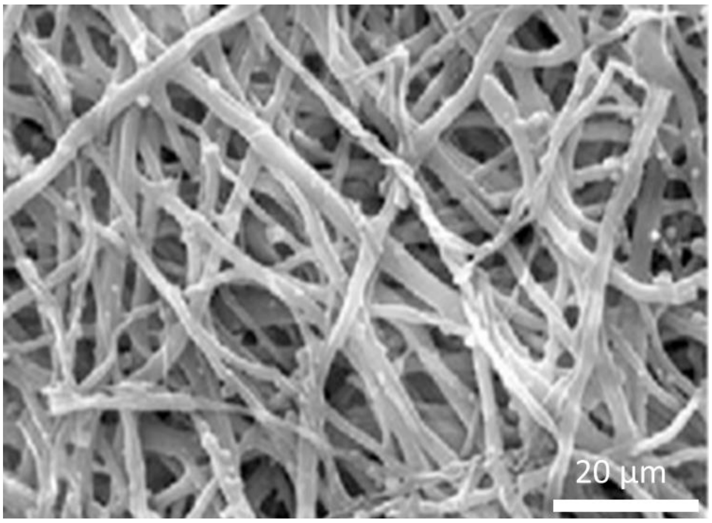


Figure 2.4. Fibrous eggshell membrane as seen on SEM (Wang *et al.*, 2009).

The membranes consist of a variety of organic material. The complex structure acts as the anchoring site for bio-mineralization to occur. Consequently, the membrane is thought to influence the initial crystals which form on nucleation sites. These then develop into mammillary cones.

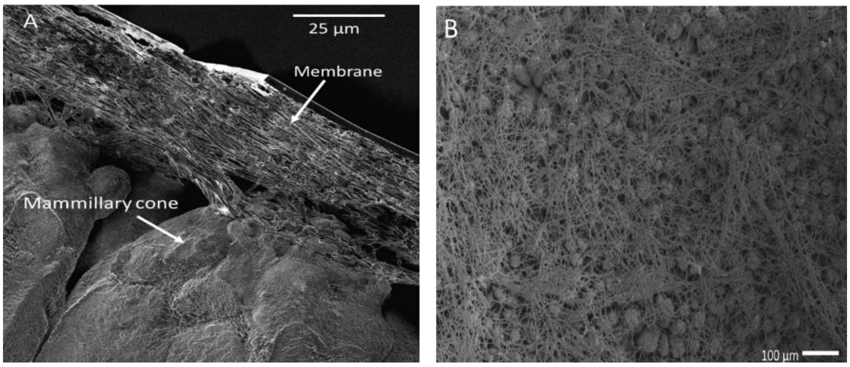


Figure 2.5. A. Fused membrane on mammillary cone. B. Membrane fibres attached to mammillary cones (project images)

Glycosaminoglycans (GAGs), are present in both inner and outer membrane at similar concentrations. Although a larger quantity of these molecules is found in the shell matrix, their presence may influence the membrane in some way. The GAGs which are present in the membranes are hyaluronic acid, keratan sulfate and chondroitin sulfate-dermatan sulfate. Sialic acid is present in the inner membrane (Nakano, Ikawa and Ozimek, 2001). Amino acid analysis suggests the membranes are rich in proline and hydroxyproline. Hydroxyproline levels are indicative of collagen fibres being present (Wong *et al.*, 1984). Collagen type I, V and X are found in the membranes, with collagen type I being the most abundant. The non-helical domains of collagen type X were found to inhibit calcite crystallization by *in-vitro* assays. Proposing that collagen type X inhibits crystallization into the membrane, where instead the crystals grow along or away from the membrane (Arias *et al.*, 1997).

The peptides present in the membrane highlights the arrangement of protective measures that the egg has in place for the developing bird. Antimicrobial peptides such as avian defensins, ovotransferrin, lysozyme and ovocalyxin are thought to be present in the membrane to inhibit bacterial and fungal infiltration. Ovomucoid and mucin are known to inhibit viral penetration and have been identified in the membrane (Nakano *et al.,* 2003).

Nakano *et al*. (2003) notes that there was an absence of osteopontin and phosvitin in the inner membrane. These two proteins are significantly linked to the calcification process of the shell. Makkar *et al.,* (2015) propose that the inner membrane has no interaction with the calcification process during the shell development in the uterus, implying that there is no requirement of osteopontin and phosvitin at this layer. This suggests that the outer membrane contains organic molecules which impact the calcification process whereas the inner membrane is independent of the shell formation.

2.3.1.2 Mammillary layer

The mammillary layer is the first section of the 'true' shell, or the base of the mineralised shell. It is composed of cone-like structures. This is the inner most layer of the shell and measures approximately 100µm thick in hens. The mammillary layer serves both a structural and functional role. The calcium dissolved from the eggshell for embryotic skeletal growth is provided, by the most part, from the mammillary layer (Arias *et al.*, 1993). The mammillary layer is a composite material of interlacing fibres from the egg membrane and calcium carbonate crystals (see **Figure 2.5**). The mammillary cones mark the start of calcite crystal formation. The cones contain keratan sulphate, which is supplied by the tubular glands located at the base of the isthmus. The diameter of the crystals which form the cones measures 20-40 Å in hen eggshells. The calcite columns extend away from the membrane and become part of the next phase; the palisade layer (Chien, Hincke and McKee, 2009). **Figure 2.6** Shows the mammillary cones observed from the inside of the shell with the membrane removed.

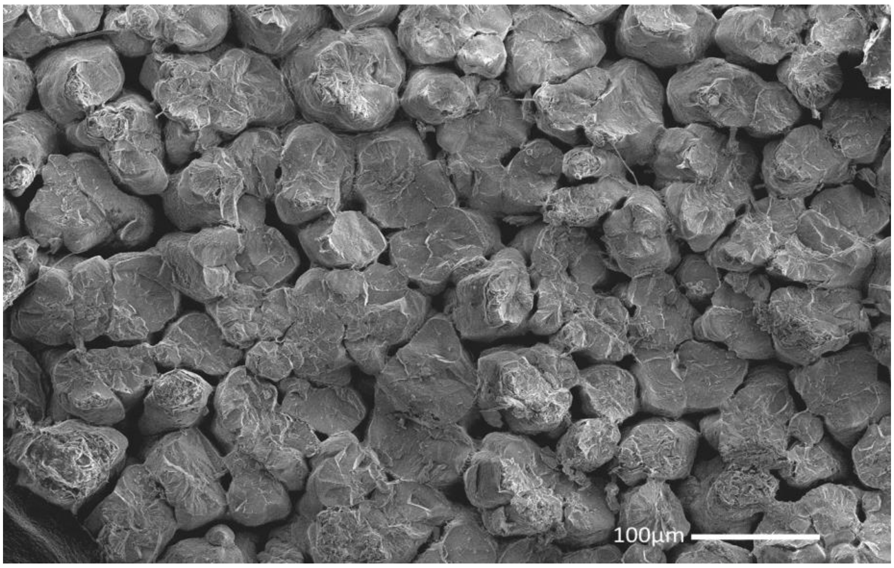


Figure 2.6 SEM image from inside the shell at mammillary cones with membrane removed (project image).

2.3.1.3 Palisade Layer

This is a columnar area that stretches across the shell, meeting the crystalline layer. Spanning most of the shell, in hen eggs it measures from 200-350 µm. This is the largest section of the shell in all avian eggshells. This can be seen in **Figure 2.2** and **Figure 2.3**. The initial position of the palisade holds a certain level of ambiguity as it starts at the region just above the mammillary layer where the crystals coming from the cones are confluent. The crystalline columns grow perpendicular to the egg itself, developing in the available free space. The crystals here are arranged in a spherulitic texture (Hincke *et al.*, 2012). Some research suggests this is to allow for crack propagation during piping (Nys *et al.*, 2004). A comparative study performed by Chen and Shen (2000) demonstrated that palisade layer of the Tsaiya duck was more compact compared to the eggshell of the domestic hen.

2.3.1.4 Vertical Crystal Layer

The vertical crystal layer (VCL) layer is deposited on top of the palisade layer measuring approximately 8 µm thick in hen eggs. This thin layer is composed of crystals which are more compact compared to the layers below (see **Figure 2.7**). This layer is observed to be in a vertical orientation between the palisade layer and the outer waxy cuticle (Fraser, Bain and Solomon, 1999). There is very little known of this layer and there has been little analyses focused on this portion of the eggshell. The structural difference of the crystal calcite orientation of this layer compared to the palisade layer does generate a question of its significance. Does the change in crystal orientation in the VCL bare any significance on the overall structure of the shell? Does this layer have any active structural or mechanical role in the overall shell? Further investigations into this layer could be beneficial to further understanding the eggshell integrity and mechanical properties.

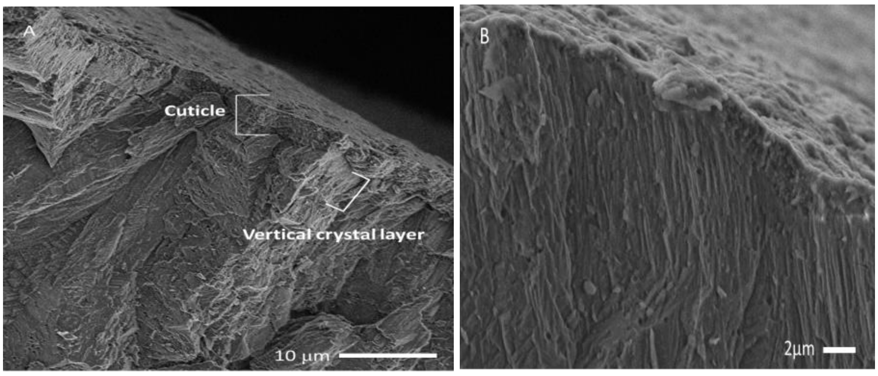


Figure 2.7.A. Vertical crystal layer of hen eggshell as seen on SEM. B. Magnified image of the vertical crystal layer. Crystal orientation is visible (project image).

2.3.1.5 Cuticle

The cuticle is the outermost layer of the shell. In hen and duck eggshell the cuticle measures approximately 5 µm and 8 µm, respectively (Chen *et al.,* 2019). This water insoluble layer is deposited directly on to the vertical crystal layer. The cuticle provides protection for the egg from pathogenic infiltration. There is an inner and an outer portion of the cuticle. The inner vesicular layer is composed of vesicles. The outer non-vesicular layer is densely compact (Fraser, Bain and Solomon, 1999). The cuticle coats most of pore entrances which cover the entirety of the shell surface. This coating impedes microbial penetration and prevents excess water loss (Nys and Guyot, 2011). Studies have confirmed that avian species, specifically altricial bird eggs, that have little to no cuticle on the eggshell surface had much higher bacterial penetration rates when compared with eggshells of birds with a thicker cuticle (Chen *et al.*, 2019).

There are some pores which are still visible even when the cuticle is fully intact (see **Figure 2.8)**. Thicker cuticles are seen in eggs of species which encounter greater microbiological challenges, such as aquatic birds like ducks. This supports the idea of the cuticle having a functional antimicrobial role (Kusuda *et al.,* 2011). Eggshell cuticle can be influenced by hormonal stress factors, such as epinephrine, which can delay oviposition and impact the quality of the cuticle layer (Butcher and Miles, 1995).

The uneven cuticle layer contains a variety of proteins, carbohydrates and lipids. Phosphorus containing hydroxyapatite crystals are also present. Hydroxyapatite is present in bone and teeth to provide rigidity (Abdulrahman *et al.*, 2014).

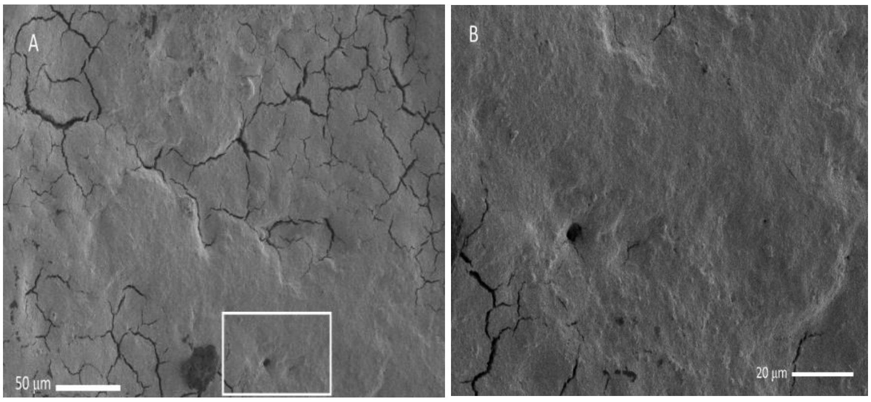


Figure 2.8. A. SEM micrograph of outer eggshell. B. Depicts a magnified area from around a pore entrance which is visible despite the cuticle being intact (project image).

2.3.2 Ultrastructure

2.3.2.1 Crystalline Structure

The calciferous crystals of the eggshell maintain the shell rigidity. The mineralisation process occurs in three phases: initiation, growth and termination. The growth of crystals at the initial stage are entwined with fibres from the outer membrane. These give rise to mammillary cones. The crystals do not form back in toward the membrane. As mentioned, there are components in the membranes, such as collagen type X, which are thought to inhibit the crystal growth. Therefore, the growth pattern of the crystals see it move radially and vertically, with no crystal growing in toward the inside of the egg itself. The radial growth is limited, as eventually the crystal contact neighbouring crystals. These neighbouring crystals are what will eventually fully form adjacent mammillary cones. This competitive growth stage leads to columnar structures, creating the palisade layer. The palisade layer eventually gives rise to the vertical crystal layer, its crystalline content is denser and with smaller subunit size (Athanasiadou *et al.*, 2018).

The calcium carbonate polymorph, calcite, is deposited on the membrane. The membrane contains molecules which are thought to promote crystalline growth. The spherulitic crystals are understood to be anisotropic as a result of the lengthened crystal columns. It has been suggested that organic components in the uterine fluid guide this process (Gautron and Nys, 2006). Crystal width increases as it progresses towards the outside of the egg. Ranging from 20 µm at the inner section to 80 µm on the outer shell (Hincke *et al.*, 2012). The crystalline surface is covered in striations and fine granulations. In the palisade layer and the vertical crystal layer, neighbouring crystals fit together in a mosaic-like fashion (Heyn, 1963).

Quantitative analyses of crystallography of the eggshell has developed over time. Initially, the crystal properties of the shell were determined by optical microscopy. Rodriguez-Navarro *et al*., (2007)(a) and Rodriguez-Navarro (2007)(b) proposed that 2 dimensional X-ray diffraction (2D-XRD) was more quantitative than standard optical microscopy when analysing microstructure of the eggshell. The authors also explain that 2D-XRD has advantages over conventional X-ray diffraction techniques due to the time and sample preparation. Crystal sizes and orientations can be detected and quantified using this technique and has proven beneficial in understanding the microarchitecture of eggshell.

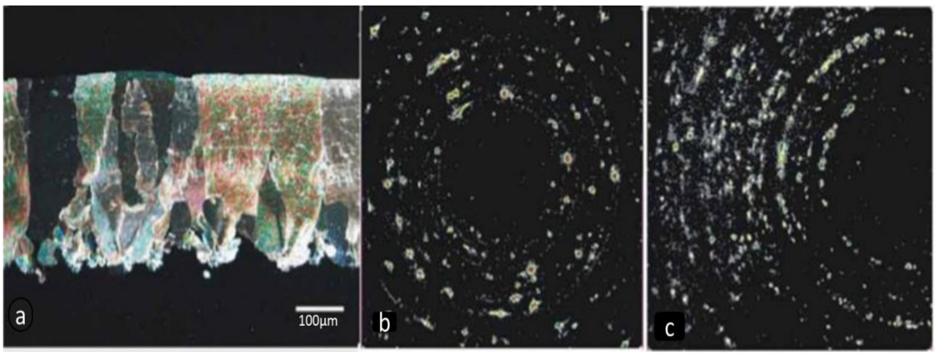


Figure 2.9. Adaptation from Rodriguez-Navarro (2007)(b) eggshell microstructural analysis. (a) Depicts a microphotograph of hen eggshell under cross-polarized light. The light extinctions show the differences in orientation

of each columnar crystal unit. (b) Shows transmission X-ray diffraction patterns and (c) shows the reflection X-ray patterns of the same eggshell. The stronger the crystal orientation, the more prominent the arcs in the reflective pattern are as the reflective spots merge closer together.

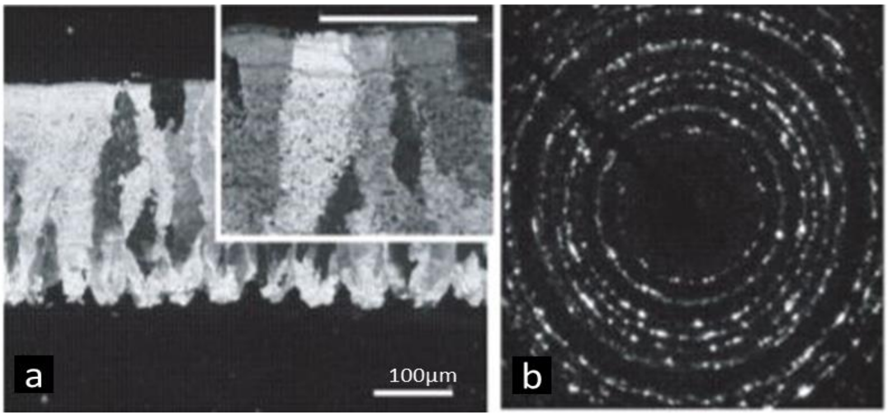


Figure 2.10. Adaptation from Rodriguez-Navarro *et al*. (2007)b. (a) Microphotograph of Muscovy duck eggshell under cross polarised light. (b) Diffraction pattern of duck eggshell.

Different avian species possess varied crystal structure. Studies using X-ray diffraction performed by Rodriguez-Navarro and team (2002) have determined the FWHM (full width half maximum) of hen eggshell crystals to be between 50º-120º. It was acknowledged, using the FWHM values as the integral of orientation dimensions, that eggshells with randomly orientated crystals are mechanically stronger than those of highly oriented crystals (Rodriguez-Navarro *et al.*, 2002) (Ahmed *et al.*, 2005). The columnar crystal formation in the palisade region is perpendicular to the surface and usually takes the orientation plane of *{001}* or *{104}* (Sharp and Silyn-Roberts, 1984) (Rodriguez-Navarro *et al.*, 2002). Athanasiadou and team (2018) using 2D-XRD on hen eggshells, confirmed the calcite crystals contain a high degree of internal misorientation. The paper concluded that the columns were not single crystals, but multiple crystals. In addition to 2D-XRD, Athanasiadou *et al.*, (2018) conducted Electron backscatter diffraction (EBSD) to assess crystallography. The team described that internal misalignment within the shell. EBSD was also used by Dalbeck and Cusack (2006) to observe preferential orientation which was described as {001}. The initial growth pattern of crystals was discussed in this study and confirms the formation of crystals at the mammillary layer in a 'fanning' pattern initially, proceeding to merge into the columnar palisade layer. Crystal misalignment refers to the angle of the grain boundaries between crystals. Grain boundaries are the interfaces between two crystals. Low angle grain boundaries have a strong dependence on the crystal misorientation (He, 2018).

There have been correlations made between egg weight and crystal size. Suggestions have been made that this may be due to the egg itself being bigger, therefore, the mammillary cones are spaced out from each other more which increases the area the crystals can move into during development (Dunn *et al.*, 2012).

2.3.2.2 Porosity

The eggshell is designed to maintain adequate conditions for the developing embryo. Indeed, the architecture of the eggshell ensures structural and pathogenic protection. In addition to structural roles, it provides a mineral supply of calcium to the growing embryo and allows for gas exchange *via* pores.

There are five main pore systems in avian eggs reported; simple pore systems, occluded pore system, plugged pore system, capped pore system and reticulate pore system. Domestic fowl have a capped pore system; this is where a portion of the cuticle is residing on the surface of the shell and can extend down into the pores. A convoluted pathway is present in hen eggshell to allow for diffusion which sees gas and water vapour pass between the internal and external surroundings of the egg (Board and Scott, 1980). Oxygen and carbon dioxide exchange is critical for respiration that the egg facilitates. The eggshell allows for respiration to occur by releasing water vapour through pores. This generates an air sac between the inner and outer membrane which prior to pipping, the bird uses for respiration. (Romanoff and Romanoff, 1949) (Wangensteen, Wilson and Rahn, 1970).

Gas and water vapour exchange increases with increased number of pores being present (Rahn, 1981). It has been reported that pore numbers on shells varies greatly among birds, even the same hen can lay eggs with varying pore numbers. 10,000-20,000 pores can exist in one egg (Baxter-Jones, 1994). **Figure 2.11** displays two types of pores found on hen eggshell. Larger pores can be seen on the surface, especially when the cuticle is removed. There are also very small voids throughout the crystals in the shell which can also be visualised. It is unclear if these small holes are also part of the gaseous and water vapour pore system.

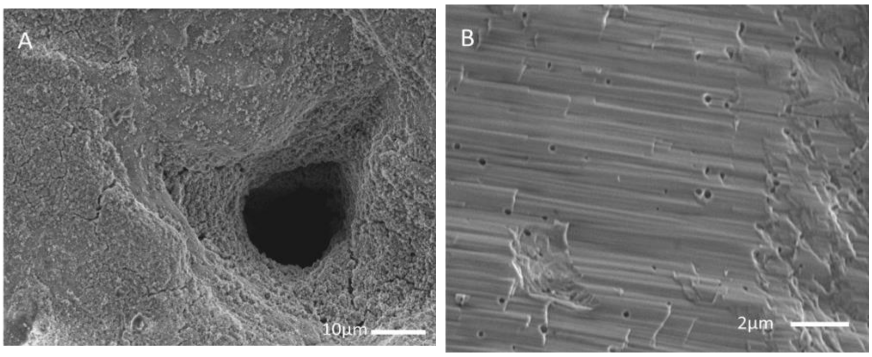


Figure 2.11. A. Entrance of large pore visible from outside shell with cuticle removed. B. Palisade layer crystals with very small voids which could be pores (project image).

The results obtained by La Scala *et al.* (2000) observed pore diameter of hen eggshells range from 1.4- 5.3 µm. These results were obtained using mercury porosimetry. This technique is limited however, as it does not give any indication into the pore shape throughout the shell. X-ray micro computed tomography (µCT) analysis gives an enhanced perception of the pore structure within an intact shell. The benefits of this technique have been that the porous pathways within the shell are better understood and visualised as was seen by Riley *et al.,* (2014). From this study, density distribution throughout the eggshell was seen using this technique, however overall it is not a method which captures quantitative data. Despite research addressing pores within eggshell, there is little inspection into the role of these pores on a structural level. Further investigation into eggshell porosity should be encouraged, especially in identifying any mechanical roles the pore sizes and distributions may play in the overall quality of the eggshell.

2.3.2.3 Organic Matrix

The organic component of the eggshell is composed of a variety of proteins and polysaccharides, composing of approximately 3.5% of the shell and referred to as the organic matrix. The matrix does not include the cuticle or the membrane. Despite its minor concentration, the importance of the organic matrix in the shell has been of research interest in recent years. There have been over 484 and 528 proteins identified in duck and hen eggshell, respectively (Zhu *et al.*, 2019) (Farinazzo *et al.*, 2009). There has been variance observed in the exact concentration and identification of proteins within eggshell and the reason for this may be due to the range of extraction and analytical methods used by researchers. Methods used for protein analyses include HPLC, iTRAQ mass spectrometry, mass spectrometry, and nanoLC mass spectrometry (Zhu *et al.*, 2019) (Farinazzo *et al.*, 2009) (Mann, Maček and Olsen, 2006).

Developmentally and structurally these components are now thought to bear some importance in the durability of the eggshell. Within the calcite shell, mostly in the palisade layer, there are microstructural voids or vesicles which are thought to contain organic material (Chien *et al.*, 2008).

Extensive analyses has occurred on proteins that are present in the fluid milieu during shell formation and proteins present in the eggshell after oviposition. Egg matrix proteins have retained their own prefix 'ovo'. Some examples include ovocleidin and ovocalyxin (Mann, Maček and Olsen, 2006). Not all of the proteins found within the matrix appear to have a mechanical role in the eggshell. Some proteins are thought to be present in the matrix due to their abundance in the fluid milieu preceding egg formation phases. Whereby, these proteins may be embedded into the eggshell during the mineral deposition process and therefore possess no active role within the eggshell itself. However, other proteins have been confirmed to reside in the matrix with their primary role believed to be the regulation of calcite crystal or for structural support of the eggshell such as osteopontin, ovocleidin-17, ovalbumin, ovotransferrin and ovocalyxin-32. Due to the abundance of various proteins residing within the eggshell, difficulty has arisen in isolating the proteins which have a functional role specifically associated to the eggshell.

Osteopontin has been identified as being one of the primary shell matrix proteins. As a member of a group of mineral binding proteins, it holds a high negative charge and flexible structure which is ideal for binding calcium. This protein is also present in bone (Fisher *et al.*, 2001). A report from Athanasiadou *et al.* (2018) confirmed osteopontin can alter structure in calcite crystal formation. Strong expression of this protein has been seen in cells located in the shell gland, where this protein is deposited into the eggshell (Pines, Knopov and Bar, 1995). This phosphorylated glycoprotein was observed in high concentrations in the outer layers of hen eggshell (the palisade and vertical crystal layer). The mammillary layer contains little osteopontin (Athanasiadou *et al.*, 2018) (Marie *et al.*, 2015). Osteopontin has been seen to largely associate with {*104*} crystallographic faces in the eggshell. Based on the understanding that this protein is found on the face of crystals parallel to the surface suggests that the protein may be involved in crystal orientation (Chien *et al.*, 2008). From genetic analysis on ducks, researchers have discovered a polymorphism within exon 7 of the

OPN gene which results in thicker and stronger eggs (Bai *et al.*, 2018). This research proposes the analogous influence the protein has on eggshell.

Reyes-Grajeda and team (2004) have proposed that ovocleidin-17 (OC-17) influences calcite crystal formation. The researchers observed that the monomeric OC-17 can transform amorphous calcium carbonate to crystalline calcite (Freeman *et al.*, 2011). This protein was seen to be abundant in the initial phase of formation and present in the mammillary cones in hen eggshell, indicating its role in the initial formation of calcite crystals (Hincke *et al.*, 1995). *REG-4*, which is another C type lecithin protein closely related to OC-17, has been identified as an abundant protein in duck eggshell (Zhu *et al.*, 2019). The C-type lecithin domain, which is a carbohydrate domain similar to that of the protein perlucin. Perculin plays a role in the mineralisation process in the nacreous layer of *Haliotis laevigata* shell. However, OC-17 has not been seen to bind to calcium in a carbohydrate inclusive process such that is seen with C-type lecithin proteins. The structural make-up of this protein has been shown to be highly polarised along with its surface being positively charged. These characteristics support the concept that it binds to calcium carbonate (Reyes-Grajeda, Moreno and Romero, 2004). OC-17 is present in the matrix, with the glycosylated form of this protein also being present in the hen eggshell matrix as ovocleidin-23 (Bradford, 1976) (Mann, 1999).

Ovalbumen and ovotransferrin are abundant in the uterine fluid during the initial phase of calcite deposition. The presence of ovalbumin and ovotransferrin in the mammillary layer suggests that they play a role in the initial stages of calcification. These two ubiquitous egg white proteins have been observed to bind Ca^{2+} ions and modify calcite crystal morphology (Gautron *et al.*, 2001) (Wang *et al.*, 2010) (Schwahn, Balz and Tremel, 2004) (Pipich *et al.*, 2008) (Zhu *et al.*, 2019).

Ovocalyxin-32 is formed in the surface epithelia of uterine tissue. It is located towards the outer palisade layer, the vertical crystal layer and the cuticle. Consequently proposing its involvement in the termination process of eggshell formation (Gautron *et al.*, 2001). Genetic analysis of this protein has seen polymorphisms associated with this 32kDa protein and some physical characteristics, such as crystal orientation and strength. Given that variations to this protein impact eggshell phenotype, or morphology, it appears intuitive that ovocalyxin-32 interacts with the mineralisation process. However, the exact mechanism of binding is unknown (Dunn *et al.*, 2009) (Dunn *et al.*, 2012) (Takahashi *et al.*, 2009) (Takahashi *et al.*, 2010).

Other proteins such as calcium binding proteins have been identified within the hen eggshell: *EDIL3 and MFGE8.* Their exact role is not fully understood, however it is hypothesised to be that of influencing the deposition and aggregation of the calcium carbonate (Marie *et al.*, 2015). Difficulty has arisen to conclude if certain proteins are in fact involved in the eggshell mineralization process. Proteins which are present in the mammillary layer, like ovotransferrin and ovalbumin could simply be there due to their high concentration in the albumin whose excess then happens to be embedded into the mammillary layer. Further research of the matrix is needed for the mineralization process to be thoroughly understood. Glycosaminoglycans (GAGs) are long negatively charged polysaccharides of repeating disaccharide units. These are large molecules and ubiquitous throughout nature. The amino sugar is usually accompanied by uronic sugar or galactose. All GAGs have a covalently bonded core protein and are known as proteoglycans, with the exception of hyaluronic acid, which contains no core protein and exists as a free polymer (Kjellén and Lindahl, 1991). Studies focusing on GAG content within the egg and eggshell have utilised various techniques such as immunohistochemistry, enzymatic reactions and HPLC-MS. The GAGs which have been reported in eggshell are chondroitin sulfate, keratan sulfate, hyaluronic acid, dermatan sulfate and heparan sulfate (Liu *et al.*, 2014). It is under consideration if dermatan sulfate and chondroitin sulfate exist as a co-polymer within the matrix, as both GAGs are present throughout the shell. (Nakano, Ikawa and Ozimek, 2001) (Liu *et al.*, 2014).

Liu and team (2014) performed extensive tests analysing the GAG composition within the eggshell organic matrix using HPLC-MS. The research performed observed that chondroitin sulfate and dermatan sulfate was the most abundant GAGs in the shell matrix. Water soluble keratan sulfate was seen to be present in high quantities in the eggshell matrix. Hyaluronic acid was observed in lower concentrations and heparan sulfate was found in the matrix at trace amounts. This research confirmed the presence of heparan sulfate in the eggshell matrix which was unknown until this study.

Dermatan sulfate was detected mainly in the palisade layer of the hen eggshell (Carrino *et al.,* 1996)(Liu *et al.,* 2014). Carrino and team (1996) tested this GAG on calcite crystals, and it responded to crystal formation, making the crystal smaller in a concentration dependant manner. Dermatan Sulphate is produced in the shell gland; this is also the location of calcium crystal deposits. This research suggests that dermatan sulfate is indeed regulatory in crystal formation. This GAG found to be present in most shells of various avian species, including duck. Keratan sulfate was observed to be released from cells in the isthmus 5.5 hours after ovulation. This coincides with the initial deposition of calcium carbonate on the nucleation sites at the mammillary layer. This proposes that keratan sulfate has a function in the initial crystallization of the eggshell (Nys *et al.*, 2004). Research performed on a variety of avian species illustrated that no keratan sulphate was present in the eggshell of duck (Panheleux *et al.*, 1999). Hyaluronic acid is an important GAG in nature and present in cartilage chondrocytes. As a particularly large molecule, it is seen to act as a shock absorber (Zhang *et al.*, 2017). This may perform the same role in eggshell, but it has not been investigated.

Although there is a low concentration of organic material within avian eggshell, their presence should not go overlooked. It is clear these components play a role in eggshell formation and perhaps that is where their function ceases. Nonetheless, the examination of a structural role these organic components could provide is of interest. The testing of GAGs and proteins in eggshell and their impact on mechanical properties has not been sufficiently examined and there is a scope for future testing to occur.

2.4 Manufactured Ceramics and Failure

Ceramics are a group of materials which are neither metallic nor organic. Manufactured ceramic, which is generally crystalline and non-reactive, is formed at high temperatures in excess of 1,400^{o}C (Bouville and Studart, 2017). The classification of a ceramic is determined by its properties. These properties include: high brittleness, high hardness, strong compressive strength and weak tensile strength (Bengisu, 2001). These characteristics are determined atomically. Ionic and covalent bonds make up ceramics (Kingery, 1976). The atomic bonds make up the crystal, these crystals are linked to the brittle nature of the material (Huang and Best, 2007). Specifically, in covalent ceramics, electrons in a covalent bond are concentrated to an area between two atoms, not delocalised like metallic bonds. Similarly, electrons in the ionic bonds are also directed. This electron concentration means that the it is more difficult to move atoms when they are in this arrangement, which results in a non-deformable, brittle material (Rösler, Bäker and Harders, 2007). Ceramics fail without having any plastic deformation, unlike metals which can undergo significant plastic deformation before failure (see **Figure 2.12**).

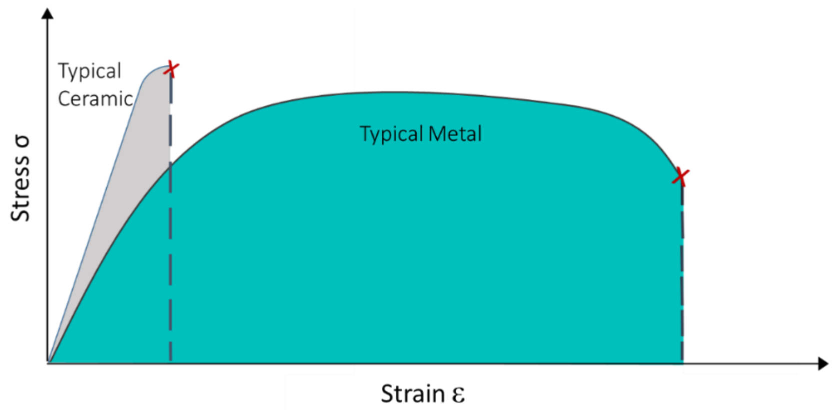


Figure 2.12 Stress-Strain curve demonstrating typical ceramic and metal behaviour under tensile stress (project image).

Ceramics can withstand high compressive loads but fail easily in tension. This is because the flaws or defects within the material act as stress concentrators. Ceramic strength is therefore determined by the defects existing in the material before failure has occurred (Rösler, Bäker and Harders, 2007). These defects can be formed during manufacturing. Pores or voids can be created during the sintering process, which is when the powder material is heated at high temperature to form the ceramic. In addition to sintering, compaction can occur which reduces the amount of pores. Although this minimises the pores, it does not completely remove them (Rösler, Bäker and Harders, 2007).

Because of the brittle nature of ceramics, they can fail rapidly with no plastic deformation. This can be an undesirable feature in industry. Consequently, understanding fracture in the material is important. Fracture toughness is the property which provides great insight into the behaviour of ceramics. This property is defined as the measure of resistance of a material to fracture when a crack is already present (Lancaster, 2005). The atomic bonds play a role in the determination of fracture toughness as it is essentially the measure required to pull these bonds apart which creates a new surface (Shukla, 2006). As mentioned, the defects within the ceramic can concentrate the stress. If there were no flaws, the stress would be evenly distributed. But when a flaw is present the stress can gather in a small area which is known as stress concentration (Rösler, Bäker and Harders, 2007). **Figure 2.13** demonstrates the stress concentration around a crack. This stress concentration is included in the measurement of fracture toughness, or K_{Ic}.

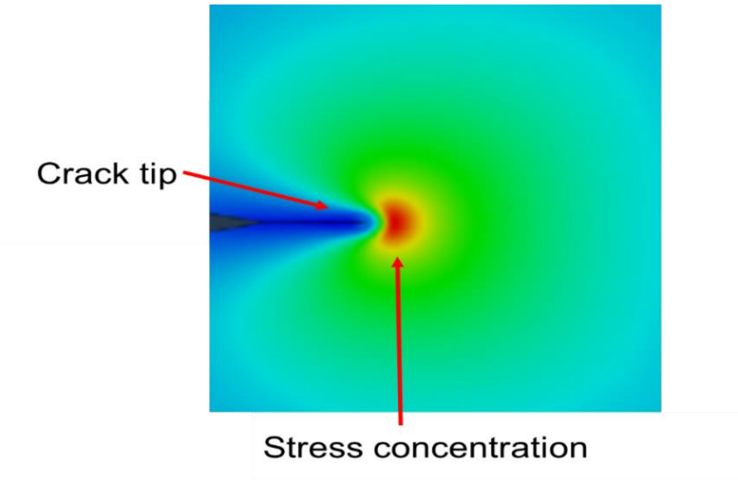


Figure 2.13 Stress concentration concentrated to the tip of the crack (Malakooti and Sodano, 2014).

A way of reducing this stress concentration at a crack tip is having particles in the ceramic which encounter the crack tip. The stress concentration is dispersed around the particle, meaning more energy is required for the crack to continue on its path. If the crack cannot go through the particle, it is forced to move along the boundaries which increases the surface area distance the crack has to travel and thus, increases the K_{Ic} of the ceramic (Rösler, Bäker and Harders, 2007). The stress intensity and crack tip has piqued the interest of many researchers. In 1921 the Griffith Criterion was established. This states that the crack will propagate when the potential energy that occurs in crack growth is greater or equal to the increase in surface energy from the creation of a new surface (Griffith and Taylor, 1921). The stress intensity factor (K) is a measure of prediction of the stressed state near the crack tip. This theoretical factor is useful in identifying if the material is likely to fail and is dependent on crack length, the size of the material and the stress applied (Sherry, 2010). It is not possible to measure every single defect within the material and so probability analyses of fracture in ceramics is regularly applied, such as Weibull probability statistics (this is be discussed further in *2.6.4 Probability Analysis*).

2.5 Calcium Biomaterials

Eggshell is not the only bioceramic material found in nature. There are many other materials which are predominantly calcium based and have protective and structural roles. The properties of shell have been widely studied in the phylum *Mollusca*, particularly bivalves (*i.e.,* oysters and mussels) and gastropods (*i.e.,* snail and conch). The strength of these shells is mainly due to the structure and the combination of the calcium carbonate (Checa, 2018). Bivalves usually have various layers which ensure that the shell has the properties to keep the organism well protected. These shells usually have an aragonite nacre layer which is an extremely rigid material. The layer above this is the prismatic layer which has a certain degree of flexibility and is itself attached to the outer most organic layer of the shell; the periostracal layer (Rousseau *et al.*, 2009). These two outer layers are more malleable and when combined with the rigid nacre layer, see that crack propagation is difficult to permeate throughout the shell as the different textures inhibit the crack's direct passage. The tough nacre layer is composed of aragonite platelets alongside organic beds. Chitin and proteins act as sites for calcium deposition. The organic deposits along with the architecture strengthens the material (Song *et al.,* 2003). The formation of the aragonite form almost in a mismatched brick and mortar appearance with the organic material between the aragonite platelets which can be seen in **Figure 2.13** (Marin, Le Roy and Marie, 2012).

The K_{Ic} of pure aragonite is 0.25 MPa√m and the K_{Ic} value reported in the of the middle layers of a conch shell was 2.26 MPa√m (Kamat *et al.*, 2000). Nacre itself was seen to have a K_{Ic} value of 3-5 MPa√m (Kakisawa and Sumitomo, 2012). One research team obtained a K_{Ic} value of 8 MPa√m for mollusc shell (Cortie *et al.*, 2016). The reason for higher K_{Ic} values within shells of bivalves and gastropods may be related to the presence of the organic matrix within the layers. The polysaccharide chitin is thought to influence the toughness of the shell. The structure of the aragonite platelets embedded into an organic matrix likely impact crack propagation. The organic component at the grain boundary may act to dissipate stress when encountering a crack. This may produce a tougher material as more energy is required for crack propagation (Khayer Dastjerdi, Rabiei and Barthelat, 2013). The organic components, along with structural orientation differences may be linked to the superior properties that shells from Mollusca have compared to basic aragonite.

The fracture toughness testing methods for nacre and other parts of shells from species in Mollusca varies. Some researchers created notches of various sizes and applied 3 point bending mechanisms, tensile stress, beam bending to observe failure (Jackson *et al.*, 1988) (Cortie *et al*., 2016) (Kamat *et al.*, 2000). The variation in testing could account for potential differences in measurements.

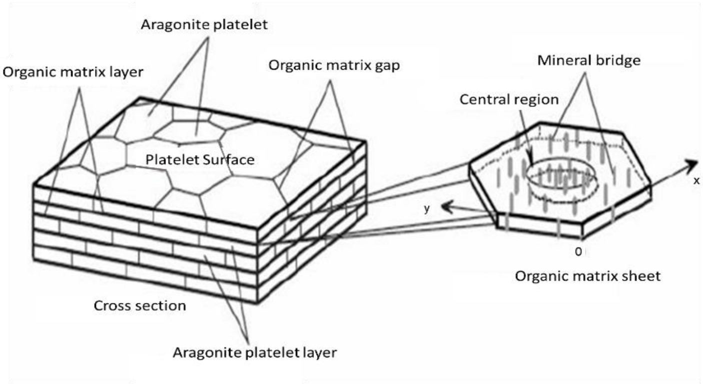


Figure 2.13 Diagram of the structure of nacre (Song, Soh and Bai, 2003).

Mollusca shells typically have a Young's modulus of approximately 50 GPa (Currey, 1976). This is a relatively high Young's modulus for a natural material.

Bone is another example of a bioceramic and is the most studied calciferous biomaterial. The properties of bone ensure that it withstands impact and protects internal organs. Bone is composed mainly of calcium based hydroxyapatite and collagen fibres (see **Figure 2.14**). Collagen fibres serve as nucleation sites for mineral crystals to form. The compressive strength of bone is high. The material is also tough, as collagen Type I has been identified as playing a role in the toughness of bone (Viguet-Carrin, Garnero and Delmas, 2006). The organic component of bone increases its tensile strength (though greatly inferior to its compressive strength). Young's modulus of human cortical bone, when measured using micro tensile tests, achieved a value of 18.6 *GPa* (Rho, Ashman and Turner, 1993). Although there are many factors which influence the property of bone such as age, diet and disease, the relationship between the hydroxyapatite crystals and the collagen fibrils is decidedly important.

Fracture mechanics analysis for bone is similar to that used in research in Mollusca shell. Single-edge notch on a specimen using three point and four point bending have been considerably popular in the fracture mechanics analysis of bone (Li, Abdel-Wahab and Silberschmidt, 2013) (Woodside and Willett, 2017) (McNerny *et al.*, 2015). There is an obvious medical interest in looking at crack propagation in bone. As a biocomposite, it is a fine example of the relationship between organic and inorganic molecules that create a 'fit for purpose' material.

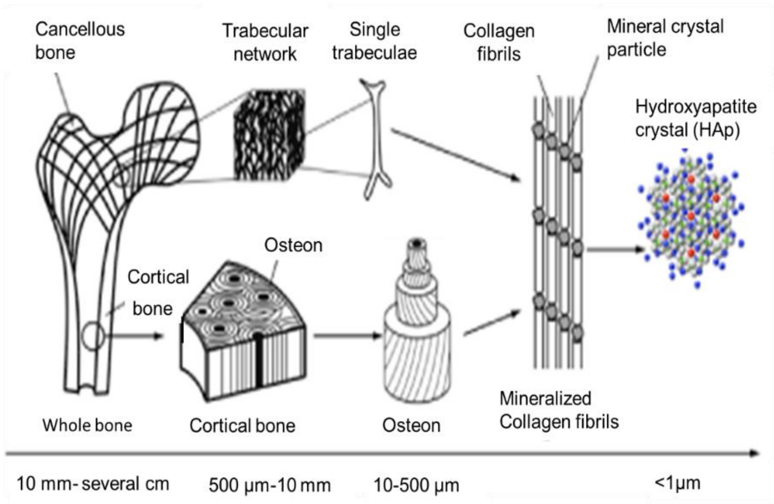


Figure 2.14. Basic depiction of both trabecular and cortical bone structure. Both bone type are essentially made of collagenous fibres and hydroxyapatite particles (Gamagedara and Ziana, 2018).

Enamel is another essential biomaterial within the body. Enamel makes up the outer layer of teeth. It is the hardest material in the body (Hayashi-Sakai *et al.*, 2012). Like bone, enamel is made of hydroxyapatite. The concentration of this organic component however is lesser than that of bone, with enamel having less than 1% organic components (Duverger, Beniash and Morasso, 2016). This makes enamel compositionally more similar to shell. Ameloblasts are the cells which are thought to be involved in the formation of enamel (Retrouvey, Goldberg and Schwartz, 2012). Enamel itself is made of enamel rods which are compact structures of hydroxyapatite (Fernandes and Chevitarese, 1991).

Due to the nature of enamel being structurally ingrained in teeth, compressive and tensile strength have proven to be difficult to obtain (Hayashi-Sakai *et al.*, 2012). Indentation tests have been performed by several research teams to provide several properties of enamel such as hardness, elasticity and fracture toughness. Microhardness techniques performed on enamel revealed fracture toughness value of 0.88 MPa√m (Biswas *et al.*, 2013).

Fracture toughness analysis on enamel is difficult to perform using standard tests such as the three-point bending technique. Because enamel is a thin film which covers dentin (which makes up the bulk of the tooth), the process of finding fracture behaviour is almost exclusively performed by indentation techniques. It was noted by a research team that irregular enamel rods which are seen to change direction increase fracture toughness values and so lead to the conclusion that irregular crystal rods increase enamel resistance to fracture (Hayashi-Sakai *et al.,* 2012). Comparisons here can be drawn between that of Mollusc shell and enamel. As mentioned, Mollusc shell have a staggered array of aragonite platelets which inhibit crack propagation and increases toughness. It is true that the Mollusc shell also has an organic component which plays a role in fracture resistance, which is not true for enamel. Nevertheless, it is clear that a similar disarray of inorganic calciferous components plays a role in increasing toughness across many bioceramic materials.

Biomaterials found in nature are complex and fully understanding how their structure dictate their properties has sometimes proven difficult. The role of organic components within these mostly inorganic materials has been researched widely in recent times and there are indications that they play some role in the properties of the biomaterials. Manufactured industrial ceramics are usually produced using high energy techniques *i.e.,* high temperature and pressure. Although manufactured ceramics often reign superior in toughness and resistance to failure compared to their natural counterparts, it is still interesting to look at the production of these natural ceramics. The study of the formation and the structure of bioceramics is an area that researchers should be willing to inspect further as their properties are desirable and could be utilised in future low energy production techniques.

2.6 Physical Properties of Eggshell

A great deal of research has occurred to understand the physical properties of the eggshell. Eggshell has unique characteristics which separate it from all other materials. It possesses the perfect balance to protect the developing embryo from external forces yet is weak enough for the pipping process. Researchers have attempted to quantify these physical attributes to help in developing a thorough understanding of the shell.

Hardness, Young's modulus, strength and fracture toughness have been tested in respect to eggshells. These features are not mutually exclusive and have been measured simultaneously with various techniques. Previous observations made have conflicted with each other. This may be due to the inconsistency of varying equipment and the physiological property of the egg, *e.g.* curvature, which makes certain accurate measurements difficult. In addition, as a biological material, variance among animal, egg, farm and diet can influence these values and create variation. Consequently, the outcome of different research has led to a degree of uncertainty.

2.6.1 Strength

Strength analysis is the most studied property of eggshell. There have been many attempts to accurately evaluate the characteristic strength of eggshell of various species. The objective of this is to understandably attempt to identify the stress which the eggshell can withstand. This knowledge can be translated to industry and farming where egg loss due to failure is an ongoing issue. Many studies attempt to quantify the strength of eggshell through similar methods. This method being contact loading tests. In 1967, Voisey and Hunt designed an experimental procedure which measured the maximum force applied on impact of the shells. This resulted in the hypothesis that compression testing may only be performed by using a steady and reasonable speed in order to generate any eggshell strength measurements. It was later discovered that deformation is point specific when placed under static load, in that when placed between two plates failure occurred at the point of contact (Carter, 1970). This contributed to future research on crack propagation in eggshells. This was also useful in industry as the most common force endured by the egg is static compression (Nedomová *et al.*, 2009).

Under axial compression, tensile forces generated radially are understood to result in material failure. This has been proposed by Hahn *et al.* (2017), who performed mechanical tests on a variety of species (quail, chicken, goose and ostrich). Axial compression tests under plate loading were performed by this team. A similar test was carried out by Nedomová *et al.* (2014) which also used plate loading, as depicted in **Figure 2.15.**

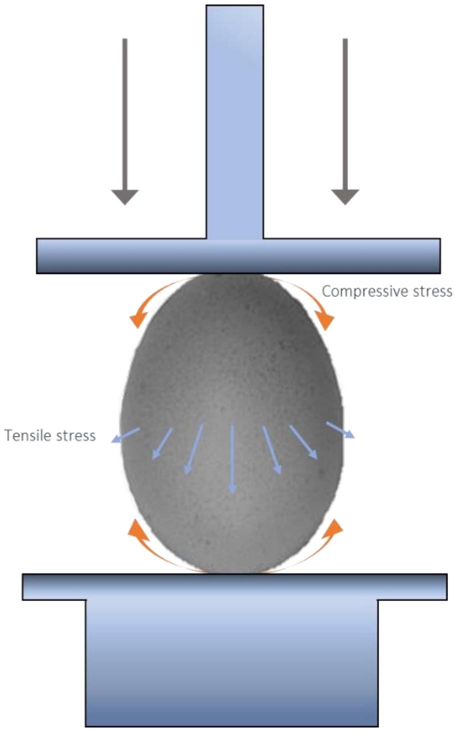


Figure 2.15 Diagram of axial loading of egg between two plates. Tensile stresses along the equator form as a result of compressive forces at each pole from the plates (project image).

The results obtained by Hahn and team (2017) indicated that failure strength obtained on a variety of chicken types ranged from 21.9- 30.9 MPa using whole egg compression. The team also tested eggs using distributed loading tests whereby there was a 'holder' for the egg at both poles. The authors discuss how this method creates a more even distribution of stress around the shell.

Using this distributed load method, it is noted that the cracks appear from the equator of the egg only. Whereas commonly during point load, the eggs fail at the contact point. This suggests that the distributed load test is more consistent as it measures tensile failure only, rather than mixed mode where stress distribution is difficult to determine. Macleod *et al.,* (2006) attained a strength of ~17 MPa of hen eggs which were measured just after oviposition and a lower value of ~14 MPa when measured at retail outlet. The reason for this change in strength was given as a result of microdefects gathering within the shell during handing, a weaker eggshell is generated. Failure by means of internal pressure catalogued as strength value of 15 MPa (Entwistle and Reddy, 1996). As the results for strength are similar for different techniques, it indicates that the simple compression testing set up is a reliable way of achieving strength of the egg.

There are other tests performed to attain strength values of eggshell. Tanaka *et al.*, (2020) performed flexural strength analysis on emu eggshell (*Dromaius novaehollandiae*). The study was interested in observing the anisotropic property of eggshell. Sections of the egg were mounted onto an *in-situ* bending stage. The team observed lower strength when sections of the shell were tested outwardly (15 MPa), this was when the cracks initiate on the inside of the egg and grow towards the outside. There were higher strength values observed when the shell was tested in an inward direction (24 MPa and 20 MPa, when tested longitudinal and latitudinal orientation, respectively). **Figure 2.16**(a) shows images of latitudinal and longitudinal orientation of the eggshell and **Figure 2.16**(b) depicts the flexural strength testing. The authors comment on the anisotropy of eggshell being as a result of the hatching bird easily escaping from the egg whilst the shell ensured protection from external forces during embryotic development. This is an evolutionary explanation for the difference in strength values across the different orientations, but is not a conclusive reason. It could be as a result of the crystal structure of the eggshell at the mammillary layer. Perhaps cracks can be initiated between mammillary cones which allows for easier initiation of crack production compared to initiation at the compact vertical crystal layer at the outer portion of the shell. Or perhaps there is greater distribution of pores towards the inside of the egg compared to the outer portion. None of this is confirmed, but is based on the morphology of the shell. It would be interesting to tests these theories in the future to identify the reason for changes in strength across the eggshell.

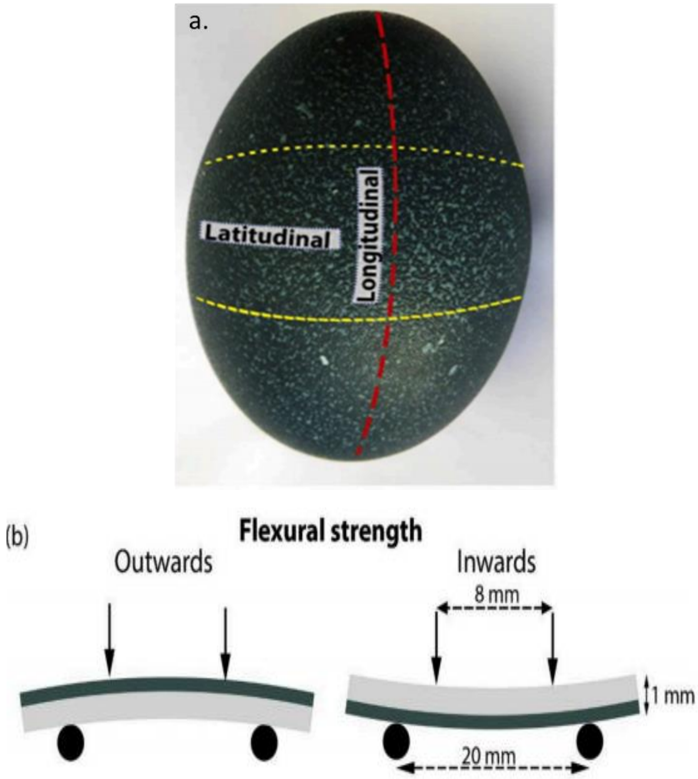


Figure 2.16(a) Image of emu egg with red lines showing longitudinal and yellow lines showing latitudinal sections. (b) Diagram of flexural strength of outwards and inwards testing of emu eggshell (Tanaka *et al.,* 2020).

Stress measurements which used loading tests were obtained through various methods. Hahn *et al.* (2017) proposes several ways in which stresses can be measured using the egg cups in place to distribute stress. The team proposes a method of obtaining the hoop stress of the egg but assume a spherical shape, as opposed to a prolate spheroid which is a more accurate representation of the shell geometries. This is presented in *Eq.2.1*.

Eq.2.1

$$\text{Hoop stress} \qquad \sigma_{\theta} = \frac{N_{\theta}}{t} = \frac{N_{\theta}}{R\,ext(1-\rho)}$$

Where N_{θ} is force per unit length, ρ is thickness ratio ($\rho = R_{int}/R_{ext} = 1-t/R_{ext}$; where R_{int} is internal radius and R_{ext} is external radius) and t is shell thickness. This calculation is further developed to take into account the egg cup in *Eq.2.2*.

Eq.2.2

$$N_\theta = \frac{P_r\, sin^2\alpha}{2\, sin^2\emptyset} \quad if\ \alpha < \emptyset \leq \frac{\pi}{2}$$

Where normal pressure P_r= Force/surface area of cup, α angle subtended from the centre of the egg to the end of the cup and $\emptyset$ the short radius of the egg. This equation assumes even hoop stress throughout the eggshell, which is not what the team presented from FEA analysis. Taylor *et al*. (2016) uses a similar test rig to Hahn *et al.* (2017) and calculates the tensile stress in a simpler fashion (see *Eq.2.3*).

Eq.2.3

$$\sigma_t = \frac{F}{2\pi bt}$$

Where F is the maximum force, b is the egg equatorial radius and t is the shell thickness. This calculation also assumes a spherical shape for the egg but does not factor in egg cup dimensions. It also assumes that the tensile stresses at the equator of the egg during this load account for all of the stresses applied to the egg, which may not be completely accurate. It would be interesting to see a stress value obtained by eggshell using the correct geometric factors (*i.e.,* prolate spheroid) and the inclusion of the stress distribution egg cups.

Commercial interest into strength testing has been ongoing in the 21st century. Technologies have been designed to measure eggshell quality specifically, such as the 'Egg Force Reader'. Many of these technologies are commonplace on farms and in egg production sites around the world. These tests are non-destructive and the eggs can continue on the supply chain after being tested. The tests operate similar to the plate load test. There is a guide provided by the manufacturers with a minimum force measurement which recommends a good quality egg should withstand. For example, the company Orka, produce an 'Egg Force Reader', states a healthy egg can withstand a force of 3.5 kg.f (34.3 N) or above. Eggs that can withstand this force are considered to have a shell which can withstand processing and transportation (Mikesell, 2021). These tests are considered adequate and are used regularly. To note, many of these force readers advertise their technology to provide strength values for eggs. This is not entirely accurate, as the only values given are force, not strength. It can of course be used to obtain strength values once coupled with the appropriate geometric measurements.

In addition to the incorrect advertisement of strength, differing egg sizes does not seem to be included in the standard force measurement that the manufacturers consider a 'healthy shell'. If the eggshell is considerably small, which can be caused by different factors such as species, the force may not be representative of the overall quality of the egg itself. This is an issue with single force requirements on a material that has many variations. One standard force measurement may not be fully representative of all eggshell sample quality. Despite these limitations, various research teams have found this device useful in the calculation of strength values and so the testing itself can be useful upon application. In addition, for functional purposes the values given as a 'healthy shell' can be useful cut off to the producer to indicate the likelihood of fracture.

Strength analysis has become an integral part of eggshell quality analysis. Researchers have kept a similar egg load model to attain stress data. The understanding of stress distribution using this load model has provided the poultry world with a better insight into the egg behaviour. Although, there is certainly more studies needed to assess loading onto different sites along the shell similar to that of Tanaka and team (2020).

2.6.2 Elasticity and Hardness

Studies on eggshell stiffness and hardness have been conducted over the years. Young's modulus was deduced by several research teams using a variety of methods which ultimately resulted in a range of values. Pure calcite has a Young's modulus value of 72 GPa (Crystran Ltd., 2012). Considering this value, despite the variance in results, the literature has shown lower Young's modulus values to that of pure calcite. A lower Young's modulus would translate to increasing flexibility of a material.

Research performed by Macleod *et al.,* (2006) on hen eggshells achieved a mean Young's modulus value of 55 GPa. The research team performed tests which included: stiffness measurements by whole egg compression between two plates; the relationship between applied load and the local hoop and meridional strains, which was measured by a strain gauge rosette placed on the egg's equator. The data presented by these experiments expressed that there were differences in Young's modulus values between polar and equatorial loading. Polar Young's modulus values were lower than equatorial Young's modulus values in the compression and strain gauge tests. The reason given was that the potential differences of spatial and microstructural difference *i.e.,* porosity and crystallinity.

This was not concluded from this research. However, other research has claimed that the microstructure of the eggshell may also impact on physical characteristics (García-Ruiz, Navarro and Kälin, 1995). Severa *et al.,* (2010) examined the Young's modulus of hen eggshells at the various regions on the shell using nanoindentation. Although Young's modulus was observed to vary with location along the eggshell, that difference was not found to be significant (47.4- 53 GPa). It was discussed that the reason for heterogeneity among Young's modulus values from the eggshell at different locations may be as a result of the microstructure, which was aligned with a similar discussion given by Macleod (2006) and García-Ruiz (1995). Nedomová *et al.,* (2009) obtained similar Young's modulus values to the aforementioned papers. Using load compression analysis, this research team concluded the Young's modulus value of hen eggshells was approximately 39 GPa.

Performing whole egg compression test, Hahn *et al.,* (2017) claimed that the Young's modulus value of hen eggshell was 27.5 GPa. Vibrational analysis measuring resonant frequency gave a lower Young's modulus value of 28.55 GPa, which is closer to the results obtained by Hahn (Kemps *et al.*, 2004).

Despite a variance among quantifying Young's modulus, eggshell still holds a higher value than most biological materials, even higher than the modulus of cortical bone, which measures 18.6 GPa (Rho, Ashman and Turner, 1993).

The differences in Young's modulus values among researchers may be as a result of influences on the eggshell or the measurement analysis itself, or both. It would be of interest to obtain results from a future study which assesses Young's modulus values using a controlled study considering other variables *i.e.,* bird age and species. Perhaps for the purpose of this material property, a larger in-depth study should be executed to provide a more accurate range of values. The nature of the eggshell being a natural bioceramic can also be suggested as a factor for result variation. Results can change from sample to sample depending on a multitude of factors relating to the bird *e.g.* age, diet and environment. Despite the variation in Young's modulus results, the question which needs to be considered is how is this related to the quality of the eggshell? Are eggshell with greater stiffness less likely to fracture? Or perhaps other factors such as micro-cracks and pores play a role. In any case, there is a not a sufficient amount of knowledge on stiffness and its relationship with eggshell. Future analysis of this could be beneficial.

Hardness assessment of eggshell has been performed *via* indentation. Several research teams have published analogous hardness results of hen eggshells. Micro-indentation

(Vickers hardness testing) of eggshell was completed by Tung *et al*., (1968) on the cross section of eggshell. The team observed that removal of the membrane made no difference to hardness of the shell. The study also observed that hardness values increased at the outer and inner portions of the shell compared with the middle section. There were two explanations given by the authors for this observation. This was that an increase in magnesium levels found on the outer portion of the shell may cause increased hardness and that the inner mammillary layer has smaller crystals and therefore, grain size is decreased which can cause increased hardness. A 2011 study by Igic *et al*. again performed microhardness testing on brood parasite eggshell. Brood parasite birds lay their eggs into the nest of others and essentially utilise the birds nest to facilitate the incubation and eventual feeding of their own offspring (Croston and Hauber, 2010).

Studies have shown that eggshell from brood parasite birds has increased ability to resist breaking by means of pecking compared to counterpart host eggshell as a result of shell thickness (Antonov *et al.*, 2009). Although this does not relate to hardness of the shell, it is still an interesting structural change observed from these species of bird. This increase in thickness thought to be an evolutionary process whereby host birds cannot reject the eggs of brood parasite bird eggs (Antonov *et al.*, 2006).

The analysis intended to assess any differences in hardness amongst brood parasite eggshell (Cuckoo) with the hardness of the bird 'hosts' eggs. The study observed that eggs from brood parasites had increased Vicker's hardness compared to host eggshell. These studies illustrate that microhardness tests are relevant when assessing eggshell behaviour.

Furthermore, nanoindentation tests were conducted and achieved hardness results in addition to Young's modulus results. Values between 2-3 GPa have been published to describe eggshell hardness which was attained by means of nanoindentation (Ramirez, 2011) (Athanasiadou *et al.*, 2018). Athanasiadou *et al*., (2018) observed higher hardness values in the VCL and the mammillary layer compared with the palisade. As the VCL is the outer part of the shell, this suggest that the outer portion of the shell is more resistant to external forces compared to the inner section. The authors observed a correlation between hardness and nanostructure within the outer VCL and upper palisade area. It was suggested that the relationship between hardness and subunit size of a nanocrystalline material adheres to the Hall-Petch relationship and is applicable in this instance. Hardness in a ceramic can be seen to 'increase with decrease of subunit size' (Athanasiadou *et al.*, 2018). The suggestion given demonstrates that the nanostructure of the outer portion of the shell impacts the properties of the material. However, this theory did not align with increase in hardness in the inner portion of the shell *i.e.,* mammillary layer. The research suggests that the reason for the mammillary layer to exhibit an increase in hardness could be as a result of less structural homogeneity or the elemental composition may play a role (Chien *et al.*, 2008).

Pure calcite was presented to hold a hardness value of 5-5.5 GPa (Lee *et al*., 2016). The decrease in hardness of eggshell compared to pure calcite, like Young's modulus results, may be as a result of the microstructural or organic components within it. The use of indentation to assess properties of the shell does prove useful. However, the association of these results to 'real life' needs to be further analysed.

2.6.3 Fracture toughness

If a material possesses a defect, a significantly lower amount of stress would be needed to initiate failure. This material property measurement can be considered important, as crack propagation is one of the most important physical properties. Due to the biological nature of eggshell, the probability of it possessing pre-existing microscopic defects is high.

Eggshell behaves like a ceramic, in that there is little to no plastic deformation upon the application of stress and it is stiff. The material does behave somewhat differently to most biomaterials in that it has a high Young's modulus and low fracture toughness (Taylor *et al*., 2016). These properties provide the ideal material to house a developing chick which requires physical protection but it is then required to break when the developed bird finally hatches free. These unique biomaterial characteristics can be visualised in **Figure 2.17.**

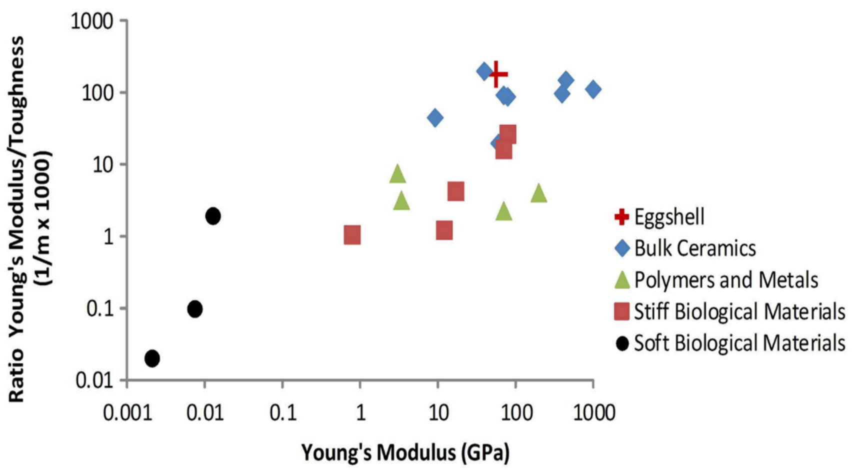


Figure 2.17 Plot adapted from Taylor *et al*., 2016. Ratio of E/K_c *vs* E for various materials (function of E plotted) where E is Young's modulus.

Obtaining a value for fracture toughness of eggshell proves difficult because of its shape. Local stresses change when encountering a crack tip. Two ways in which fracture toughness can be measured are the energy required to propagate a crack (G_c) and as a parameter of the stress required to propagate a crack of a given length (K_{Ic}). They are related to each other by Young's modulus. This calculation is a component of linear elastic fracture mechanics (LEFM). Both of these measurements are combined from Janssen *et al*., (2005) to give a derivation of:

Eq. 2.4

$$K_{Ic} = \sqrt{EGc}$$

Fracture toughness values of hen eggs have been observed through various methods. Different research teams have presented conflicting K_{Ic} values. Research teams obtained a K_{Ic} measurement of eggshell, however these values range from 0.3 – 12.6 MPa√m, indicating a large degree of variance amongst analysis (Taylor *et al.*, 2016) (Xiao *et al.*, 2014) (Mabe *et al.*, 2003).

Mabe *et al*., (2003) performed fracture toughness analysis on whole hen eggs by placing the egg between two plates and analysed the propagated cracks. The team did not perform physical fracture toughness tests, there was no preformed crack introduced to the eggshell and therefore no intentional surface flaws of known dimensions were present. The authors obtained the K_{Ic} by using a calculation derived by Bain (1999). The following formula was used:

Eq. 2.5

$$K_{Ic} = K_{nd}\left(\frac{F}{T}^{2/3}\right)$$

Mabe *et al*., (2003) equation for fracture toughness which was extracted from Bain (1999). Where, K_{nd} is stress intensity factor, F is breaking strength (N) and T is thickness of shell. To

Note: K_{nd} is taken as 0.77 (2.388 + (2.9934(6/R)), R is the radius of curvature (mm). It is unclear if the values used here have been derived from experimentation or from Bain (1999).

Xiao *et al*. (2014) used the very same methods as Mabe and team (2003), including calculations. Both Xiao and Mabe's group obtained a K_{Ic} value of 11.1 MPa√m and 12.6 MPa√m, respectively. The fracture toughness values obtained by these two teams are quite high considering the value of pure calcite is 0.2-1.8 MPa√m and that the fracture toughness of industrial ceramics rarely exceeds 10 MPa√m (Lv, Jiang and Zhang, 2015) (Vavro and Soucek, 2013). Because the results of both of these papers are higher than expected, the values should be considered. Both tests did not introduce preformed cracks into the sample and used an equation to attempt to extract the K_{Ic}. Perhaps, this was the reason for obtaining such a high value.

Zhang and team (2017) attempted to achieve fracture toughness values for hen eggshell which were undergoing dietary changes. The team performed calculations from the same equation as Mabe *et al*., (2003). The result reported was ~346 $N/mm^{2/3}$. This was an average taken from the different eggshells receiving different diets. When converted, this achieved a K_{Ic} value of 10.9 MPa√m. Similarly, Zhang *et al*., (2022) performed the same calculation for fracture toughness on duck eggshell and reported a value of 512 $N/mm^{2/3}$. When converted, this equates to 16.189 MPa√m. It is clear that these fracture toughness result seems quite high for any ceramic and that the calculations being performed here are not achieving accurate fracture toughness results.

In a 2016 paper, Taylor and team conducted fracture toughness analysis which resulted in a K_{Ic} that contradicted previous results. This research estimated that the fracture toughness of hen eggshell was 0.3 MPa√m. The results of their experimentation fall within a range similar to that of pure mineral calcite: 0.2-1.8 MPa√m (Vavro and Soucek, 2013) (Lv, Jiang and Zhang, 2015). Taylor's team devised a test to apply an axial compression load whereby the applied stress was not localised to a small load area, unlike previous compressive analysis which created high local stress where the compressive plates contacted the eggshell (Mabe *et al.*, 2003) (Xiao *et al.*, 2014). The experimental set up details that the stresses are distributed along the equator of the shell. The distribution of stress during the application of axial loading was applied later verified by Hahn and team (2017). However, the team reported that the highest stresses occur just below the contact loading area, not directly at the equator. This analysis was performed computationally. Perhaps the distribution of these stresses are dependent on the size of the cup which the egg sits in during loading.

In 2020, Tanaka *et al*., conducted fracture toughness testing of emu eggshell. The testing was performed on different sections of the shell (see **Figure 2.18**). The team applied preformed notches to different parts of the shell (latitudinal, longitudinal (see previous **Figure 2.16**) and radial sections, which is the cross section of the egg) using a single edge V notch beam (SEVNB) method. Results from the latitudinal and longitudinal sections presented with a value of 0.3 MPa√m. However, the test performed on the radial direction were considerably lower (0.06 MPa√m).

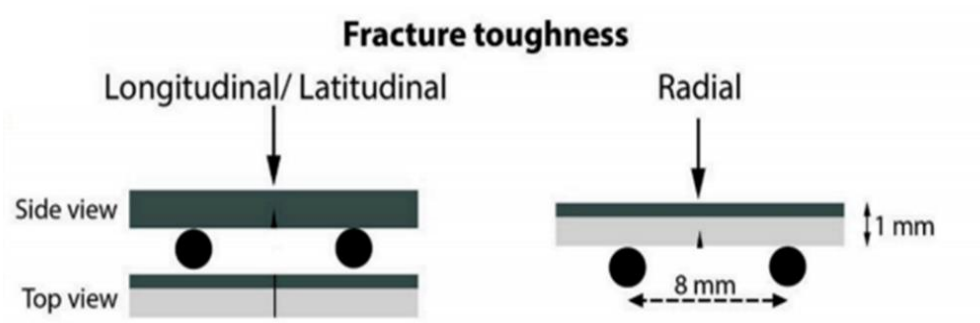


Figure 2.18 Schematic depicts the notches on eggshell from Tanaka *et al*., (2020)

According to the authors, the difference observed in fracture toughness for the shells tested radially was understood to be associated with the separation of crystals mammillary layer which are organised in a similar direction. It was suspected that the decrease in fracture toughness in this layer was directly related to the evolutionary process of the hatchling birds breaking free from the shell. The material behaved in a manner which allowed for internal forces *i.e.,* the hatchling to break out of the shell relatively easily, compared to external forces disrupting the shell.

Both Taylor (2016) and Tanaka (2020) both relay similar K_{Ic} results despite using different testing methods. The observations also align with the measurements of that of pure calcite. There were obvious differences. The teams examined different species of birds and focused on different parts of the shell. It would be of interest to perform a cross species analysis of eggshell using these test methods and examine differences observed. Fracture toughness measurements are especially important to in the area of eggshell as the main issue within the poultry industry is egg fracture.

As the shell naturally contains pores and defects, greater understanding this property and developing research in this area could be of great importance to the overall development of reducing failure in eggshell which is a great burden to the industry. In addition to this, the production of a ceramic material in low temperature environment may give way to a more sustainable or natural production of ceramics in the future. Understanding the fracture mechanics of eggshell could benefit in understanding the fundamentals of bioceramic production which could be translated into other ceramic manufacturing industries.

2.6.4 Theoretical Analysis

The material properties of the eggshell indicate that it behaves like a ceramic and fails through brittle fracture. Due to the biological production of eggshell, variation between samples is high. This variation leads to difficulty in gaining a tight distribution of results. A wide range of innate flaws within the shell can be present. This distribution of flaws can lead to distribution of mechanical test results (*i.e.,* strength). As a result of these variations, there has been increased interest into research of failure probability analysis. Computational methods for theoretical probability analysis have been performed to examine eggshell failure. Although, most of these have been conducted in tandem with physical experimental techniques.

Hahn and team (2017) performed finite element analysis (FEA) to incorporate the applied compressive forces, which was obtained through experimental methods and, from this, attempted to obtain the tensile stress at failure. The team explored the integration of FEA with experimental methods to obtain strength data for eggshell. Entwistle and Reddy (1996) tested the accuracy of using probability methods by comparing their strength results from physical experimentation with predicted values. The strength values were obtained by increasing internal pressure of the egg until failure occurred and by plate loading tests. The team calculated stress distribution on the eggshell (Entwistle, Silyn-Roberts and Ochieng Abuodha, 1995) (Entwistle and Reddy 1996) using the following statistical method:

Eq. 2.6

$$\sigma_p = \bar{\sigma}^{m}{}_{f} A_E$$

Where σ_p is the predicted strength, A_E is effective area of the egg, $\bar{\sigma}_f$ is the average strength obtained through experimental methods and m the Weibull modulus. The Weibull modulus was obtained by plotting a Weibull distribution curve from the experimental results (Weibull, 1951). By using this method of calculation, the team deduced that the average strength prediction value obtained from the loading test would be 12.6 MPa, the values they obtained from the actual study was reported to be 15.2 MPa. The authors claim that the differences in results are sufficient to assume that the predicted calculation model operated sufficiently and that both methods captured a similar overall strength value.

Weibull modulus is a parameter extracted from a Weibull distribution model which is obtained by scatter in strength data of a brittle material (Afferrante, Ciavarella and Valenza, 2006). Eq.2.7 displays the calculation for Weibull modulus. This dimensionless parameter is beneficial as it provides an understanding into the probability of failure compared to physical experimentations which may not provide significant result variation.

Eq.2.7

$$Pf(\sigma) = 1 - exp\{ -(\frac{\sigma}{\sigma_0})^m \}$$

Where $Pf(\sigma)$ is the cumulative probability of failure below a stress σ, σ_0 is a constant with the dimensions of stress, and m is the dimensionless Weibull modulus.

Traditional ceramics (*i.e.,* clay) hold Weibull moduli below 3. Technical or engineered ceramics typically fall between 5-10 (Meyers and Chawla, 2008). Weibull modulus of emu eggshell was obtained by Tanaka *et al*., (2020). The team could fit flexural strength data on to a distribution using FEA software. The value enabled the team to conduct failure probability analysis on the various orientation tests performed (inwards and outwards) testing of the eggshell. The team achieved Weibull modulus values of between 7.2 – 11.5, depending on the loading orientations.

Table 2.1 *Weibull modulus values for materials* (Meyers and Chawla, 2009)

Material	*Weibull modulus*
Traditional ceramics (chalk, clay)	<3
Engineered ceramics	5-10
Metals	90-100

Strength measurements are seen to be proportional to the function $V_s^{1/m}$, whereby V_s is the volume of a material under an applied stress and m is the Weibull modulus obtained from scatter analysis of measured strength data. This value is not determined as a constant due to several factors; surface area and volume expectedly leads to variations as there will be

less defects in a smaller amount of material; the dependence on variable values *e.g.,* crack length, which will be individual in each case. Nonetheless, the Weibull modulus obtained from multiple research teams of eggshell was presented to be between 5.5 – 11.5 (Macleod, Bain and Hancock, 2006) (Entwistle and Reddy, 1996) (Hahn *et al.*, 2017) (Macleod, Bain and Hancock, 2006) (Tanaka *et al.,(*2020). These values consider eggshell as a 'technical ceramic'. Taylor *et al.* (2016) discuss the beneficial use of theoretical analysis in determining eggshell failure mechanics, which they feel aligns reasonably with the actual experimental results. The authors proposed the use of the theory of critical distances (TCD) which is a group of methods applied to predict failure. The method is derived from LEFM (Taylor, 2007). The importance of pre-existing cracks plays a huge role in the failure of eggshell and the application of this theory potentially allows for a greater understanding of the behaviour of the eggshell by the nature of the defect *i.e.,* size and shape of crack or notch. By comparing TCD analysis to the actual experimental values obtained, the team observed similar results.
The approach used the Line Method, which is used to predict brittle fracture. This method uses the elastic stress which is located linearly beyond the point of maximum stress in the direction of crack propagation. Here, the point of maximum stress is the pre-formed crack tip. Failure is predicted if the average stress is greater or equal to the tensile stress of a sample of the material 'containing no stress concentration features' (Taylor *et al.,* 2016) (Taylor, 2004). The team examined through this theoretical approach, the strength of the eggshell if the preformed notch was changed (*e.g.,* length and shape). Through the TCD method, the team obtained results which helped identify the theoretical behaviour of the shell if it was subjected to a stress with a flaw. The authors suggest that predictive models could be used to gather mechanical data of the shell successfully.
Theoretical probability analysis has proven to support the experimental observations made by researchers. It can be compared with practical data and support findings obtained.

2.7 Mechanical property influences

Recognising which factors influence the eggshell is necessary, because by doing so it can ensure better egg quality, bird health and produce less egg waste. As eggshell is produced from a biological organism, ideal engineering does not occur. There will undoubtedly be defects and differences from one egg to another. Influences on the bird itself can have consequences on the structural behaviour of the eggshell. Factors such as diet and age of the hen can influence eggshell quality.

2.7.1 Structural influences

The structure of the eggshell may be reflected in its mechanical properties. Researchers have discovered many features within the eggshell which may contribute to the shell performance and quality. There has been an interest in the mammillary layer, which is where shell growth is initiated, and eggshell behaviour. The mammillary cones form on the nucleation site entwined with the membrane on the interior of the shell. Research by Bain (1992) using computational models and quasi-static compression testing observed that the mammillary layer influenced crack propagation and predicted that crack formation occured from this layer. The research also observed cracks within the mammillary layer near mammillary cones which displayed poor morphology. Thus, suggesting that the cracks within the egg were more prominent at areas within the mammillary layer which were poorly formed.

Other research on structural influences which may impact on the physical properties of the eggshell is the crystal arrangement. It has been suggested that the orientation of crystals has an impact in strength. It was proposed that the egg was stronger when crystallinity was misaligned. The author discusses that this is due to the change in grain boundaries, which creates an obstacle for cracks propagation and the crack tip has to move around crystals which requires more energy to propagate the crack (Rodriguez Navarro *et al.*, 2002). The research from Athanasiadou *et al.* (2018) showed the change in hardness across the various layers of the shell through nanoindentation techniques. As mentioned, the team proposed the Hall-Petch theory for the outermost section of the shell (VCL) but could not fully explain why the inner portion of the shell (mammillary layer) also had a higher hardness result than the middle (palisade layer) which displayed the lowest hardness.

Porosity is a factor in mechanical properties of eggshell which perhaps has not been fully examined. Pores could present material flaws within the eggshell. It is likely that voids within the brittle material can impact the mechanical properties (Munz *et al.*, 1986). The void size can impact the failure stress of a material as observed in **Figure 2.19.** It is understood that increased void sizes decreases failure stress of a material (Freim *et al.*, 1996). Therefore, it may be significant to understand void distribution in eggshell in order to thoroughly understand the failure mechanisms.

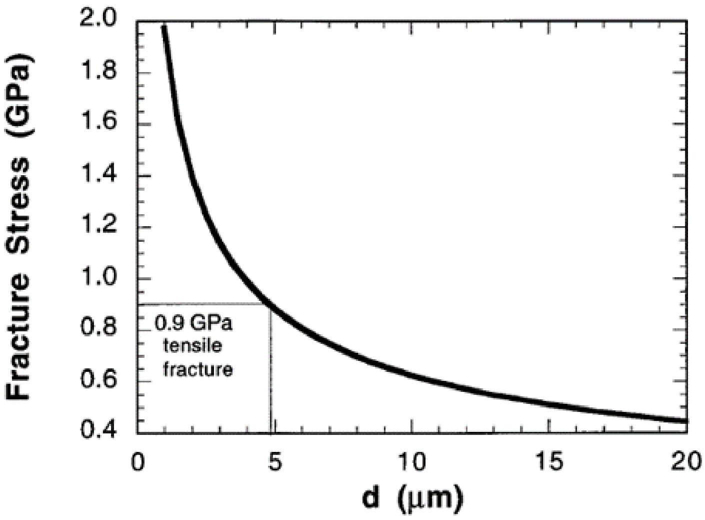


Figure 2.19 Plot of fracture strength against flaw (or pore) size for a material (Freim *et al.,* 1996).

Hahn *et al.* (2017) observed decrease in eggshell strength and a larger distribution of surface pores in quail eggshell compared to other species. However, the porosity analysis of quail eggs in this regard was tested looking at surface voids and potential pores observed from microscopy analysis, but thorough pore analysis was not performed *e.g.,* to identify through pores. It is therefore difficult to conclude any substantial evidence from this research due to inconclusive pore quantification. Nevertheless, the research does leave an open question in relation to porosity which would be interesting to assess in future research.

Crack tip blunting was observed in a study by Salvini, Pandolfelli and Spinelli (2018) (see **Figure 2.20**). It was noted that an increase in external load is required for crack propagation once the crack tip is blunted by a pore, as the stress concentration at the tip decreases (Deng *et al.*, 2004). However, this report is conflicting as in this mechanism where there is a material with many pores, there is less energy required because there are less new surfaces to create and hence, a lower energy for crack propagation.

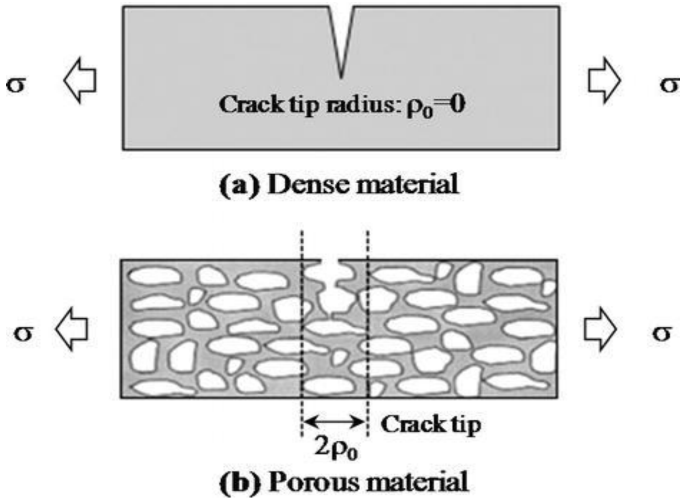


Figure 2.20. Schematic of a (a) dense material and (b) a porous material both under tensile stress (Salvini, Pandolfelli and Spinelli, 2018)

Based on the literature, it is clear that structural factors within the eggshell can are involved in its mechanical properties. Perhaps it is a case that multiple structural factors playing a role in the overall behaviour of the eggshell. There is definite scope for confirming these factors and understanding their roles, if there truly are any.

2.7.2 Organic matrix influence

The organic components of eggshell is thought to influence the eggshell. Consequently, the organic components could play a role in the mechanical profile of the eggshell. Despite the modest concentration of organic material within eggshell, its importance in the mineralisation process is becoming evident.

Osteopontin is one such protein which has been analysed in relation to eggshell properties. As previously mentioned, this glycoprotein is associated with the eggshell formation and it is thought to play a part in the calcification process of the palisade and vertical crystal layer. Athanasiadou *et al.*, (2018) synthesised calcite crystals in the presence of increasing osteopontin concentration. In topographical analysis using atomic force microscopy (AFM), higher levels of osteopontin was seen to induce greater nanostructure within the calcite crystals. The team discovered increased hardness of calcite crystals that were incorporated with osteopontin compared with pure calcite control. In addition, osteopontin was seen to be present mostly in the palisade layer of hen eggshell as seen by immunohistochemistry (Athanasiadou *et al.*, 2018). Other observations within the eggshell were made using colloidal gold labelling and observation on SEM. This research identified occluded osteopontin present on the {104} calcite crystal face, which is thought to be its natural cleavage site. The authors suggest that the protein within this specific face and region may have an influence on the eggshell structure and in turn may also have an effect on the fracture properties during eggshell failure (Hincke *et al.*, 2008). Other proteins within the matrix have not been seen to directly influence mechanical properties. However, their role in crystallization during eggshell formation has shown the importance of proteins within the shell.

GAGs within egg membrane has been suggested to influence eggshell strength. Keratan sulfate has been seen to increase egg strength with an increase in hen eggshell weight (Ha *et al.*, 2007). Bronsch and Diamantstein (1965) saw a correlation between breaking strength and uronic acid concentration, the sugar in present in most GAGs. Chondroitin sulfate concentration was seen to influence hen eggshell breaking strength from a study by Liu *et al.* (2014). However, from this study, no other GAGs were observed to correlate with breaking strength.

There is some degree of difficulty in determining if, and how, organic components influence the eggshell structure and properties. Identifying which role each organic component plays, be it in mineralisation, structure or fracture properties is not without challenges and researchers have struggled throughout the years to thoroughly discern the function of each component within the organic matrix. It could be feasible that the organic matrix within the eggshell adopts a structural role, much like that of osteons in bone or the organic matrix in nacre.

2.7.3 Age

There have been many claims that eggshell quality decreases as the bird ages. Eggs produced from older birds are larger and the shell is thinner (Lee *et al.*, 2016) (El-Hanoun *et al.*, 2012). These eggs are seen to be more susceptible to damage. Selling eggs of older birds is not feasible due to their delicate nature. As birds age, the commercial interest in their egg produce decreases due to defects and losses. Consequently, at a certain age, the birds are taken off production duty and they themselves are used in feed. However, larger eggs, which come from older birds, are more profitable for producers. As a result of this, producing higher quality eggs from older birds is commercially desirable. This has caused producers to seek out ways to improve animal health to ensure maximum supply of larger, better quality eggs. It should be noted that despite the various claims of eggshell quality decreasing as the bird ages, a question which does not seem to be answered is this: do eggshell mechanical properties change as the bird ages, or does the shell remain the same but become thinner? Despite this question, various explanations have been proposed to determine the change in eggshell from ageing birds. Calcium regulation and histological changes in the oviduct are thought to be linked to the change in eggshell over time. The efficiency of calcium reuptake within the kidney is impacted due to the depletion of a regulatory molecule 1,25-dihydroxycholecalciferol (Joyner, Peddie and Taylor, 1987). This renal molecule regulates calcium reabsorption within the kidneys of birds (Kenny, 1976). The decrease in 1,25-dihydroxycholecalciferol concentration within the kidney of hens is thought to impact the concentration levels of calcium within the ageing bird.

As reported by Rodriguez Navarro *et al.* (2002), hens aged 58 weeks had less than half the breaking 'strength' of eggshells produced from hens aged 30 weeks. The authors claim to eliminate the difference in egg size the eggs were weighed from the two groups. The authors also present breaking strength in kg, which is not representable as a stress, only as a force. This can be visualised in **Figure 2.21**. This was measured using whole intact egg loading. Based on the inaccuracy of the strength claim, it is difficult to conclude that breaking strength does in fact increase with thicker eggs from younger birds. It could simply be related to the increased amount of material rather than being a change in intrinsic shell property.

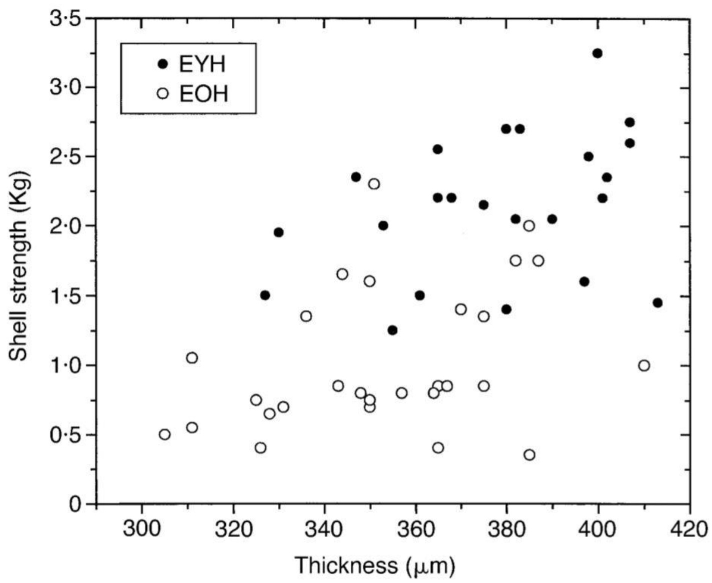


Figure 2.21 Thickness against force values (not 'shell strength', despite being proposed as such) of eggshells from young hens (EYH) and eggshells of older hens (EOH) from Rodriguez-Navarro *et al.* (2002). The data

suggested to the author that eggshells of younger hen were thicker and required a higher load to fracture when tested using loading tests.

In addition to this however, the study did also measure crystallographic properties of the younger and older hen egg groups using XRD. From this same study, microstructural observations on eggshells of older hens compared with younger hens saw an increase in crystal size with hen age using XRD. For younger hen eggs, there was a higher FWHM. The study suggested that nonaligned crystal orientations, related to the FWHM values, may correlate to increased fracture load. The author proposed that this correlation may be due to the cracks being unable to propagate in a direct path. Older hen eggs showed highly orientated crystals that are tilted with their *c*-axis at 44^{o}. Older eggs were seen to possess two crystal orientations; {001} and {104}, compared to crystals in eggs of younger hens having mainly one crystal orientation, {001}. Suggesting that the {001} orientation is more favourable as crystals in younger hen eggs as increased force was required for failure. However, from the study performed by Hincke *et al*. (2008), which observed occluded osteopontin on the {104} calcite crystal face, the researchers favoured the idea that this enhanced eggshell strength. This contradicts the data presented by Rodriguez-Navarro and team which suggest the {001} orientations are favourable for eggshell breaking force. Due to the difference in force measurements and lack of definitive strength data from this study, it is difficult to fully acknowledge the increase in breaking strength being anything other than an increase in thickness. However, the changes in crystallographic XRD results do suggest that there may be a structural difference between younger and older hens which could influence failure.

A change in morphology in the organic matrix was seen by Fraser *et al.* (1998) as the hen aged. An increase in microstructural voids within the palisade layer were observed using TEM. The authors suggest that the increase in voids which hold part of the organic matrix may result in a weaker shell. The organic matrix has been linked to crystal orientation and eggshell organisation. Morphological changes observed in ageing hen eggshell propose the potential organic matrix involvement in the quality decline of the eggshell.

Like any biological organism, changes occur within the bird's reproductive system. The fibrosis, atrophy and deterioration of microvilli of endometrial cells have been seen to occur within the uterus as a result of age (Park and Sohn, 2018). Larger eggs from older birds spend longer developing within the oviduct. This may be due to the lower concentration of calcium, or that the endometrial cells within the oviduct are not operating as efficiently as they had previously (Melek, Morris and Jennings, 1973). As the endometrial cells deteriorate with age, so too do the eggs produced. Telomeric DNA was seen to decrease in endometrial cells of aging hens and the length of the endometrial cilia increased (Park, 2018) (Woudstra and Thomson, 2002). This implies that over time the bird's ability for natural regeneration deteriorated.

A study performed by Park and Sohn (2018) saw that with increasing age, levels of Ca^{2+} and S^{2-} decreased in the bird. These ions are usually seen in high concentrations in the oviduct during the crystallisation process. Decrease in concentration may impact on eggshell crystallization. The researchers also observed a significant decrease in mammillary cone density (see **Figure 2.22**). The decline in calcium concentration is linked to the formation of atypical mammillary cones (An, Kim and An, 2016).

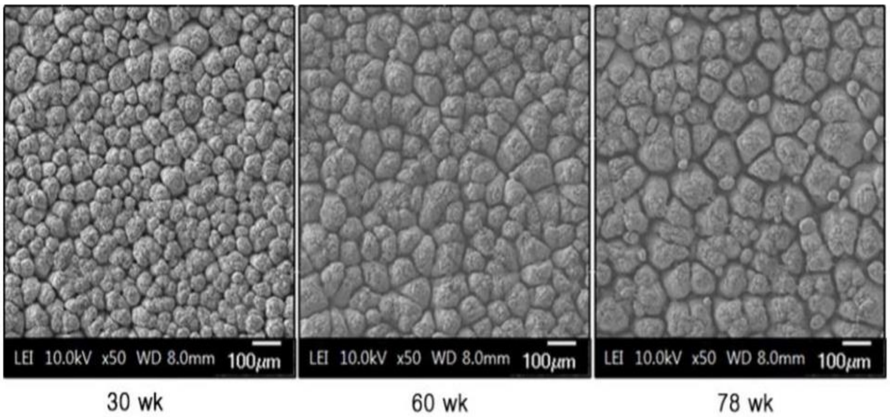


Figure 2.22 SEM micrographs of mammillary cones with the membrane removed of eggshells from aging birds. The authors noted significant differences in mammillary cone density in eggshells from aging hens. The cones appear to get larger (Park and Sohn, 2018).

Many studies have shown that ageing critically influence the oviduct and in turn, eggshell formation. Furthermore, bird age is a challenge for egg producers and stopping the natural physiological deterioration has proven difficult.

2.7.4 Diet and Minerals

Age is seen to influence eggshell formation through defective endometrial cells impacting on ion concentrations within the oviduct. Similarly, bird feed has been important in the poultry industry to ensure birds remain healthy, grow large and produce better quality eggs. Dietary minerals and vitamins have been shown to influence the eggshell.

As the eggshell is made of mostly calcium carbonate, it is correct to assume that calcium in the diet is of considerable importance. Calcium deficiency in the diet of laying birds can lead to the bird recruiting calcium from its medullary bone in order to provide the required amount of calcium for egg formation (Kerschnitzki *et al.*, 2014). The use of calcium from bone is not an ideal source of calcium as it will ultimately impact on bird health (Hartel, 1987). Consequently, sufficient concentrations of the mineral must be incorporated into the daily diet of birds to achieve healthy egg production. Increasing calcium concentration in the diet of aging birds is recommended as it has been proven that it enhances bird health. A study performed by An *et al.* (2016) on layer hens noted that increasing the daily intake of calcium significantly influences the eggshell strength and thickness. There was also a slight increase in tibial breaking strength which would imply that calcium can improve bone health of birds. Calcium intake is dependent on breed of bird and optimum concentrations differ amongst various strains and species (Huang and Lin, 2011). Altogether, calcium is a key element in the formation of eggshells and is essential component in maintaining good egg production and bird health.

There are other dietary components which are beneficial to egg quality. Vitamins and minerals have been seen to influence the overall quality of the egg and have been incorporated into bird feed. Vitamin D_3 is effective in birds and plays as active role in calcium absorption within birds. The liver and the kidney play a considerable role in the metabolism of calcium. Molecules within these organs facilitate the metabolism of calcium whereby it can be utilised by the bird in the shell gland or the bone. The metabolism of Vitamin D within birds can be seen in **Figure 2.23.** 1.25 dihydroxy-cholecalciferol is an important molecule in calcium processing (Nys, 1995) (De Matos, 2008). As previously mentioned, age can have an effect on the concentration of this molecule within the kidney, which can have an impact on bird health and eggshell production.

The incorporation of 1,25 dihydroxy-cholecalciferol, and 25-hydroxycholecalciferol, is common in poultry feed as it has proven to optimise calcium metabolism in the hen which has downstream influence on bird bone health and eggshell, specifically thickness (Bar, Vax and Striem, 1999) (Nascimento *et al.*, 2014). Dietary Vitamin D_3 levels should remain constant throughout the bird's life in order for maximum calcium absorption to occur (Nys, 1999).

Healthy egg production requires an array of compounds integrated into the diet for optimum production. Like most animals, the correct concentrations of nutrients are essential in the diet of birds. Some elements are required in trace quantities and if these elements are ingested in excess, it can be detrimental to the egg and bird health. These include chlorine, sodium and phosphorus (Hughes, 1988) (Nys, 1999).

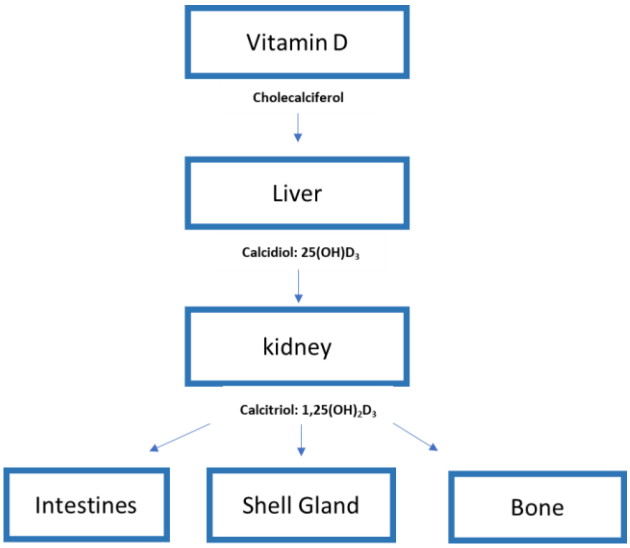


Figure 2.23 The simplified process of vitamin D metabolism in birds (project image).

Mineral elements have been related to eggshell properties. Copper deficiency disrupts lysine-derived cross links which are present in the membrane. This may influence the nucleation sites within the mammillary layer (Chowdhury, 1990). Manganese deficiency has been seen to produce thinner shells and irregular mammillary cones that appeared to have fused together during formation in hen eggshell (Leach and Gross, 1983). Manganese has also been seen to act in the synthesis of GAGs. Alterations to GAG formation may lead to the organic matrix being compromised (Leeson and Summers, 2001). Magnesium is needed in the diet at trace amounts. Anything lower than the required concentration (<0.021%) can result in a magnesium deficiency and minimise egg production rates (Waddell *et al.*, 1989). Kim *et al.*, (2013) saw an increase in hen eggshell strength when concentrations of magnesium in the diet were kept at 3.0 g/kg. This high concentration of magnesium appeared to have no detrimental effect on the bird itself.

There are conflicting results regarding mineral supplementation and its importance in eggshell structure. Stefanello *et al.* (2014) observed an alteration in mammillary cone distribution when a hen received no dietary mineral supplementation. The author observed cluttered and uneven morphology. These mammillary cones had a higher density, which they claim is associated with decreased eggshell strength. However, Rodriguez Navarro *et al.* (2010) did not observe any change in density of mammillary cones in the absence of mineral supplementation. Despite the disputed results, mineral elements are still thought to impact shell quality. Mineral element supplementation is routinely endorsed in poultry feed as an eggshell and bird enhancement.

There has been a recent interest in understanding the exact mechanism of action through which these trace elements can influence the eggshell ultrastructure and properties. Testing of minerals from different sources has been shown to generate various responses in the egg. Minerals that are bound to peptides have become a recent area of interest. Substantial levels of inorganic minerals eaten by the bird are excreted. A research team has proposed that by enhancing the uptake of these trace minerals, eggshell quality may be enforced (Zamani, Rahmani and Pourreza, 2005). It is proposed that the organic trace minerals may influence the eggshell. Increased uptake of trace minerals with an organic chelate compared to unbound inorganic minerals in birds. Stable bonding between the mineral and the organic molecule was seen to aid in the uptake of these products (Murphy, 2018).

There has been an increased attraction to analysing the influence that the organic mineral sources have on the eggshell compared with their inorganic counterparts *i.e.,* oxides, sulphates, carbonates and phosphates (Zamani, Rahmani and Pourreza, 2005). However, there is some disagreement throughout the literature regarding the validity of these proposals; if indeed these two sources (organic and inorganic) impact the eggshell. Mabe *et al.* (2003) found no difference in hen eggshell quality between the organic bound mineral supplement and inorganic bound mineral supplement diets. Zamani *et al*. (2005) suggested that organic minerals influence mechanical properties of the egg significantly. This team used zinc and manganese and combined both into corn-bean diet. The team performed quasi-static load to achieve mechanical results. The team however incorrectly refer to strength in Newton (N); which is a force not a strength measurement. Again, this does not conclusively disclose the intrinsic material property of the shells in the test, as the eggshell may be increasing only in thickness, hence the failure force increases. Another study similarly proposed that organic zinc and manganese were found to influence eggshell strength. Again, the data was measured as resistance force (Kg), not strength and so it is difficult to conclusively suggest that these diet changes impact the mechanical properties of the eggshell (Stefanello *et al.*, 2014).

More research in the area of organic supplementation must occur to conclude any possibility of these bound minerals reigning superior to their inorganic counterparts with respect to eggshell quality. Unfortunately, there are studies which allude to dietary changes causing intrinsic improvements to the shell due to incorrect measurements of 'strength'.

2.8 Literature review conclusion

There are still many mechanisms which are not fully understood and improvements which can be made to understand eggshell properties. As eggs are a biological product, it is difficult to manufacture perfect shells. Likewise, gathering representative data from various studies on samples where there is large variance proves difficult. This review focused on age and diet supplementation as a means to improve eggshell quality. It is unclear from the literature if this has occurred. From this comprehensive review of eggshell, what is obvious is that there is no decisive understanding of the influence various factors has on the shell. Structural, dietary and age influences have not drawn any complete conclusive results. In addition to this, many papers incorrectly confuse eggshell strength with force measurements. This indicates the dire need to thoroughly examine the factors which influence the eggshell without simple errors. The widespread indication that bird age negatively influences eggshell quality, rather than simply quantity (*i.e.,* thickness) is another question which has not been conclusively answered.

Furthermore, advancements in the area of avian eggshell has accelerated in recent years but there are still many unanswered questions. Research performed is almost exclusively associated with hen eggshells. Meagre investigations have commenced on eggshells from other species of bird. As there is a growing demand for eggs consumption from alternative bird species such as duck, there is an increased demand for broader understanding of eggshell from these species.

The complexity of the production of eggs illustrates that more research into external influences must occur to obtain a better grasp on how the poultry industry reduce eggshell waste which has proven such a burden on the industry.

Chapter 3. Methods

3.1 Overview

Information regarding the processes and studies which took place for the duration of the project are summarised in this section. Examinations were performed on eggshells from two sources; 'store purchased' eggs and 'farm supplier' eggs.

The 'store purchased eggs' were used for the optimisation of test procedures and for the analysis of varying egg sizes as advertised (medium, large and very large). Table 3.1.1 relays the tests and sample size used. Eggs are defined by these labels (medium, large and very large) based on weight. Small eggs were unable to be sourced from retailers. The smallest that could be sourced were medium eggs. The size differences correspond with the following measurements; medium (53-63g), large (63-73g) and very large (73g+) (British Lion Eggs, 2021). Eggs purchased from store were from caged birds.

It is of interest to note that there are significant limitations when working with store purchased eggs.

No information is available on the biological or environmental factors under which the eggs where produced *i.e.,* bird age and feed, which may influence results and variability within the study. In addition, the eggs provided would be considered good quality, the defected eggs would not be sold. This could easily skew results. The only variable that is known is the egg weight. This large degree of uncertainty is not ideal. However, obtaining eggs from the same retailer and brand keeps a certain degree of consistency.

Table 3.1.1 *Information and sample number of Size analysis study. This study used store purchased eggs of varying sizes.*

Size	*Fracture toughness n=egg*	*Quasi-static test n=egg*
Medium	*18*	*24*
Large	*21*	*22*
Very Large	*21*	*24*

The farm supplier eggs were used as part of a larger study focusing on bird age and diet and the impacts these factors can have on the eggs. There were several farm supplier studies performed over the entirety of the project. Table 3.1.2 described these studies and the various tests which were carried out. The Age & Diet study 1 was the primary study group in this project. The short duration and limited analysis of some studies (*e.g.* Age & Diet Study 2 and Age & Diet Study 3) were as a result of disruptions due to Covid-19 and an avian influenza. These unprecedented circumstances caused multiple studies to be stopped, and as a results impacted assessing eggshell from birds over time. Each of these studies assessed eggs when birds were given different diets. Organic mineral supplement was given to one group and a generic bird feed given to the other. The organic mineral supplement was referred to as a metal proteinate which is a product resulting from the chelation of soluble salts with amino acids and hydrolysed proteins.

Age & Diet Study 1 was separated out based on different sheds for hens. These eggs were obtained from the same supplier at the same time. There were 4 sheds; *Shed A, Shed B*, *Shed C* & *Shed D*. *Shed A* and *Shed B* consisted of Hyline-plus breed and *Shed C* and *Shed D* were Lohmann classic breed of hens. All of these sheds were operating under the same environmental conditions and, in each of these sheds, the birds were separated out based on diet. Eggs were collected from each of these groups over a period of time.

The remaining Age & Diet studies (2 and 3) were obtained from different farm sources at different times. Each of these remaining studies were similarly separated out according to diet; one group receiving organic mineral supplement feed and the other received generic feed.

Table 3.1.2 *Information on mechanical tests (fracture toughness, quasi-static and nanoindentation) for Age and Diet studies. Diet 'Min' is mineral supplement and 'Gen' is generic feed. The project consisted of three separate Age and Diet studies. Study 1 is split into 4 different subsets based on breed and shed (Shed A Shed B, Shed C & Shed D). Study 2 consisted of duck breeder eggs. Study 3 comprised of hen eggs.*

Study	*Bird*	*Age (weeks)*	*Diet*	*Fracture Toughness n=egg*	*Quasi static n=egg*	*Nanoindentation n=egg i=indents*
Age & Diet	Hen *(A)*	*22*	Min	*12*	*12*	*n=3 i=20*
Study **1**		*22*	Gen	*12*	*12*	*n=3 i=20*
Shed A		*29*	Min	*11*	*12*	*n=3 i=20*
		29	Gen	*12*	*12*	*n=3 i=20*
		35	Min	*12*	*11*	*n=3 i=20*
		35	Gen	*10*	*12*	*n=3 i=20*
		43	Min	*12*	*11*	*n=3 i=20*
		43	Gen	*12*	*11*	*n=3 i=20*
Age & Diet	Hen *(B)*	*22*	Min	*12*	*12*	*n=3 i=20*
Study **1**		*22*	Gen	*11*	*12*	*n=3 i=20*
Shed B		*28*	Min	*12*	*12*	*n=3 i=20*
		28	Gen	*12*	*12*	*n=3 i=20*
		36	Min	*10*	*11*	*n=3 i=20*
		36	Gen	*12*	*11*	*n=3 i=20*
Age & Diet	Hen *(C)*	*22*	Min	*11*	*12*	*n=3 i=20*
Study **1**		*22*	Gen	*12*	*12*	*n=3 i=20*
Shed C		*29*	Min	*12*	*12*	*n=3 i=20*
		29	Gen	*12*	*11*	*n=3 i=20*
		36	Min	*11*	*12*	*n=3 i=20*
		36	Gen	*11*	*12*	*n=3 i=20*
		43	Min	*12*	*12*	*n=3 i=20*
		43	Gen	*12*	*11*	*n=3 i=20*
Age & Diet	Hen *(D)*	*24*	Min	*11*	*12*	*n=3 i=20*
Study **1**		*24*	Gen	*12*	*12*	*n=3 i=20*
Shed D		*35*	Min	*12*	*12*	*n=3 i=20*
		35	Gen	*11*	*12*	*n=3 i=20*
		43	Min	*12*	*11*	*n=3 i=20*
		43	Gen	*11*	*11*	*n=3 i=20*
Age & Diet	Hen	*68*	Min	*0*	*0*	*n=3 i=20*
Study **2**		*68*	Gen	*0*	*0*	*n=3 i=20*
		71	Min	*0*	*0*	*n=4 i=20*
		71	Gen	*0*	*0*	*n=3 i=20*
		74	Min	*0*	*0*	*n=3 i=20*
		74	Gen	*0*	*0*	*n=2 i=20*
		77	Min	*0*	*0*	*n=3 i=20*
		77	Gen	*0*	*0*	*n=3 i=20*
Age & Diet	Duck	*40*	Min	*12*	*12*	*0*
Study **3**		*40*	Gen	*11*	*12*	*0*
		45	Min	*11*	*11*	*0*
		45	Gen	*10*	*12*	*0*

All egg samples were stored at 18-20^{o} C. All samples were weighed and measured on day of collection.

The width was measured from the widest point of the minor axis and height was measured as the widest part of the major axis using digital *Vernier* callipers (Hilka Tools Ltd, Surrey, UK) with an accuracy of 0.02mm. Eggs received from supplier farms were sourced through Alltech® (Alltech Biosciences Centre, Co. Meath, Ireland).

3.2 Loading apparatus

Previous loading methods noted that plate loads created localised stresses at point of contact (Mabe *et al*., 2006) (Xiao *et al*., 2014) (Hahn *et al*., 2017). The theory behind the compressive apparatus used in this project comes firstly from Taylor *et al*. (2016) and was also used by Hahn and team in 2017, whereby an even distribution of stress is applied to the eggshell by way of a cup at both poles. Therefore, the highest stress applied to the egg would not be at the point of contact, but rather be distributed around the egg. Taylor and team (2016) state that a biaxial stress state arises at the equator of a thin walled sphere undergoing axial compression loading. Holders were designed which the egg were placed into. These sample cup holders were used for eggs in both fracture toughness tests and quasi-static tests. Two cups were designed to hold both poles of the egg in place while leaving the central equator region exposed. An average measurement of egg width and height was used for cup dimensions. The cups were 3D-printed (see Figure 3.1). A low stiffness sponge was used which was placed in between the cups and the egg itself in order to aide in the distribution of stresses and minimise any intense contact load stresses. This was made of absorbent cellulose sponge material.

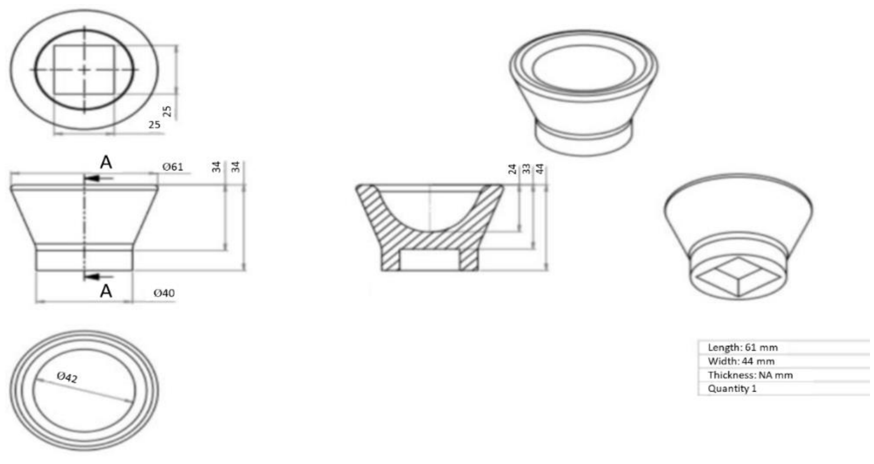


Figure 3.1 Technical drawing of cups that were created for axial compression of eggs (project image).

A Zwick Z5 Universal Test Machine (Hertfordshire, UK) was used for quasi-static and fracture toughness tests. The machine is operated using the testXpert V11.01 software. The machine can be used in compressive and tensile loading. A constant loading rate was achieved which was desirable for the application (Voisey and Hunt, 1969). The force and deformation of the sample can be detected and recorded. The cups used for egg tests were attached to the load cell on the crosshead of the machine. From here, the sample could be loaded and the appropriate settings on the software are applied (See **Figure 3.2**). The load cell is a transducer which converts force into a signal which is quantified (Hernandez, 2006).

The load cell was changed depending on the test. For quasi-static compression and fracture toughness tests, load cells of 5kN and 500N were used, respectfully. The reason for this was to use a load cell which corresponded to the force being applied (see Supplemental Experiment 3.10.2).

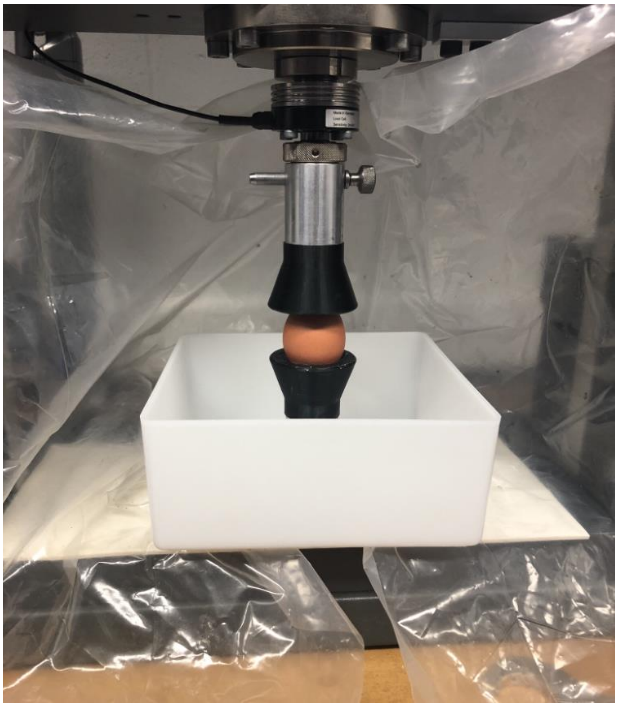


Figure 3.2 Image of loading test set up of the egg. Cups attached to load cell on crosshead visible with egg sample placed in between. Contents of whole eggs were expelled once fracture occurred therefore collection and cleaning of residue occurred as appropriate.

3.3 Imaging

White light microscopy was performed using a Keyence 3D Digital Microscope (Milton Keynes, UK). This microscope operates at high magnification of up to 5000x and provides high resolution images of samples. There is a high-speed auto focus stage which allows for easy adjustments. Notches, cracks and thickness were examined and measured using this microscope. White light microscopy is limited by lens quality and also by the wavelength of light. There is a limit to magnification resolution as a result of this (Lauterbach, 2012). Therefore, increased magnification of images was performed using SEM imaging to assess morphology and examine various layers of the shell. This method is superior to white light microscopy due to the higher magnification and resolution. It is more time consuming and can require sample preparation (*e.g.,* sputter coating). SEM operates by bombarding a sample with high energy electrons under a vacuum. The vacuum is required to minimise the electrons from interacting with molecules present between the sample and the beam (Dunlap and Adaskaveg, 1997). Once the electrons interact with the sample, there can be secondary electrons, backscatter electrons or X-rays produced. These interact with detectors and a high quality image is computed (Pawley and Schatten, 2007).

White regions may appear, known as charging, when the sample is non-conductive and the electrons are essentially trapped in the material. To mitigate against this, low conductivity samples were prepared by applying a thin layer of a conductive material on the surface which allowed for better conductivity of electrons and minimised charging (Kim *et al.*, 2010). When examining the cross section of the eggshell to examine the internal layers, high quality images were obtained without the sputter coating. However, when imaging the surface with the cuticle undisturbed, a coating of gold was applied to the sample as charging was evident.

A voltage of between 2-5kV were used. Higher voltages resulted in excess charging on the images. Working distance of 12-13mm and probe currents of 20-30A were used.

3.4 Fracture toughness testing

The fracture toughness *mode I* stress intensity factor was used. The protocol followed was that described by Taylor *et al*., (2016) which was created to achieve an accurate K^{Ic} value. This method is derived from LEFM (linear elastic fracture mechanics). The nominal stress at the location of the preformed notch was calculated with the assumption that the sample was spherical and that all the stress induced vertically by the test was translated to the horizontal axis at the egg equator.

To calculate stress at failure and fracture toughness (K_{Ic}) the following equations were used, which were adapted from Taylor *et al*. (2016).

Eq. 3.1

$$\sigma_f = \frac{F}{2\pi r t}$$

Where stress at failure is σf; f is force; r is radius and t is thickness.

Eq. 3.2.

$$K_{Ic} = P\sigma f\sqrt{\pi a}$$

Fracture toughness K_{Ic} was obtained using this equation. P is geometric parameter of K_{Ic} (taken to be 1) and a is the preformed notch half length (see **Figure 3.3**). Contents were removed by puncturing two small holes (approximately 1mm in diameter) in each end of the egg (*i.e.,* both poles) with a hypodermal needle (22 gauge). The contents were removed from the punctured holes using compressed air and water. The preformed notch necessary for fracture toughness testing was applied to the equator of the egg. A small hole was placed into the central equator location using a hypodermal needle (22 gauge). Notches on either side of the hole were cut into the eggshell using a scalpel (blade size 11; Swann Morton Sheffield, UK). The notch measured approximately 4mm from tip to tip. **Figure 3.3** illustrates the notches created for fracture toughness testing.

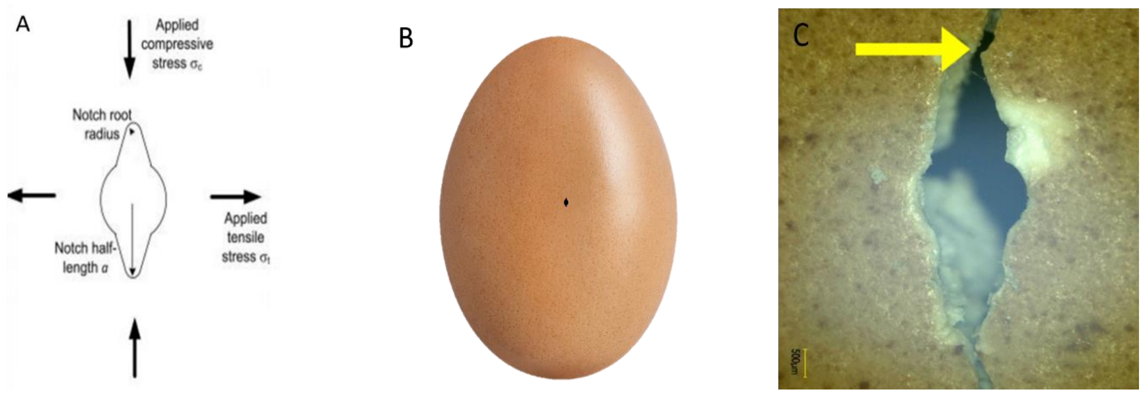


Figure 3.3 (A) Schematic of notch and stresses applied to notch (Taylor *et al*., 2016). (B) Image of hen eggshell with a preformed notch placed along centre of egg. (C)

Digital photograph taken on white light microscope of crack propagation from notch tips.

Eggs were placed into the 3D-printed cups which were attached to the 500N load cell on the Zwick loading machine. A loading rate of 0.5mm/min was used. A pre-load of 30N was performed at a rate of 10mm/min. Failure detection was set at 15%. Eggs were loaded into the cups and tests proceeded. Once a crack occurred, the system was stopped, the crack was inspected, and the force was recorded. The thickness of the shell was then measured using a callipers at the site close to the crack initiation site. See Table 3.1.1 and Table 3.1.2 which illustrate the various studies which underwent fracture toughness along with the sample number for each test.

3.5 Quasi-static test

Tensile strength analysis was performed using a quasi-static compression test. An axial compressive force was applied onto the egg, distributed about the poles, such that the stress field maximum at the equator of the egg consists of zero compressive stress but rather a diametral tensile stress of magnitude that is approximately an equal to the compressive force applied at the poles. The force at failure was measured and the stress at failure could be calculated. The egg was placed into the cup on the Zwick loading machine. As mentioned, cups were used to create a more even load distribution onto the egg. The theoretical understanding of this methodology is that the compressive stresses which are applied to the eggshell were translated into equatorial tensile stresses. This assumes that all stresses are tensile and located at the egg equator, which may not be fully representative. A loading rate of 1 mm/min was used. Once a crack occurred, the test was stopped. Force at failure was recorded and eggshell thickness was measured using a *Vernier* callipers at the site close to the crack initiation site. See Table 3.1.1 and Table 3.1 2 for information on the various groups of eggs that were studied using this test method. Stress was calculated using Eq.3.1. Note that unemptied eggs were used in quasi-static tests. However, a study was completed to assess differences between whole eggs and emptied eggs on quasi-static tests and no significance difference was seen. See Supplemental Experimentation 3.10.3.
Weibull probability analysis of eggshell failure was carried out on the quasi-static compression test results. It was reported that a sample number of at least 30 values is required for the Weibull modulus to be calculated (Quinn and Quinn, 2010). This was performed by gathering stress data and ranking it from lowest to highest. From this, values could be plotted and Weibull modulus

and characteristic strength attained. The Weibull probability function is as follows (Quinn and Quinn, 2010):

Eq. 3.3

$$F=1-\exp\{-(\frac{\sigma}{\sigma_0})^m\}$$

Where F is the probability of failure (see Eq.3.4), σ is stress at failure, σ_0 is the characteristic failure strength and m is the Weibull modulus.
The probability of failure F was achieved using the following equation:

Eq.3.4

$$F= (r - 0.5)/n$$

Where r is the rank number and n is the total number of samples in the distribution. The x axis and y axis is plotted using $ln(\sigma)$ and $ln(ln(1/1-F)))$, respectively. Once plotting the curve, the equation of the line can be determined with the slope indicating the Weibull modulus (Weilbull, 1939):

Eq.3.5

$$y = mx + b$$

as

$$ln(ln(\frac{1}{1-F})) = m\, ln\sigma - m\, ln\sigma_0$$

Where the slope, m, is the Weibull modulus and b is the intercept. The characteristic strength can be determined from this equation in the following manner:

Eq.3.6

$$b = - m\, ln\sigma_0$$

This is further rearranged in terms of σ_0 as the following:

Eq 3.7

$$\sigma_0 = e^{-b/m}$$

From this, the characteristic strength value of the material can be achieved.

In addition, volume and area was factored into this equation to establish the effects that they could have on the overall probability analysis. The introduction of these parameters into this equation is as follows:

Eq 3.8

$$ln(ln(\frac{1}{1-F})\frac{v_0}{V}) = m\ ln\ \sigma - m\ ln\sigma_0$$

Where V_0 is the smallest volume in the cohort.

Eq. 3.9.

$$ln\ (ln\ (\frac{1}{1-F})\frac{Ao}{A}) = m\ ln\ \sigma - m\ ln\sigma_0$$

Where A_0 is smallest area of an egg in the cohort.

3.6 Nanoindentation Test

Nanoindentation was preformed using the Hysitron TI Premier (Bruker, Germany) to assess hardness and stiffness of eggshell. These results were obtained through the methodology used by Oliver and Pharr (1992). This method uses the load-displacement curve from indentation to achieve these parameters. Three measurements were obtained upon the loading and unloading of a sample; the maximum load (P_{max}), the maximum displacement (h_{max}) and the stiffness of elastic unloading ($S=dP/dh$). This method used a diamond Berkovic indenter. The nanoindenter used in the eggshell analysis provided stiffness results by means of reduced modulus. The reduced modulus is the elastic deformation which occurs in both the sample and the indenter tip (Rodríguez, Alcalá and Souza, 2012). The reduced modulus is related to Young's modulus in Eq.3.10. This equation was used in the calculation of Young's modulus from the reduced modulus data provided.

Eq.3.10

$$Er = E/(1-v^2)$$

Equation relating reduced modulus to Young's modulus. Er is reduced modulus, E is Young's modulus, v is the Poisson ratio of the indentor (Kontomaris *et al.*, 2018).

A Poisson ratio value of 0.3 was used in this calculation as this was the result Hahn *et al*., (2017) used for eggshell. The authors used this value as it was representative of a homogenous-isotropic linear elastic material. In addition to this, the Poisson ratio of most materials falls between 0.1 and 0.45. Therefore, the dimensionless value used in the calculation is acceptable as it falls within this range (Belyadi, Fathi and Belyadi, 2019). It should be noted that nanoindentation itself is useful but there are external factors which can influence the result such as roughness, cleanliness and pile-up (Santo and Davim, 2012). The rates used were 20 seconds loading, 5 seconds hold time and 20 seconds unloading. The maximum loading force (Pmax) used for testing was 8000 µN. Therefore, a loading rate of 400 µN/S was used. This Pmax was identified as the optimum load for this test (see Supplemental Experiment 3.10.5).

In-situ scanning probe microscopy was performed to obtain images of indents. The technique utilises the indenter tip probe to scan a designated area and obtain high resolution images of the surface pre-indent and post-indent. The indent can be visualised and assessed, *i.e.,* for fracture or pile up. This process was completed for several indents. The majority of indents were performed using an array setting which performs multiple indents in a selected area and gathers quantifiable data from the sample. 5x4 indents at 1µm apart were examined in the array setting. For *Age & Diet Study 1* (see Table 3.1.2) array indents were performed on three layers of the eggshell: mammillary, palisade and upper palisade layer. Ideally the outermost vertical crystal layer was of interest to examine, however, it was difficult to pick the exact location of indent. Therefore, the closest to the outer portion of the shell was examined through indentation *i.e.,* upper palisade.

Preparation of samples for nanoindentation was required. Samples needed to be as smooth and flat as possible. Sections of the eggshell were broken off and embedded in cold mount epoxy resin by Akasel (Roskilde, Denmark). The eggshell samples were placed in clips to keep them in the correct position within moulds where cold mount resin was poured in (see **Figure 3.4**). These were set for 24 hours at 18-20^{o}C. Once solidified, the embedded samples were removed from the mould where they were ground. The grinding process consisted of using Rhaco Grit silicon carbide (Akasel, Denmark) paper of varying grit sizes (P800, P1200 and P2400). The grinding of samples was followed by polishing using suspension and polishing cloth. Suspensions from MetPrep (Coventry, UK) used in order of decreasing particle size: 9µm, 6 µm, 3 µm, 1 µm diamond solution, followed by colloidal silica 0.06 µm.

Roughness of samples were measured and examined using an optical profiler in addition to the Keyence Digital 3D light microscope (Milton Keynes, UK). Roughness average (RA) was obtained from the Contour GT 3D optical profiler (Bruker, Germany) which offered a quantifiable metrology result. This technique was noncontact and fast. An RA of between 50-80 nm was achieved on the samples.

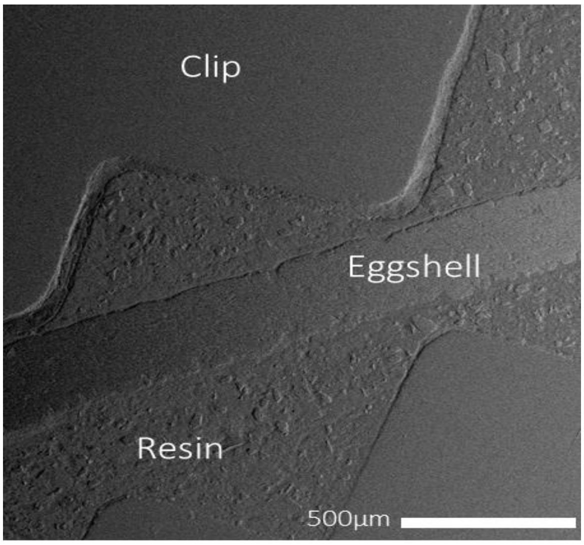


Figure 3.4. SEM micrograph of cross section eggshell embedded in resin (project image).

3.7 Two Dimensional X-ray Diffraction analysis

Two Dimensional X-ray Diffraction analysis or 2D-XRD has been used to identify and quantify eggshell microstructure. The principle of this technique operates by exposing a sample to an X-ray beam. Spacing between atoms act as a diffraction grating. Once the incident beam interacts with atoms in the sample, scattered intensities can be measured using detectors. Spacing between atoms and the angle of incidents of the X-ray beam can interact or 'interfere' with each other. Constructive interference occurs when two waves are in phase and are said to fulfil Braggs law (Bruce, O'Hare and Walton, 2014). Equation for Bragg's law can be seen in Eq. 3.11.

Eq 3.11

$$2d\sin\theta = n\lambda$$

Where d refers to the space between diffracting planes, θ is the incident angle, n in an integer and λ is the wavelength.

When constructive interference occurs, the diffracted beam of X-rays is detected in a form of a reflection spot (Bruce, O'Hare and Walton, 2014). In doing so, crystallographic information can be extracted from the sample, such as phase and orientation. 2D-XRD uses an area detector rather than a point detector and so can gather a more information from the sample. This process is also quite quick (Ahmed *et al.*, 2005). In addition, concentric rings of the diffraction spots, called Debye-Scherrer rings, can illustrate preferential orientation of crystals. These spotted rings can also divulge relative size information of the crystal, if the crystal is larger, the rings will have increased prevalence of spots (Rodriguez-Navarro *et al.*, 2007).

Crystal orientation and size have been associated with eggshell quality. Specifically, researches have suspected that with decrease in orientation and crystal size, it is more difficult for a crack to propagate through. This is believed to be because of the increased number of surfaces and boundaries a crack will encounter which means it requires more energy to travel through the sample (Rodriguez Navarro *et al.*, 2002) (Rodriguez-Navarro *et al.*, 2007). It is to be noted that the authors assume intergranular fracture.

2D-XRD testing was carried out in the Crystallographic Facility, School of Chemistry Trinity College, Dublin, Ireland, under the guidance of Dr Brendan Twamley. The test was performed using the Apex-Duo (Bruker, Germany). The eggs used were from Age & Diet Study 1 *Shed A* (generic feed group), and so there were 4 time points being analysed. From each group (or time point) 5 eggs were examined using transmission setting and 1 egg from each group was also tested in the reflection setting *i.e.,* transmission n=5, reflection n=1. The two types were performed for crystal orientation. As the difference between samples was not expected to be great, only one sample per time point was tested in reflection.

The 2D-XRD technique was modelled on the procedure completed by Rodriguez-Navarro and team (2007). Sample size used was approximately 1x1mm. Transmission parameters were as follows: distance from sample was 60mm, 2Theta 0^o, Omega 0^o, Phi 90^o and Chi 45^o. Changes to parameters for reflection were as follows: Omega 10^o, 2Theta 20^o. The detection of X-rays was recorded and analysed using the software XRD2DScan4.1.1 (Grenada, Spain, 2011). This software was generously provided free of charge by Dr Alejandro Rodriguez-Navarro.

3.8 X-ray micro-computed tomography

X-ray micro-computed tomography (X-ray µCT) is a technique which can be used to assess pores and voids within an eggshell. It operates by imaging a material slice by slice in high resolution in a non-destructive manner. An X-ray is emitted by a micro-focused X-ray tube through the sample. The rays are then detected by a 2D X-ray detector (see **Figure 3.5**). The focus tube shoots X-rays through the sample. The sample can be rotated at 360° where a beam can be directed all around the sample. The 2D detector distinguishes the X-rays as pixels, which can be made into an image. Each pixel can be processed through software through which a 2D and 3D digital image of the sample can be achieved (du Plessis *et al.*, 2017). Both 2D and 3D images can be obtained through this analysis.

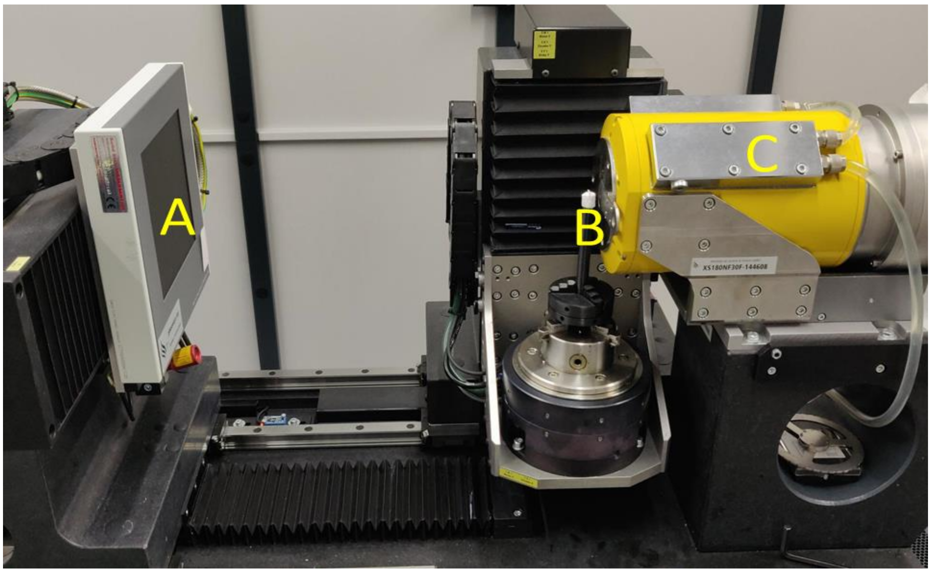


Figure 3.4 *A* is the 2D detector which collects the X-rays as pixels. *B* is the sample mounted. *C* is the X-ray beam source. Image courtesy of Mr Brendan Phelan, SEAM Research Centre, Waterford Institute of Technology.

The objective of this technique was to gather quantitative information on pore and void size and the distribution of these through the eggshell. This test was performed by Mr Brendan Phelan and Dr Ramesh Raghavendra of SEAM (South Eastern Applied Materials) Research centre, Waterford Institute of Technology, Waterford, Ireland. The X-ray µCT equipment used was the Nanotom, Waygate Technologies, Wunstorf, Germany. 3 eggs used were from Age & Diet Study 1 (generic feed group), each from the 4 time points being analysed. The eggshell sample of 1.5 mm^2 size was mounted into polystyrene foam which has very low X-ray absorbance and can be easily separated from the sample in images post processing (Meftah *et al.*, 2019). Once

mounted, the following parameters were followed: voltage 75 kV; current 190 µA; distance from detector FOD 12.44 mm and FDD 400 mm. Approximately 1,500 images per shell (averaging 3 frames per integration and 1 skip image). Software used was as following: Segmentation - Avizo (Thermofisher, France). Volume and thickness analysis - VG Studio Max (Volume Graphics (Hexagon), Germany).
Pore diameter was calculated from data obtained by the X-ray µCT. This was performed using the volume of a cylinder equation:

Eq. 3.12

$$V = \pi r^2 h$$

Where V is volume of the pore, r is radius and h is pore height. This was performed assuming full pore shape was cylindrical, which may not be fully representative of the pore shape. Based off the images acquired, the entrance of the pores appeared to be almost conical in shape (**Figure 4.3.10** in Results section). This variation was not included in this calculation but should be considered when interpreting the results.

3.9 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) is an elemental analysis which provides information on the surface of a material. The surface technique works by irradiating the sample with an X-ray beam and measuring the energy emitted from the atoms on the surface of the sample. This kinetic energy provides valuable information regarding element identification and can be used in the quantification of atomic states on the sample surface (Engelhard, Droubay and Du, 2017). The objective of this technique was to identify elemental composition in the eggshell and specifically attempt to identify this at the various layers of the eggshell.
This technique was performed in the Bernal Institute, University of Limerick, Limerick, Ireland by Dr Fathima Laffir. Before testing, cuticle and membrane removal was performed as per Deeming (1987). This involved washing the eggs in 14.7 mM solution of sodium hypochlorite (*Emplura*) at 45°C for 2-3 minutes and rinsed well with deionised water. For the XPS, the Kratos AXIS ULTRA spectrometer was used. The eggs used were from Age & Diet Study 1 *Shed A* (generic feed group), and so there were 4 time points being analysed. The following parameters were used for the set up: X-ray gun mono Al K 1486.58 eV; 300 W (20 mA, 15kV); Step- 1.0 eV (survey)

and 0.05 eV (regions); Dwell- 50 ms (survey), 100 ms (regions); Sweeps- survey (~3), narrow regions (8-18). The spectra were collected in the normal to the surface direction; XPS detection limit is estimated to be ~0.1 at %. The inside surface and outside surface sections of the egg were measured *i.e* inside-where mammillary cones are and outside where the cuticle is (although cuticle was removed).

3.10 Supplemental Experimentation

Supplemental studies were performed to support and validate primary experiments in the project. Various parameters were unknown and needed to be confirmed by performing analysis which aided in the execution of the primary experiments.

3.10.1 Supplemental Study 1: Loading Rate

The rate at which the load is applied is of importance to eggshell failure as mentioned by Voisey and Hunt (1969). This paper outlined high load rates can effect strain-rate sensitivity of eggshells. To identify which rates would work best for the loading tests in the project, a test was designed to obverse differences in failure of eggshell upon different loading rates. Loading rate analysis was performed for fracture toughness and quasi-static tests.

In the fracture toughness rate analysis and quasi-static load rate analysis, three loading rates were examined: 0.5mm/min, 1mm/min and 2mm/min. Store purchased medium hen eggs (*Ballyfree*) were used in both tests. 6 eggs were used to examine each load rate. These numbers were the same for both fracture toughness (n=6) and quasi-static tests (n=6). Values were recorded and calculated.

3.10.2 Supplemental Study 2: Load cell

When performing tests using the Zwick machine, the load cell is important. The maximum force required for failure of a sample should be lower than the load cell limit. Two load cells were available for the testing machine: 5kN and 500N. With quasi-static tests, the maximum force at failure sometimes exceeded 500 N, therefore the 5kN was the load cell used. Ideally, a lower load cell would be used (~1000N) but this was not available. However, in the case of fracture toughness testing, a lower force at failure occurred (<500 N) and therefore, a lower load cell could be used. This load cell was 500N.

To assess differences in results of fracture toughness of eggshell when using different load cells, an experiment was conducted. Fracture toughness experiments were carried out in accordance to section *3.4 Fracture toughness testing*. 10 samples were tested using the 5kN load cell and 10 samples were tested using the 500N load cell. Store purchased eggs were used (*Ballyfree*). Fracture toughness (K_{Ic}) measurements were calculated and recorded.

3.10.3 Supplemental Study 3: Quasi-static content analysis

A study by Hahn *et al*., (2017) examined the differences in stress of whole eggs and compared it to the stress values of emptied eggs. Emptied eggs had two holes applied to each pole and the contents were removed. Whole eggs remained undisturbed. Upon testing, the team concluded that there was no significant difference in stress of empty eggs compared with whole eggs.
In order to test if emptying the eggs had any impact on the mechanical properties a repeat of the test performed by Hahn and team (2017) was conducted in order to confirm this result. Store purchased size large hen eggs (*Ballyfree*) were used for this test with 12 eggs emptied and 12 eggs untouched. Contents were removed by puncturing two small holes (diameter approximately 1mm) in each end of the egg with a hypodermal needle (22 gauge). The contents were removed from the punctured holes using compressed air and deionised water. Quasi-static tests were completed as per methods *3.5 Quasi-static test*. Values were recorded and stress was calculated.

3.10.4 Supplemental Study 4: Cuticle analysis

The cuticle provides several functions including physical protection from microbial infiltration, UV protection and plays a role in water conductance. It is a considerably thin layer (approximately 6µm in hen eggshell) and can be unevenly distributed across the eggshell surface (D'Alba *et al.*, 2017).
It was decided to analyse the eggshell stress with and without the cuticle to examine if the cuticle had any mechanical influence on the shell. To test this, a protocol was created which examined a cuticle free group of eggs and a cuticle intact group of eggs under quasi-static compression. Eggs were kept whole for this test. Store bought large hen eggs were used (*Ballyfree*). 12 cuticle free eggs and 12 intact cuticle hen eggs were tested. Cuticle removal was performed as per Deeming (1987). This involved washing the eggs in 14.7 mM solution of

sodium hypochlorite (*Emplura*) at 45°C for 2-3 minutes and rinsed well with water. Quasi-static tests as per section 3.5 Quasi-static tests.

3.10.5 Supplemental Study 4: Nanoindentation maximum force

The maximum force (Pmax) was discussed in research performed by Severa *et al*., (2010) which examined hen eggshell using nanoindentation. The team discovered that when indenter penetration depth exceeded 500nm, fluctuations in results occurred.

It was decided that a study on the appropriate Pmax for testing was required. Sample preparation occurred as per methods *3.6 Nanoindentation Test*; where 3 medium store purchased hen eggs (*Ballyfree*) were used (n=3). Each sample was tested along the palisade layer (middle layer) of the eggshell. An array of 5x4 indents was used. The Pmax was examined using 2000, 4000, 8000, 10000, and 12000 µN per array. Hardness and Young's modulus (calculated from the given reduced modulus see Eq. 3.10) were obtained.

3.11 Statistical Analysis

Statistical analysis was performed on all tests. Mean average and standard deviation was calculated in any group. These calculations were performed using Microsoft Excel software (Microsoft Office Professional Plus 2016). Comparative and significance analysis was completed using SPSS Statistics software (IBM SPSS Statistics 27). Analysis of variance (ANOVA) was performed to understand if statistical significance occurred between groups in the form of p value being less than 0.05. Post Hoc tests were performed to observe differences between multiple groups and understand where the difference is specifically occurring. Tukey HSD was the Post Hoc test used in this project. The student T-test was performed when comparing two groups of data from each other. The means of two variables were compared to see if the null hypothesis can be accepted or rejected.

3.12 Probability Analysis

Probability analysis was conducted modelling the TCD approach derived from the Line Method in LEFM.

Deriving a relationship between three material constants L, σ_0 and K_{Ic} can occur using the following:

Eq.3.13

$$L = \frac{1}{\pi}\left(\frac{K1c}{\sigma_0}\right)$$

Where L is the critical distance, $\sigma 0$ is the characteristic stress of the material containing no defects and K_{IC} is the fracture toughness. From the fracture toughness tests, K_{IC} of eggshell is discovered. σ_0 is more challenging as it is impossible to obtain a stress value for a perfect eggshell with no defects. This parameter is therefore taken from the results obtained from experimentation but should be noted that it is challenging to obtain this value for eggshell.

To predict failure stress (σ_{tf}) using the TCD method, the following equation was used:

Eq.3.14

$$\sigma_{tf} = \frac{\sigma_0}{\left[1 + \frac{a}{2L}\left(1 - \frac{1}{(1+2L/a)^3}\right)\right]}$$

With a being the length of the given crack. The crack length value was altered to assess the prediction of failure when crack lengths changed.

In addition, the same method was applied using TCD with a correction factor obtained using Weibull analysis. The Weibull modulus of eggshell was obtained from Weibull prediction based off strength values as previously demonstrating in the above section 3.5 Quasi-static tests. The correction was performed by putting the TCD prediction over the Weibull stress reduction factor. The Weibull stress reduction factor was achieved as the following:

Eq.3.15

$$m\sigma = a^{\left(\frac{2}{m}\right)}$$

Where $m\sigma$ is the Weibull stress reduction factor, m is the Weibull modulus of eggshell and a is the notch length.

Chapter 4. Results

The various egg studies are explained in *Chapter 3*, section *3.1 Test Information*. Table 4.1 in this chapter illustrates the tests performed in each of these studies. This chapter will be separated out with regards to each study: Supplementary Experimentation, Age & Diet study, Size Analysis and Statistical and Comparative analysis.

The objectives of the project methods are as follows:

- The Supplementary Experimentation was performed to validate processes used in mechanical test methods.
- There were three Age & Diet studies performed. Age & Diet Study 1 consisted of 4 sheds tested separately. All of the eggs from these sheds were tested for their mechanical properties. One of these sheds, *Shed A*, was analysed further by means of structural tests. *Shed A* received increased inspection based on the interesting mechanical test results obtained (fracture toughness). The other 3 sheds (*Shed B, C* &*D*) were not examined further. In addition, Age & Diet Study 2 and Age & Diet Study 3 were only tested for mechanical properties and further structural analysis was not performed.
- Size analysis was performed on store purchased eggs as a result of limited supply of eggs from farms due to Covid-19 and an avian influenza. This study is relevant to the overall project as it provides fundamental information on commercial eggs.
- Statistical and Comparative analysis provides fundamental observations on eggshell which helps solidify an understanding into the behavior of this biological material.

Table 4.1. *Information on studies and type of testing performed*

Study	*Egg Source*	*Mechanical tests performed*	*Other tests performed*
Supplemental Study 1			
Loading rate	Store purchased- hen	Fracture toughness	
		Quasi-static	
Supplemental Study 2			
Load cell	Store purchased- hen	Fracture toughness	
Supplemental Study 3			
Content analysis	Store purchased- hen	Quasi-static	
Supplemental Study 4			
Cuticle analysis	Store purchased- hen	Quasi-static	Cuticle removal
Supplemental Study 5			
Nanoindentation max force	Store purchased- hen	Nanoindentation	
Size analysis	Store purchased- hen	Fracture toughness	
		Quasi-static	
Age & Diet Study 1	Farm source- hen	Fracture toughness	2D-XRD
Shed A		Quasi-static	XPS
		Nanoindentation	X-ray µCT
Age & Diet Study 1	Farm source- hen	Fracture toughness	
Shed B		Quasi-static	
		Nanoindentation	
Age & Diet Study 1	Farm source- hen	Fracture toughness	
Shed C		Quasi-static	
		Nanoindentation	
Age & Diet Study 1	Farm source-hen	Fracture toughness	
Shed D		Quasi-static	
		Nanoindentation	
Age & Diet Study 2	Farm source-hen	Nanoindentation	
Age & Diet Study 3	Farm source- duck	Fracture toughness	
		Nanoindentation	

4.1 Supplemental tests

4.1.1 Supplemental Study 1: Loading Rate

When comparing different load rates on hen eggshell, there was no difference in results for either fracture toughness or quasi-static tests. No statistical difference was found when using three loading rates (0.5, 1 and 2 mm/min) (see **Figure 4.1.1)**. What was observed in these tests when using the higher rates (2mm/min) was that the egg failed more catastrophically where there was a complete break of shell? Ideally, the loading machine stops before complete failure of the egg such that cracks can be observed. This is particularly important for fracture toughness tests where confirmation that the crack is propagating from the preformed notch is essential. When using the higher rates this increased the likelihood of full catastrophic failure of the egg. It was therefore concluded to use the lower rates as the failure was somewhat more controllable.

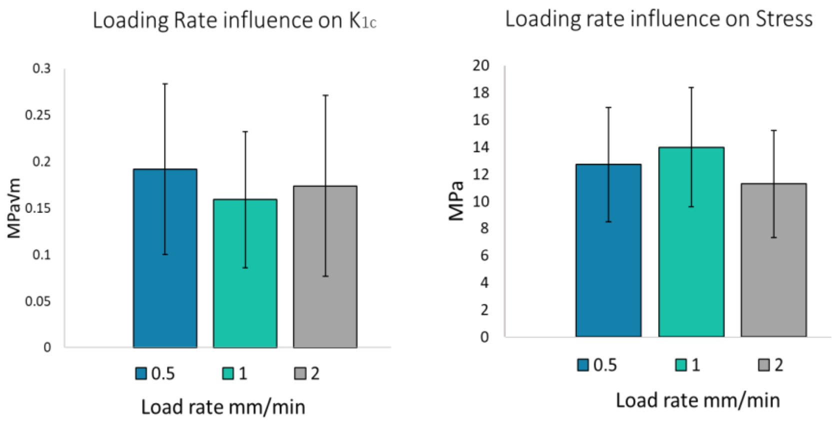


Figure 4.1.1 Loading rate influence on fracture toughness and quasi-static tests on store purchased eggs. No statistical significance was obtained when performing an ANOVA.

4.1.1 Supplemental Study 2: Load Cell

The load cell was altered for fracture toughness testing as there was a lower force required for failure in the fracture toughness tests compared to the quasi-static tests. Ideally, the load cell is lower to match the force being used. Thus a 500N load cell was tested to examine its accuracy. This was tested against the 5KN load cell in fracture toughness tests of hen eggs. The results illustrated that there was a difference seen when using the different load cell (see **Figure 4.1.2**). However, it was not seen to be statistically significant. The difference here could be as a result of the lower load cell received the force more accurately than the 5KN load cell, which would be less sensitive to low forces. Despite the variation in results, because there was no statistical difference seen between the two load cells, it was decided the 500N could be used in fracture toughness analysis due to its accuracy.

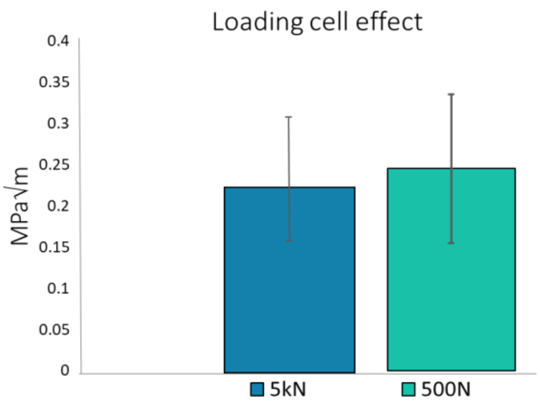


Figure 4.1.2 Load cell effect on fracture toughness results on store purchased hen eggshell. No statistical significance was obtained when performing a student t-test.

4.1.3 Supplemental Study 3: Content Analysis

In order to evaluate if contents had an effect the eggshell, stress analysis was performed. It was determined that there was no difference between failure stress values of undisturbed eggs and emptied eggs (see **Figure 4.1.3**). Although, there was greater distribution of failure stress in emptied eggs. It is not fully understood why the standard deviation was increased for emptied eggs. It could be as a result of flaws being created in the shell as a result of the holes applied to the shell for emptying. This could disturb the otherwise even distribution of stress being distributed around the shell during the loading tests. However, the failure crack did not initiate from these holes so it cannot be fully confirmed if these holes are responsible for the variation in strength results. It could be suggested that the contents may have act to more evenly distribute the stress ensuring a hoop stress about the equator. This is a potential explanation for the observations made, although not confirmed.

From the tests performed it was determined that whole unemptied eggs were more suitable for mechanical loading tests. Future analysis could be performed in the focusing on content effect with fracture toughness tests.

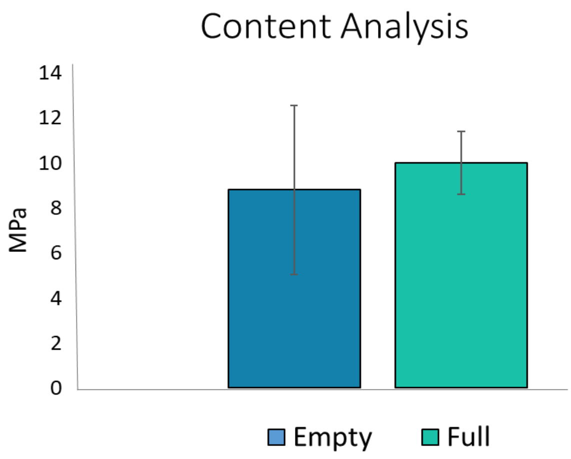


Figure 4.1.3 Effect of content on store purchased eggshell in relation to quasi-static tests. No statistical significance was obtained when performing a student t-test.

4.1.4 Supplemental Study 4: Cuticle Analysis

The cuticle is the organic waxy layer on the outside of the egg and is not thought to have a structural role. The cuticle can be seen in **Figure 4.1.4** (A).

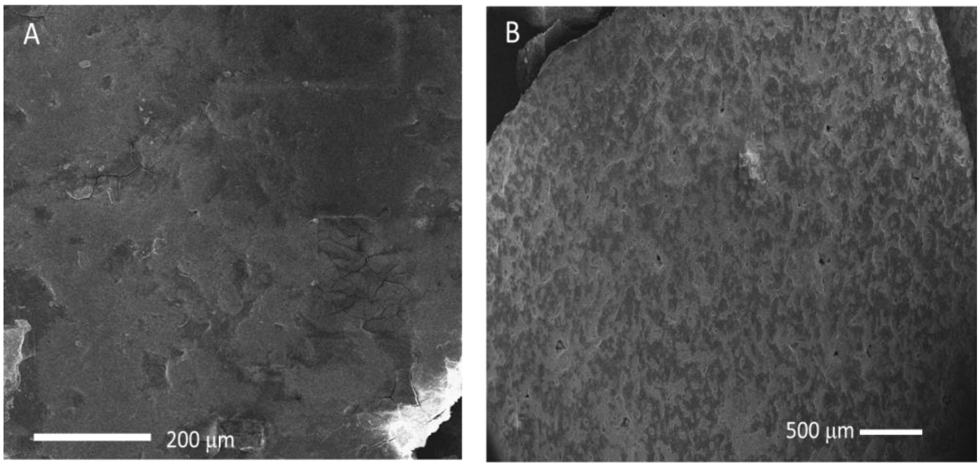


Figure 4.1.4 A. Hen eggshell surface with cuticle still intact. B. Hen eggshell with cuticle removed. Note pore openings are visible on the surface (project image).

Although the cuticle was not believed to have a structural role, a study was performed to assess if the cuticle altered the mechanical properties of the eggshell. From the quasi-static test performed with and without the cuticle, it was apparent that the cuticle had no significant impact on the strength on the shell. This can be seen in **Figure 4.1.5.**

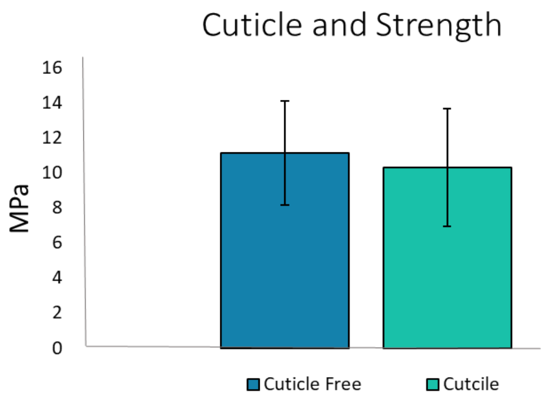


Figure 4.1.5 Cuticle influence on eggshell stress values on store purchased eggs. No statistical significance was obtained when performing a student t-test.

4.1.5 Supplemental Study 5: Nanoindentation Force

An experiment was completed to identify the optimum forces applied on eggshell in nanoindentation.

The reasoning was to identify if there were forces which gave inadequate results if the indentation was too shallow or too deep. The results from the study indicated that there was an increase in variance with the lowest and the highest forces. This is specifically evident in max force (Pmax) 2000µN and 12000µN (see **Figure 4.1.5**). The standard deviation of hardness values decreases between 6000µN and 8000µN. From this set of results, it was established that using a force to the approximate value of 6000µN to 8000µN was preferable.

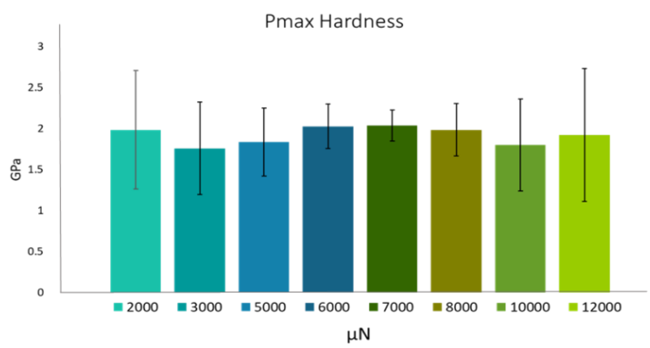


Figure 4.1.5 Pmax on hardness of store purchased hen eggshell. No statistical significance was obtained when performing an ANOVA.

In the study, variation occurred at 10,000µN which corresponds to a Hmax of approximately 480nm. Severa *et al* (2010) describes a fluctuation in results when indentation depth exceeds 500nm. This indicates that the results from this study are relatively in agreement with the range provided by Severa *et al*., (2010). A value of 8000µN was used in nanoindentation tests in this project.

4.2 Size Analysis

Store purchased eggs were analysed to evaluate differences in mechanical properties of eggs of various sizes. Specifically looking at 'medium', 'large' and 'very large'. Both egg weight and height (major axis) were related to egg size. Height was used as a size parameter rather than egg diameter or radius because radius was used in mechanical test calculations. In order to compare egg size with mechanical test results, it was therefore important to keep the values used independent of each other.

4.2.1 Fracture Toughness Test

Sizes were categorized as labelled during purchase. The fundamental difference between these three groups was evident in the weight differences (see Table 4.2.1). There was a clear increase in weight from smaller to bigger eggs as expected. Likewise, height also increased. The relationship between height and weight was measured using a regression analysis. This presented with an R^2 value of 0.78. This showed the proportion of variance of the weight variable, that can be explained by the height variable. R^2 values are measured between 0 and 1, with 1 showing a prediction of a strong relationship. A coefficient of correlation of 0.86 was achieved when measuring height and weight against each other. The Pearson's correlation coefficient determines if there is a linear relationship between two variables in a regression curve and is measured between -1 and 1. Getting a value at either end of this range indicates a strong relationship between two variables and value of 0 indicates very weak correlation. These results indicate a strong relationship between the measurements *i.e.,* increase in weight equated to an increase in length.
Contradictory to the literature, thickness did not change between groups. A decrease in thickness was expected with larger eggs, this was not identified.

Table 4.2.1 *Fracture toughness test results of different sized eggs.*

	Medium		*Large*		*Very Large*	
	Mean	Sd	Mean	Sd	Mean	Sd
Weight (g)	60.95*[a]	3.39	68.07*[a]	2.41	76.05*[a]	2.66
Thickness (mm)	0.40	0.03	0.40	0.03	0.40	0.03
Height (mm)	55.95	1.69	59.25	1.57	61.28*[b]	1.7
K_{Ic} (MPa√m)	0.21	0.05	0.30*[c]	0.08	0.27	0.09

Sd: Standard deviation. The ANOVA statistical test was performed. Significant difference*[a] in weight in weight observed between size groups as it expectedly increased with egg size. A significant difference was seen in between medium height and very large egg height. Significant difference*[c] observed in K_{Ic} , large eggs statistically different to medium eggs (p value <0.05).

Performing fracture toughness tests on eggs of various sizes saw differences obtained in K_{IC}. This can be seen from **Figure 4.2.1**. The medium sized eggs had the lowest K_{Ic} value. The very large egg group presented an increase in K_{Ic}, with the large egg group having the highest value. This illustrates that the smaller eggs are less resistant to crack propagation than their larger counterparts. The difference observed in material property from eggs of varying sizes is thought provoking. There appears to be a difference in the material as the egg size change, but this could be as a factor of bird age *e.g.* older birds producing larger eggs. From this, it is clear there is a difference between the groups and the reason for this could be as a result of porosity, crystallographic grain changes or influences in crack propagation due to organic content. Perhaps the older birds producing the larger eggs are reaching peak health, the reproductive muscles are improving and producing shells under greater force; which corresponds with decreased porosity and decrease in grain size thus enhancing fracture toughness. This is a theory and as it is unknown the ages of the birds where these larger eggs are coming from. It is not conclusive to say this theory is the mechanism for increase fracture toughness, although, it could very well be the development.

An R^2 value of 0.14 was obtained when comparing weight to $K_{Ic.}$ The value obtained in this case was a low R^2 value and implies that weight and K_{Ic} are not likely to be related to each other. When performing a Pearson's correlation coefficient examination on weight and K_{Ic}, a value of 0.2 was given. The value obtained by this test on the samples in question signifies a weak relationship between K_{Ic} and weight. Fracture from notch can be observed in **Figure 4.2.2.**

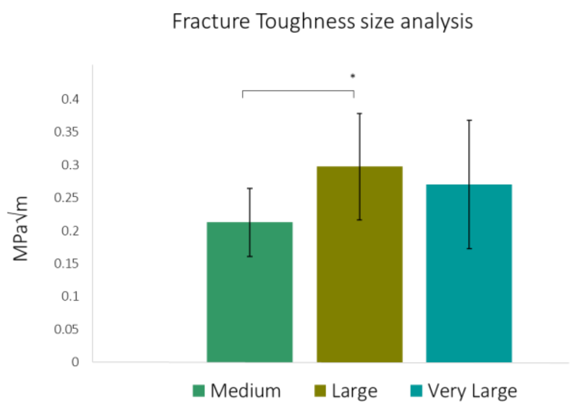


Figure 4.2.1 Fracture toughness results between various sized store purchased eggs. Large eggs had significantly higher fracture toughness than medium eggs.

Figure 4.2.2. Fracture in eggshell occurring vertically along from preformed notch.

4.2.2 Quasi-static Test

Quasi-static test results revealed failure stress information regarding the different sized eggs. Table 4.2.2 shows relevant information regarding this experiment. Similar to the fracture toughness samples, eggs significantly increased in weight as per the size label *i.e.,* medium, large and very large. Thickness measurements were higher in medium eggs than the corresponding larger eggs, but only slightly and the difference was not significant.

Table 4.2.2 *Quasi-static test results for different sized eggs. Expected increase in weight from smallest to largest which was statistically significant.*

	Medium		*Large*		*Very Large*	
	Mean	Sd	Mean	Sd	Mean	Sd
Weight (g)	58.97*[a]	2.64	66.07	1.51	74.30	2.22
Thickness (mm)	0.41	0.04	0.40	0.03	0.40	0.02
Strength (MPa)	15.09*[b]	4.99	10.18	5.33	7.63	3.86

Sd: Standard deviation. Weight*[a] increased as expected. Strength (MPa)*[b] shows medium eggs had increased strength values significantly different to that of large or very large eggs.

Failure stress measurements expressed in MPa shows a definite decrease with increasing egg weight seen in **Figure 4.2.3**. Medium eggs, which were the lowest weights, possessed the highest failure stress values, followed by large eggs and the lowest was very large eggs. A low R^2 value was obtained following analysis (0.26) of weight and failure stress (**Figure 4.2.4**). However, upon performing a Pearson's correlation coefficient calculation on these two variables a value of -0.51 was achieved. This suggests a moderate relationship between failure stress and weight. Eggshell failure can be observed in **Figure 4.2.5**.

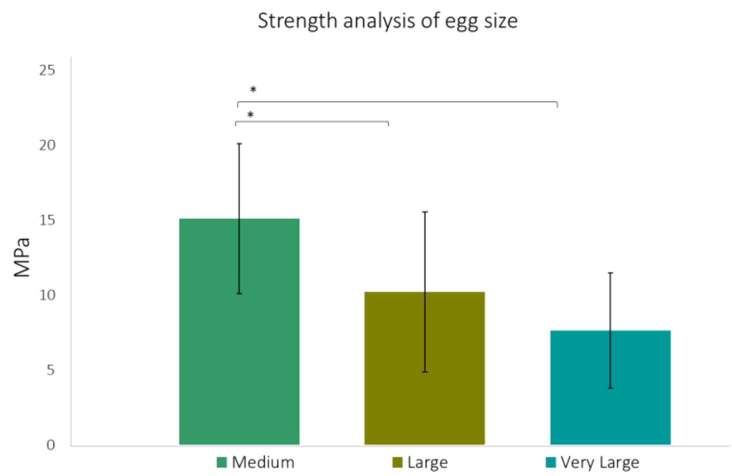


Figure 4.2.3 Strength differences seen in varying store purchased egg sizes from quasi-static tests. Medium eggs were significantly stronger than large and very large eggs.

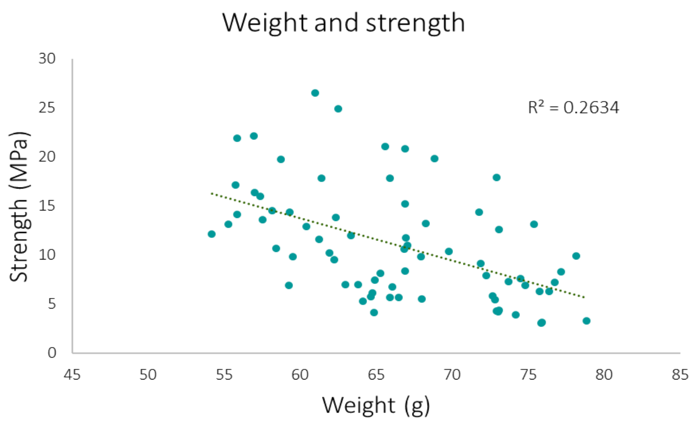


Figure 4.2.4 Correlation analysis of weight and stress of eggshell.

In this Size Analysis study (store purchased), egg size had an impact on mechanical properties *i.e.,* strength and fracture toughness. Strength decreased with increased egg size increase which was expected as this is the main plight of the poultry industry with larger eggs. Fracture toughness increased with large eggs and lowered slightly with very large eggs. This was an interesting result as it indicates a material property change occurred with the varying sized eggs. Further discussion on this in relation to failure probability analysis was complete for this study and can be found in *4.5.2 Probability Analysis*.

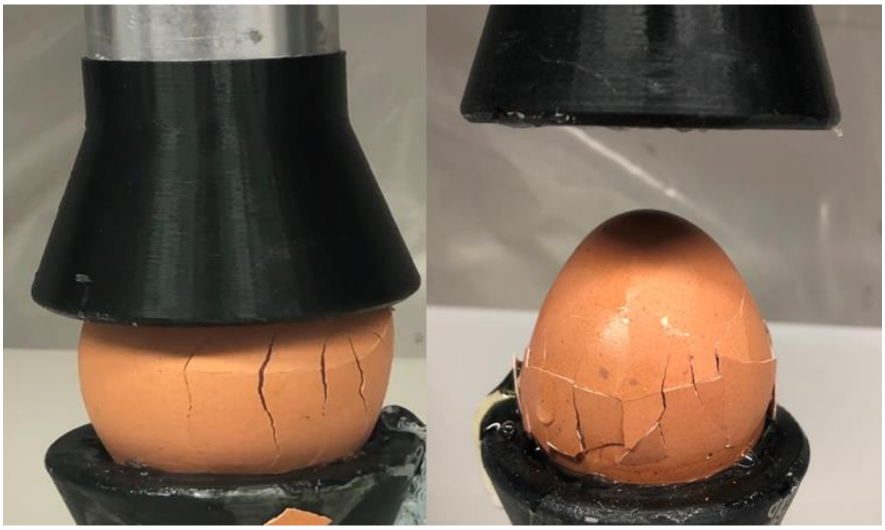


Figure 4.2.5 Eggshell failure under axial compression tests. Fracture occurred vertically along equator of egg.

4.3 Age & Diet Studies

Refer to Table 4.1 for an overview of studies which took place to analyse age and diet impact on eggshell.

4.3.1 Age & Diet Study 1 *Shed A*

4.3.1.1 Mechanical analysis

This study comprised of the most in-depth examination of a group of eggs in this project and is focused on eggs from *Shed A*. Mechanical tests were completed along with continued analysis of crystallography, porosity and elemental analysis. Table 4.3.1 provides information regarding the test results from both quasi-static and fracture toughness tests.

Table 4.3.1 *Sample information along with quasi-static and fracture toughness data.*

		Weight (g)		*Thickness (mm)*		*Stress (MPa)*		K_{IC} *(MPa√m)*	
Week	Feed	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
22	Mineral Supplement	52.60*a	4.35	0.37	0.02	5.30	2.40	0.15*b	0.04
22	Generic Feed	52.37	5.86	0.35	0.02	7.52	4.34	0.13	0.03
29	Mineral Supplement	59.74	2.59	0.39	0.04	9.03	1.39	0.22	0.06
29	Generic Feed	63.53	6.03	0.39	0.05	5.12	1.25	0.29	0.05
35	Mineral Supplement	62.76	3.16	0.39	0.02	5.34	2.67	0.37	0.07
35	Generic Feed	63.30	3.78	0.38	0.02	4.19	2.09	0.31	0.09
43	Mineral Supplement	60.44	4.48	0.36	0.04	5.29	2.19	0.57	0.18
43	Generic Feed	59.715	4.54	0.39	0.02	4.53	2.00	0.62	0.07

Sd: Standard deviation. Weight*a increases with time for both diets which was statistically significant (p value <0.05). K_{IC} [b] for both diets presented a significant increase over time (p value <0.001 both mineral supplement and generic feed diets).

From these tests, fracture toughness appeared to increase over time significantly regardless of which diet was provided (see **Figure 4.3.1**).

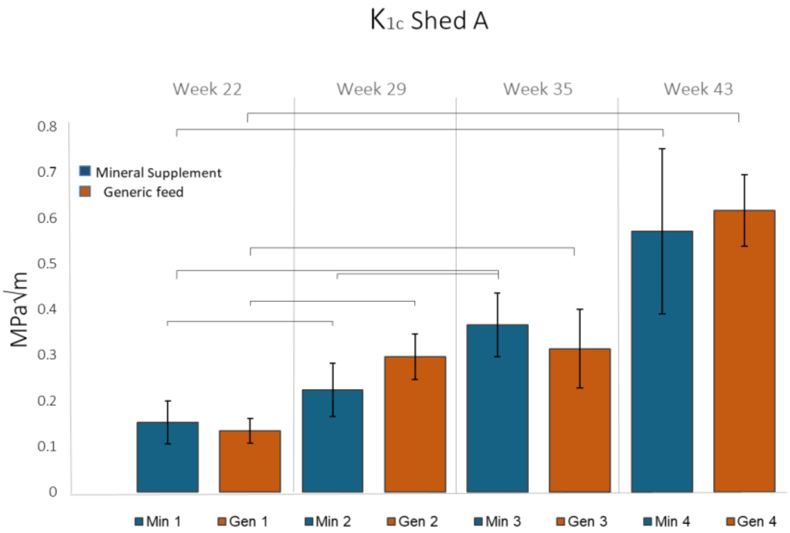


Figure 4.3.1 Fracture toughness of both diets over time of farm supplier eggs. Eggs significantly increase in fracture toughness over time.

There was no difference between mineral supplement and generic feed diets observed nor was there a difference statistically. When comparing fracture toughness results and weight, a weak relationship was displayed (R^2 of 0.02 and a correlation coefficient value of 0.2).

Strength results did not display any trend over time (see **Figure 4.3.2**). Week 29 mineral supplement group shows the highest strength value which was different to subsequent tests of this group over time. This increase in MPa is not correlated with egg weight or thickness. However, after Week 29 the values decreases and fall in line with the generic feed diet values. No clear differences observed between mineral supplement and generic feed diet groups over time. There is no trend occurring and appears that the mineral supplement creates no difference in failure stress over time compared to the generic feed. The results for stress in this study is generally unremarkable.

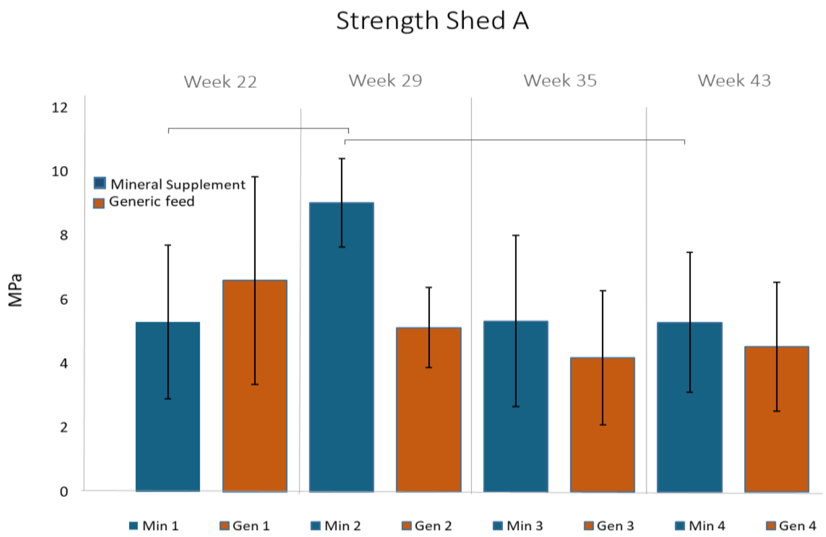


Figure 4.3.2 Stress at failure results from quasi-static test on Age & Diet study of farm supplier eggs. Mineral supplement samples from week 29 were significantly higher than other weeks.

Nanoindentation was conducted on eggs from the two diets over 4 time-points. From the test, hardness and Young's modulus was obtained (Table 4.3.2). The measurements for hardness between diet groups displayed no obvious trend occurring between the group *i.e.,* no clear indication that mineral supplement was actively impacting the eggshell in a specific way (see **Figure 4.3.3**). These results indicate that there was no clear change in overall stiffness or hardness with the introduction of the mineral supplement in this study. The generic feed results, for both stiffness and hardness results, it appeared to increase over time and decreased in the final time point. There were significant differences observed in both hardness and Young's modulus, however these differences were not forming any trend. For both hardness and Young's modulus, the final time-point (week 43) displayed a drop in both measurements. This decrease in stiffness and hardness could be significant in that it demonstrates the eggs become less hard and stiff when produced from an older bird.

Table 4.3.2 *Nanoindentation results for Age & Diet study 1.1.*

		Hardness (GPa)		*Young's Modulus (GPa)*	
Week	Diet	Mean	Sd	Mean	Sd
22	Mineral supplement	2.28	0.17	45.49	5.26
22	Generic feed	1.86	0.25	41.52	4.21
29	Mineral supplement	2.84^{*a}	0.88	45.87	6.76
29	Generic feed	2.37	0.45	45.53	5.06
35	Mineral supplement	2.59	0.59	50.57	6.13
35	Generic feed	2.75^{*b}	0.28	55.19^{*c}	4.15
43	Mineral supplement	2.18	0.22	37.98^{*d}	9.46
43	Generic feed	2.21	0.53	45.03	7.39

Sd: Standard deviation. Hardness *a week 29 mineral supplement was significantly different compared to week 22 and 43. Hardness *b generic feed week 35 was significantly different to week 22. Modulus^{*c} generic feed week 35 significantly different to week 22 and week 43. Modulus^{*d} sees mineral supplement decrease in from week 22 to week 43.

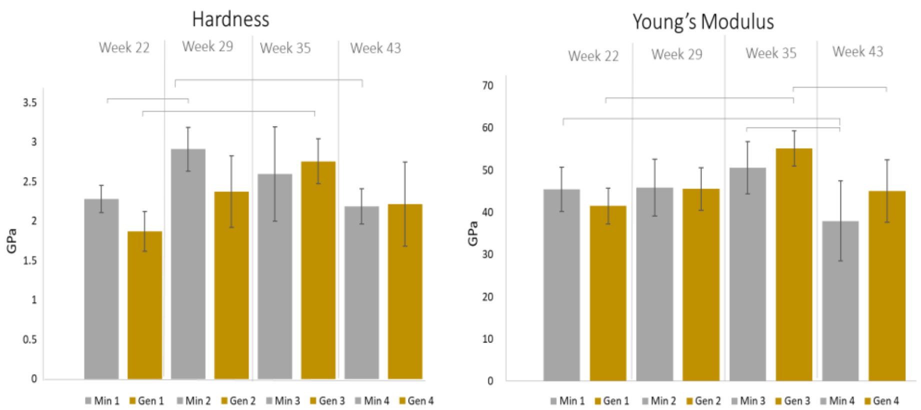


Figure 4.3.3 Hardness and Young's modulus results from nanoindentation of farm supplier eggs. 'Min' represents Mineral Supplement; 'Gen' represents Generic Feed.

The mechanical tests from this study provided an interesting result. Specifically, the increase in fracture toughness over time was not expected and was specific to this group of eggs. This prompted further analysis on this particular group of eggs. The further analysis which will be discussed was performed on generic feed eggs from *Shed A*.

4.3.1.2 Crystallography analysis

2D-XRD was performed on samples in an attempt to quantify crystal orientation. This microstructural examination technique was performed in transmission and in reflection. A 2Theta scan of eggshell can be observed in **Figure 4.3.4**. Crystal size and orientation is said to have an impact on the mechanical properties of the eggshell. It is theorized that when encountering smaller, more randomly orientated crystals, more energy is required for the crack to propagate through and so eggs with random orientation could be less likely to fail (Rodriguez-Navarro *et al.*, 2007) (Rodriguez Navarro *et al.*, 2002) (Ahmed *et al.*, 2005). The scans were run through the ICDD database (International Centre for Diffraction Data, Pennsylvania, USA) which identified the peaks that corresponded with calcite only *i.e.,* no other crystallographic phases were present.

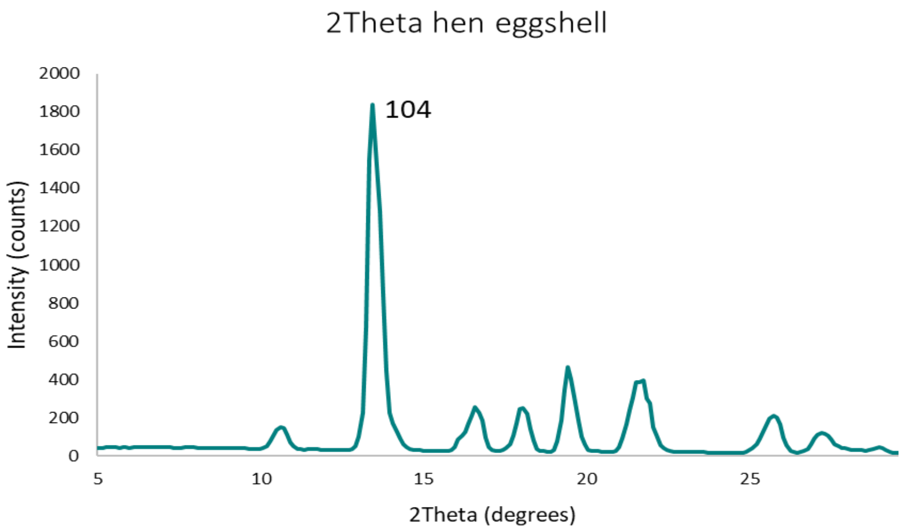


Figure 4.3.4 2Theta scan of hen eggshell.

The preferential orientation of the eggshells was understood to be 104 plane as seen on the 2Theta scan (see **Figure 4.3.4**). The 2Theta linear scan was created by integrating pixel intensity from the 2D diffraction pattern. Transmission and reflection scans can be observed in **Figure 4.3.5**. Orientation can be obtained by the ratio of 110 and 104 *hkl* planes. This ratio is said to increase with preferential orientation of the crystal. In addition, the higher the FWHM angular band breadth value, the more random the orientation of crystals (Rodriguez-Navarro *et al.*, 2007). Table 4.3.3 provides results from 2D-XRD analysis of hen eggshells from various time-points.

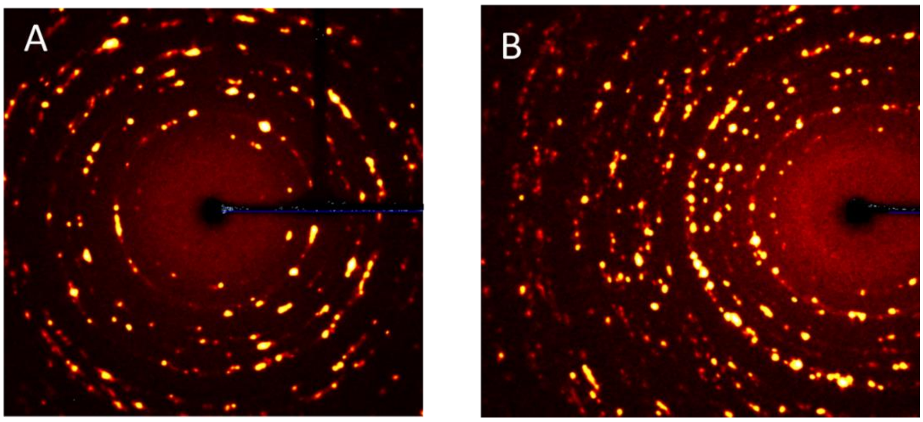


Figure 4.3.5 Debye Scherrer rings from 2D-XRD. A is a transmission scan and B is from a reflection scan of farm supplier eggs (Shed A).

FWHM increases with week 29 and 35 but decreases again in the final time-point. Intensities (I_{110}/I_{104}) increase in week 29 and 35 and decrease slightly in week 43. Both FWHM and intensity ratio measurement depict a changing situation between eggs from different age groups. However, there does not appear to be a consistent trend in relation to crystal orientation. **Figure 4.3.6** presents the diffraction pattern of eggshells from each of the four time-points. Visually, it can be observed that there is a decrease in spotting intensity in week 29 and 35. The intensity increases in week 43.

Table 4.3.3 *2D-XRD analysis of hen eggshell over 4 time points. T is transmission scans and R is reflection scans. I_{110}/I_{104} is integrated intensity of the representative peak.*

Week	*104 Intensity (counts)*		*FWHM (degree)*	*I_{110}/I_{104}*	
	Mean (*T*)	Sd	(*R*)	Mean (*T*)	Sd
22	108,760	65,603.3	42.78	0.28	0.05
29	77,631	32,139.9	57.11	0.38	0.12
35	73,488	49,293.8	74.81	0.37	0.13
43	97,030	83,031.6	27.89	0.33	0.16

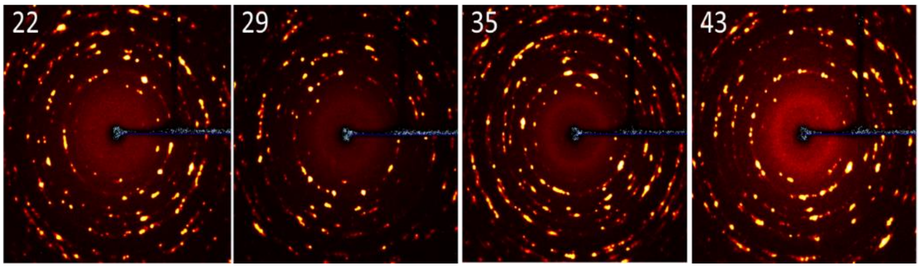


Figure 4.3.6 Transmission scans of eggs from each of the 4 time points. The representative weeks is displayed on each scan of farm supplier eggs (Shed A).

4.3.1.3 Porosity analysis (X-ray µCT)

Porosity analysis was performed by using X-ray µCT. Information from the test is visible below in Table 4.3.4. From the test, sections of the eggshell were examined to assess the pore distribution and size ranges from the 4 time points. The frequency of the pores per area (mm^2) was relatively unchanging between groups, it decreased in week 29 to increase again in week 35 and 43. It appeared the pores from the eggs display a higher frequency when compared to Riley *et al*., (2014). This research team observed 1.64 pores/ mm^2 from the eggshell equator. The current study displayed almost double this figure per area.

Overall pore volume presented with an increase over time, but this did not demonstrate any statistical significance (**Figure 4.3.7**). The increase in range of pore volume may suggest that the pores in eggs from the older birds are more varied. The pores may be less uniform in eggs from the older time points.

Assessing the largest pore present was important as the largest pore could be the largest defect within the material and therefore impact the materials behavior under stress. The largest pore volume and pore diameter are visible in **Figure 4.3.8**. From this data, it appeared that pore volume of the largest pores present in the samples increased over time. There was no statistical significance observed upon ANOVA and Post hoc tests. Regardless of significance, an increase overtime is apparent. Pore diameter also appeared to increase although the increase is not as apparent.

Table 4.3.4 *X-ray µCT results off eggshells over 4 time points. Pore volume appears to increase over time but is not statistically different from other weeks. Largest pore diameter and volume both increase over time.*

Week	*Pore Frequency*	*Pore Volume*		*Largest pore Diameter*		*Largest Pore Volume*	
	Pore/mm^2	Mean (µm^3)	Sd	Mean (µm)	Sd	Mean (µm^3)	Sd
22	2.796	0.128	0.06	27.74	3.99	0.169	0.03
29	1.720	0.168	0.07	29.93	4.41	0.227	0.06
35	2.151	0.168	0.08	28.87	5.11	0.201	0.07
43	2.366	0.202	0.14	36.15	9.76	0.351	0.18

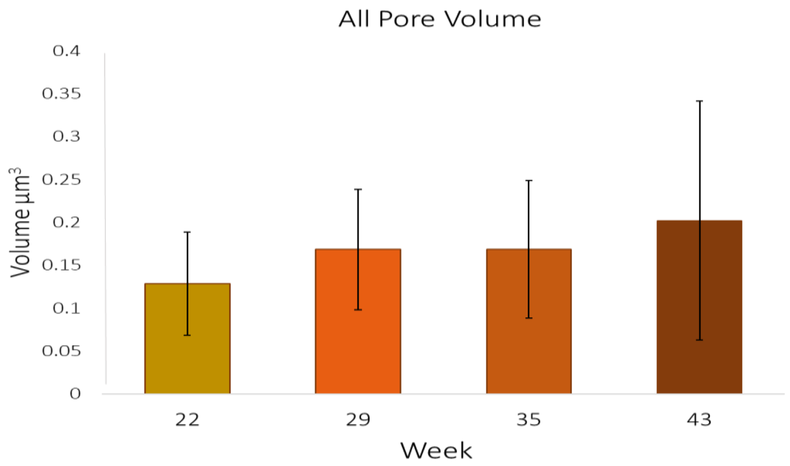


Figure 4.3.7 Average volume of all pore in a given sample over time. No significance was seen between groups of farm supplier eggs (Shed A).

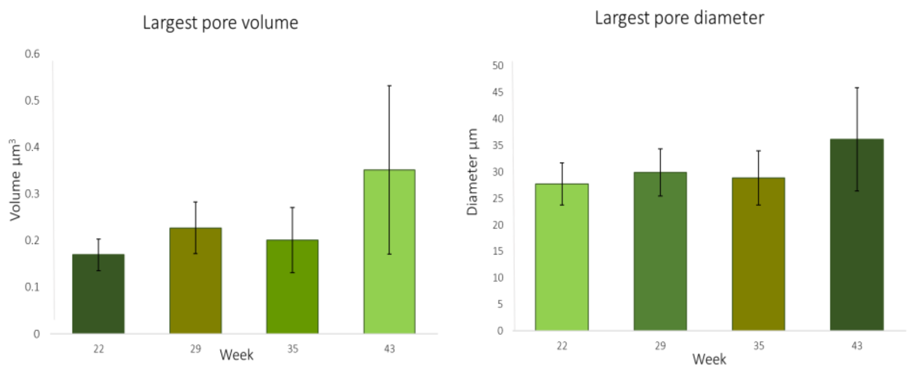


Figure 4.3.8 Largest pore data of farm supplier eggs (Shed A). Increase observed in both volume and diameter however no significance seen in AVOVA or post hoc test.

Pore diameter and eggshell thickness were correlated against each other. The relationship between the two was determined to be weak (R^2 of 0.099). This indicated that in these shells the change in pore diameter over time is not linked to any thickness change.

There are limitations to these results. The assumption that the largest volume is indicative of the largest pore may not be fully accurate. In addition, the average pore diameter was measured assuming the pore is cylindrical in shape. From the images from X-ray µCT, the opening of the pores appeared to be conical in shape and therefore the diameter appears to be different at this entrance (see **Figure 4.3.10**).

Images of the cross section of the shell along with images of pores were obtained (**Figure 4.3.9**, **Figure 4.3.10** and **Figure 4.3.11**).

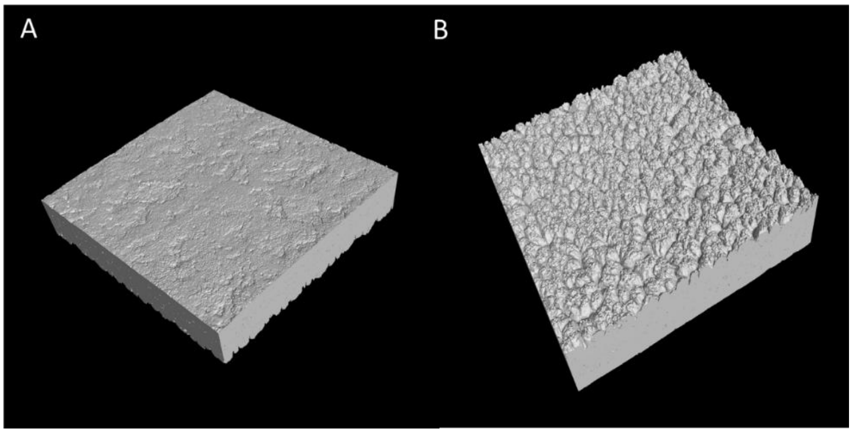


Figure 4.3.9 Computed images of A. illustrating the surface of the eggshell (Shed A) and B. displaying the eggshell from the inner mammillary layer (Shed A). (Image courtesy of Mr Brendan Phelan of SEAM Research centre, Waterford Institute of Technology).

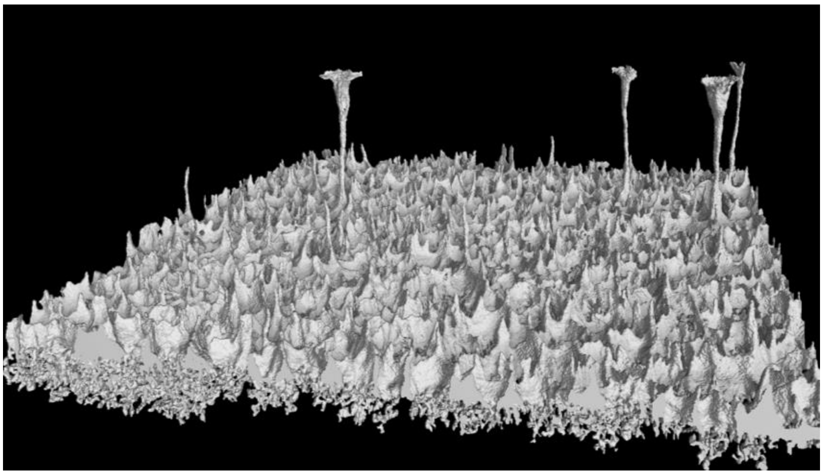


Figure 4.3.10 Pores in eggshell sample. Pores were observed through the shell. Through pores are visible passing through entire shell. Surface pores are also evident although they do not appear to pass through full shell (image courtesy of Mr Brendan Phelan of SEAM Research centre, Waterford Institute of Technology).

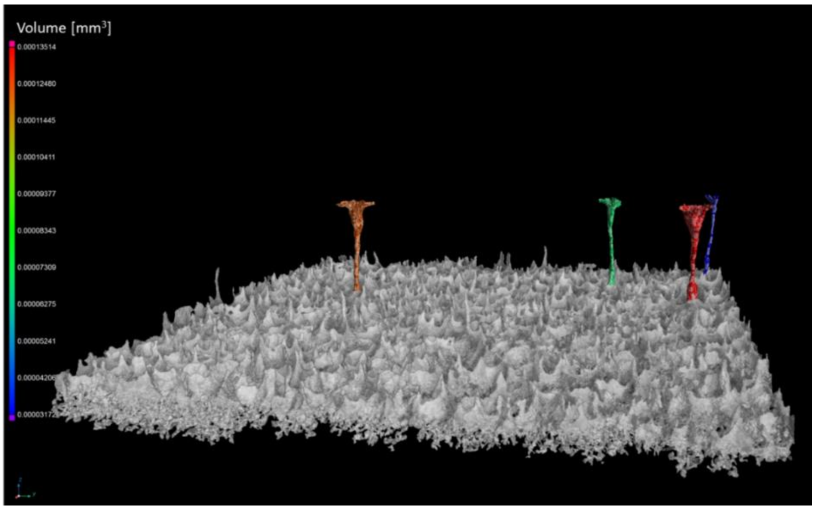


Figure 4.3.11 Computed image of voids and pores in eggshell. The pore volume was obtained and can be visualised from the key (image courtesy of Mr Brendan Phelan of SEAM Research centre, Waterford Institute of Technology).

4.3.1.4 Elemental analysis (XPS)

The following results were presented by the XPS test. The inner and outer portion of the shell were examined after the cuticle and membrane were removed. Table 4.3.5 provides information regarding the elemental composition for the inner and outer portion of shell across 4 time points. **Figure 4.3.12** presents the spectra focusing on the carbon content of the shell. From this, it was observed that there was a higher peak for the inner portion compared to the outer egg portion. This particular peak is indicative of an ester, acid or amide. Thus, the inner portion of the shell, which would be the mammillary layer, possesses different carbon species than the outer vertical crystal layer.

Table 4.3.5 *XPS results for inner and outer portion of the shell.*

		Atomic %							
Week	Location	O 1s	C 1s	N 1s	P 2p	Cl 2p	Mg 1s	Ca 2p	Na 1s
22	Inner	45.5	35.3	1.6	1.6	0.3	1.4	14.0	0.4
22	Outer	39.4	30.0	1.9	4.5	4.5	2.4	11.1	6.3
29	Inner	46.8	33.0	1.5	1.8	0.3	1.3	14.2	1.0
29	Outer	44.6	34.0	1.3	4.5	0.2	0.6	14.1	0.8
35	Inner	43.6	37.6	1.5	1.8	0.5	1.2	13.3	0.5
35	Outer	44.4	32.7	0.8	6.0	0.1	0.8	14.7	0.3
43	Inner	45.8	35.5	1.1	1.4	0.1	1.4	14.1	0.6
43	Outer	45.7	31.9	1.4	4.7	0.2	0.7	15.1	0.3

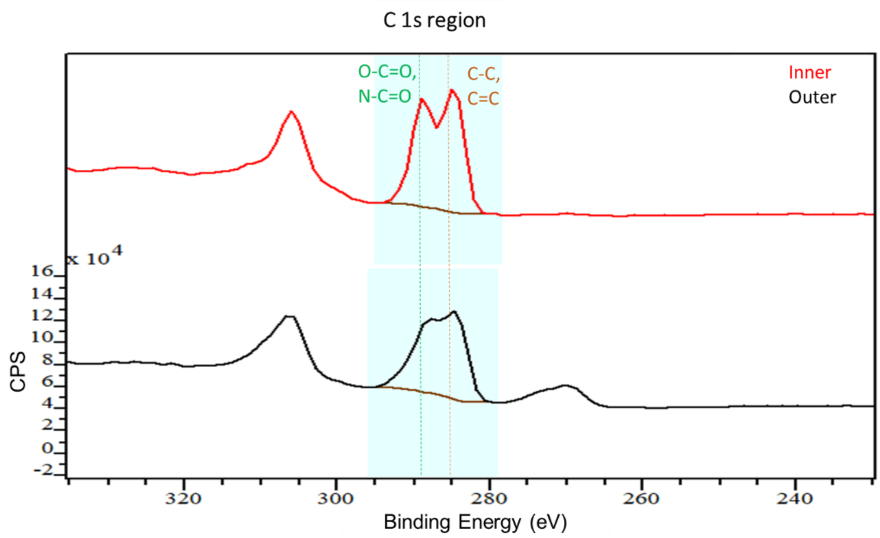


Figure 4.3.12 Carbon 1s region in the survey spectra for the inner and outer portion of the eggshell of farm supplier eggs (Shed A). Outer region missing peak which corresponds to amides, esters and acids.

From assessing the 4 different time points it was observed that the inner shell appeared to have homogenous elemental composition over all 4 time points (see **Figure 4.3.13**).

Magnesium appears to be slightly higher in the inner portion of the eggshell compared to the outer portion, with the exception of week 22.

Both inner and outer portions of the shell appear to have a similar trend in composition, with the exception of week 22 in the outer portion. However, phosphorus (2p) in the outer shell is higher overall when compared with the inner shell. This result coincides with (Cusack *et al.*, 2003) work where similarly, phosphorus appeared in higher concentrations on the outer portion of the eggshell.

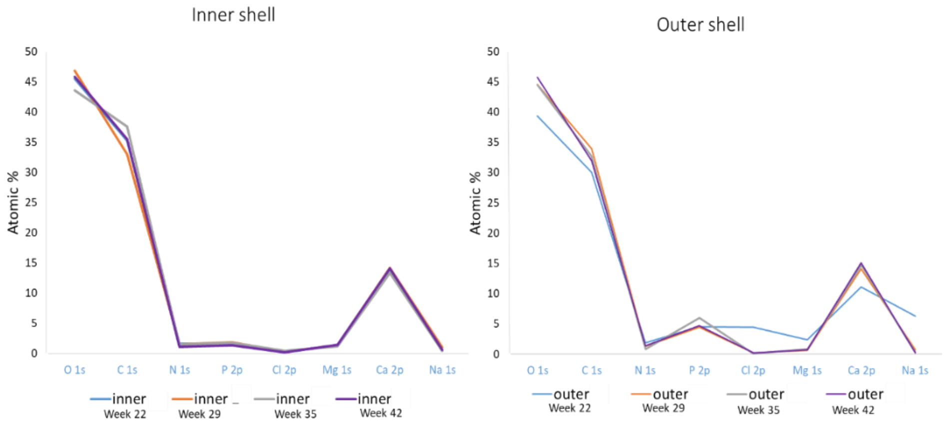


Figure 4.3.13 Elemental analysis of inner and outer portion of the eggshell from 4 different time points.

When assessing the outer portion of the shell, week 22 (time point 1) presented with differences across a range of elements (see above **Figure 4.3.13**). Carbon, chlorine, oxygen, magnesium, calcium and sodium concentration in the outer eggshell of week 22 appear varied compared to the other time points of the outer eggshell. There was a decrease in calcium and carbon, which could allude to a decreased concentration of calcium carbonate in the eggshell. Chlorine and sodium levels appeared to have increased. These two elements are generally seen together in the form of sodium chloride (NaCl) and perhaps there was an increase of this ionic compound in the outer portion of the shell. Finally, magnesium levels were slightly higher than the other weeks.

The following Age & Diet studies (Age& Diet Study 1 Shed B, Shed C and Shed D) were not followed with additional tests as there was no clear trend or indication of differences seen in any of the groups.

4.3.2 Age & Diet Study 1 *Shed B*

This study was comprised of the same diet as the previous Study 1 *Shed B*. The birds were also the same breed as the previous study (Hyline-plus). The mechanical tests performed on this group were the same tests performed on the previous study (Fracture toughness, quasi-static and nanoindentation). There were 3 time-points in this study; eggs were tested from birds aged 22, 28 and 36 weeks. Table 6.2.1 and Table 6.2.2 in Appendix section provides detailed results on fracture toughness, quasi-static and nanoindentation for this group of eggs.

The failure stress results indicated that the eggs decreased in strength over time (see **Figure 4.3.14**). This was the case for both diets. This result is in agreement with other literature which states that eggshell strength decreases over time. The values for failure stress, similar to that of *Shed A*, are lower than values reported and of those obtained from the Size Analysis study (store purchased eggs), which used commercially sourced eggs. Fracture toughness results showed little change in behavior over time. Eggs from hens on the generic diet had a lower K_{Ic} than average in Week 28, this increased again in the final week. It is observed, coinciding with the previous study in *Shed A*, where the K_{Ic} range is much higher than eggs supplied commercially.

From nanoindentation tests, hardness values for mineral supplement diet demonstrates an increase in week 28 which decreases in week 36. Young's modulus presents with a difference between diets. Here, the mineral supplement diet sees an increase in stiffness and the generic feed presents with a decrease in stiffness over time. Perhaps this mineral supplement did increase stiffness in this group of eggs. However, based on the variation of data from other egg studies in this project, it would be difficult to definitely conclude this diet is affecting the eggshell and if this trend is not just biological variation. Increased time-points would be really beneficial to deduce this.

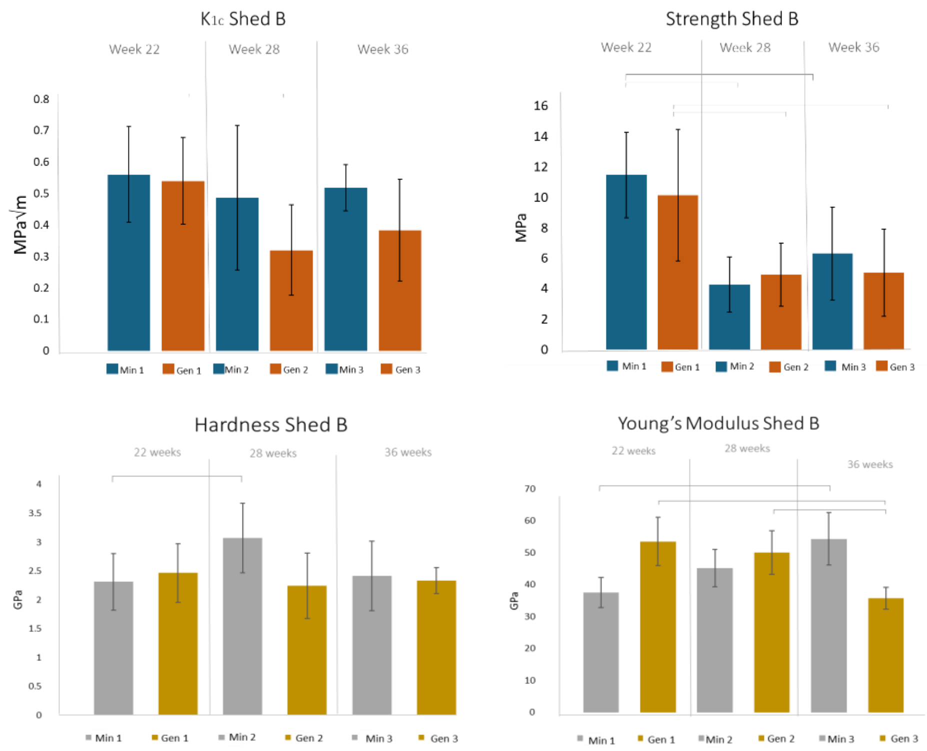


Figure 4.3.14 Fracture toughness, Strength, Hardness and Young's Modulus results from farm supplier eggs of *Shed B*. Min: Mineral supplement; Gen: Generic Feed. Strength decreased significantly for both diets. Young's modulus increased over time with the mineral supplement diet and decreased over time with the generic diet.

4.3.3 Age & Diet Study 1 *Shed C*

An additional study on the same farm as Study 1 *Shed A* and *Shed B*. The birds in this specific study are of a different breed (Lohmann). All data from this study can be seen in Table 6.2.3 and Table 6.2.4 in the Chapter 6 Appendix section. All test results from this group can be seen in **Figure 4.3.15.**

In the study, which consisted of 4 time points, weight was seen to incrementally increase over time. Thickness appeared to decrease in the final time point. Fracture toughness appeared to increase with time in both diet groups. However, the variation is high. There was significance seen between the first and final time point in generic feed as provided by a Tukey HSD statistical test. Similar to *Shed A* and *B*, the K_{Ic} results range was higher than other studies using commercially sourced eggs.

In strength tests, there was a significantly high failure stress value for generic feed diet Week 22 compared to all other weeks. This decreased with the subsequent time points.

Nanoindentation results pose a difference between diet groups. Both hardness and stiffness decreased in the mineral supplement diet; and both hardness and Young's modulus increased in the generic feed diet over time.

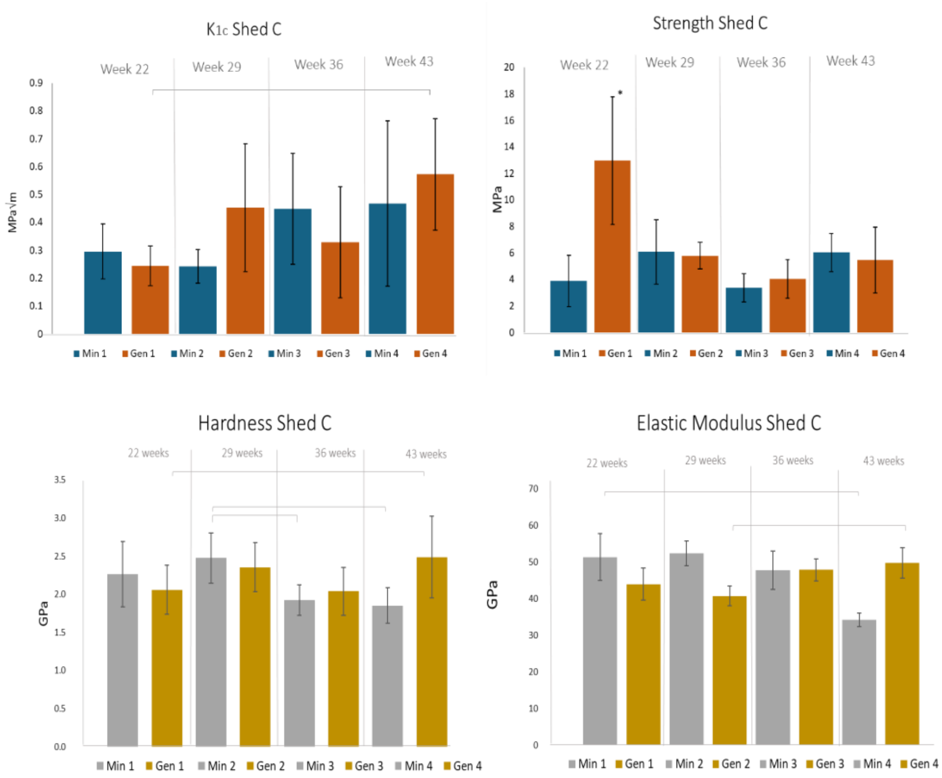


Figure 4.3.15 Mechanical test results from farm supplier eggs in Shed C. Min: mineral supplement and Gen: Generic feed. K_{Ic} increases significantly in Gen 4. Strength is highest in Gen1 and decreases significantly in subsequent time points. Both hardness and elastic modulus decrease for mineral supplement diet and increase for generic diet.

4.3.4 Age & Diet Study 1 *Shed D*

This is the final group in this Age & Diet study from the same farm supplier. This study was composed of three time points. The eggs from Shed D is Lohmann breed, which is the same as the previous study (*Shed C*). Table 6.2.6 and Table 6.2.6 in displays all results from mechanical tests for *Shed D* which can be found in the Appendix section. **Figure 4.3.16** represents all mechanical test results for this egg group.

For fracture toughness and quasi-static tests, there was no clear trend emerging. There was no difference seen between diet groups. Fracture toughness results had large variation within test groups, especially Week 43 generic feed. Strength results displayed a decrease for both diets which increased again in the third time-point.

In nanoindentation tests, there is an increase in hardness in the final time point for both diet groups, but only the increase in generic feed was seen to be statistically significant. Young's Modulus results for both diet groups see a decline in stiffness which increases again in the final time point. These changes in results may be as a result of unknown environmental factors or stress affecting the birds.

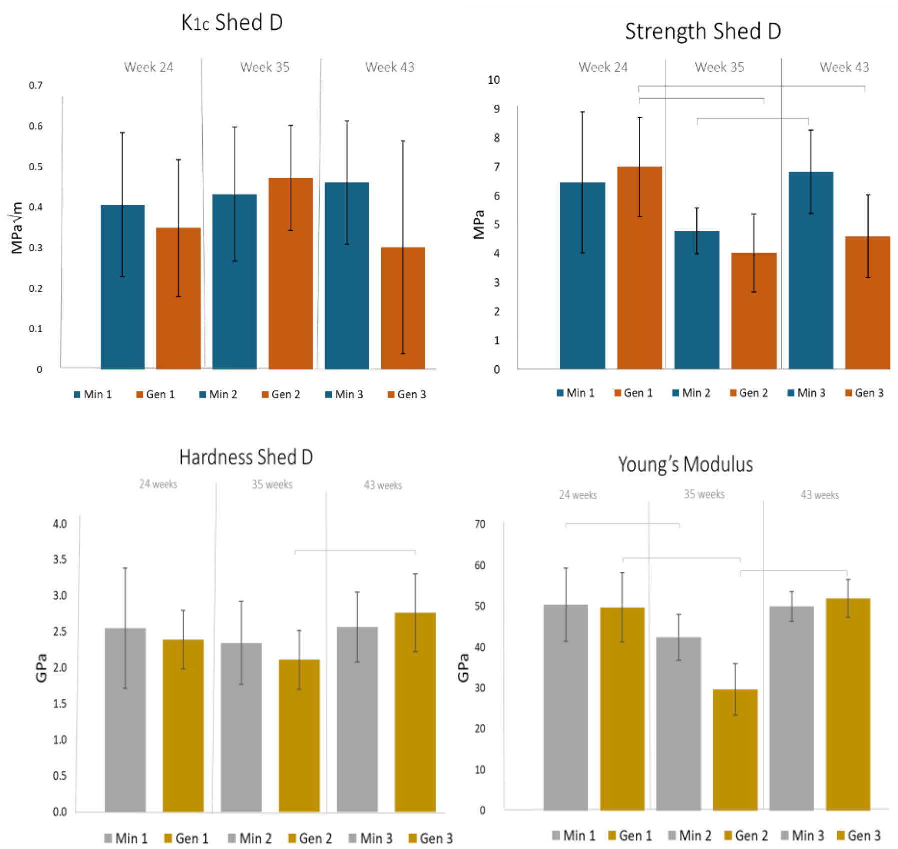


Figure 4.3.16 Mechanical test results for farm supplier eggs *Shed D*. 'Min' is Mineral Supplement. 'Gen' is Generic feed. Strength decreases significantly in generic diet group. Young's modulus decreases significantly in generic feed and mineral supplement feed in week 35.

All of the analyses from every shed in Age & Diet Study 1 indicate that there was high variation amongst birds. There were no definitive or consistent trends occurring between sheds. Fracture toughness increased significantly over time in *Shed A.* The rise in fracture toughness in *Shed C* was observed at the final time point, although this is not as definite as with *Shed A.* None of the other sheds displayed this trend.

Strength results in the Age & Diet studies did not present with a homogenous trend. It was proposed that eggs from younger birds were stronger than eggs from older birds from previous research. *Shed B* was the only study which displayed a decrease over time. The remaining sheds did not.

In the nanoindentation results, hardness and Young's modulus was measured. Both results demonstrated a large degree of fluctuations over time. Potential differences in diet can be seen in hardness results in *Shed C* where mineral supplement has a decrease in hardness and generic feed has an increase. This was also apparent for Young's modulus results with this group. In contrast, a difference between diets was also seen in Young's modulus in *Shed B* where mineral supplement diet group displayed an increase over time and generic feed displayed a decrease over time. These results express that there are lots of changes occurring between groups and between diets over time. Yet these may just be as a result of biological variation rather than a definite environmental or age factor causing a consistent trend. Analyzing these eggs over a longer period of time is optimal to conclusively evaluate these properties.

4.3.5 Age & Diet Study 2

This study assessed age and diet in hen eggshells. The birds were on the same diet as the previous study (generic feed and mineral supplement). The eggs were supplied from a different supplier farm than the previous study. This study consisted of four time points. Nanoindentation was the only mechanical test performed. Results can be observed in **Figure 4.3.17.**

This study presented with a similar narrative to the previous Age & Diet study 1; the mineral supplement and generic feed are not impacting the eggshell differently in a consistent manner. Hardness results provided by indentation tests indicate that both diet groups increased in hardness at Week 74, to decrease at the final time point. Stiffness results indicate that the mineral supplement group tested with more variation over the study time. When comparing this to the generic feed group however, there was no clear difference or trend emerging. Over time the increase in stiffness decrease for the mineral supplement group. Weight does not appear to increase significantly. Thickness measurements are consistent to previous studies and do not change significantly.

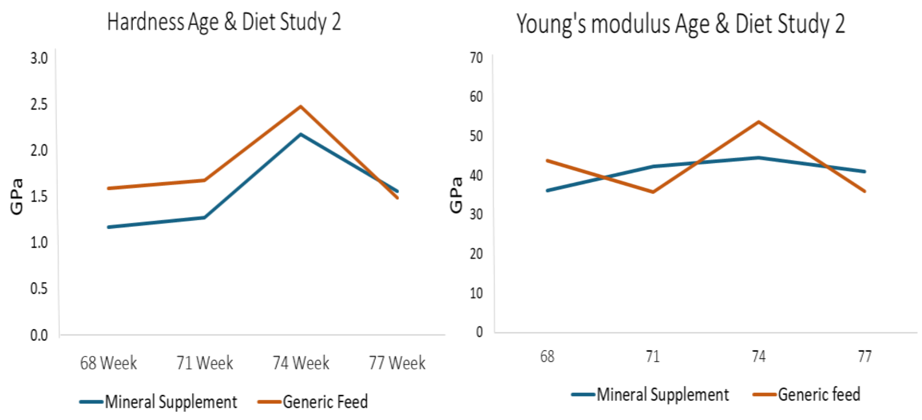


Figure 4.3.17 Young's modulus and Hardness results for both diet groups over time for farm suppler eggs (Age & Diet Study 2).

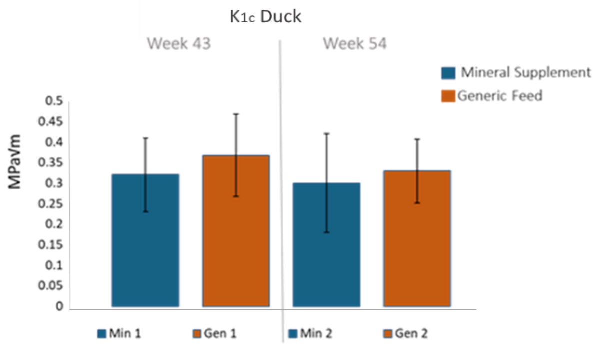
4.3.6 Age & Diet Study 3 Duck Eggshell

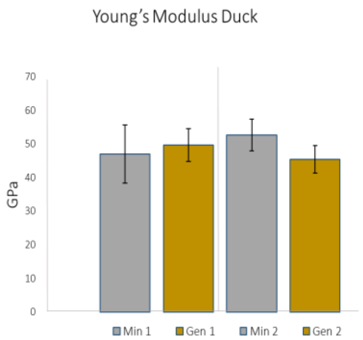
A study was completed to assess the mineral supplement feed in breeder duck eggshell. The same mineral feed supplement was used as in Age & Diet Study 1 and 2. Furthermore, this test group was shortened due to Covid-19 implications. As it was only based on two time-points, no observations over time could be made. Nevertheless, the study is still relevant to include as it is assessing a different avian species. Table 4.3.6 and **Figure 4.3.18** present the results from the mechanical tests performed.

Table 4.3.6 *Duck eggshell results from fracture toughness and nanoindentation tests.*

		Weight (g)		*Thickness (mm)*		*K_{Ic} (MPa√m)*		*Hardness (GPa)*		*Young's modulus (GPa)*	
Week	Feed	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
43	Min	93.6	4.6	0.42	0.05	0.32	0.09	1.63	0.4	47.1	4.1
43	Gen	90.7	3.9	0.44	0.04	0.36	0.08	1.89	0.3	49.6	6.3
54	Min	92.5	6.7	0.45	0.02	0.31	0.1	1.74	0.5	52.6	3.8
54	Gen	90.19	5.3	0.46	0.04	0.33	0.07	1.87	0.3	45.4	4.8

Based on the data from Table 4.3.6, there was no statistically significant difference across all mechanical test results obtained. **Figure 4.3.19** displays surface images of duck eggshell obtained from nanoindenter.





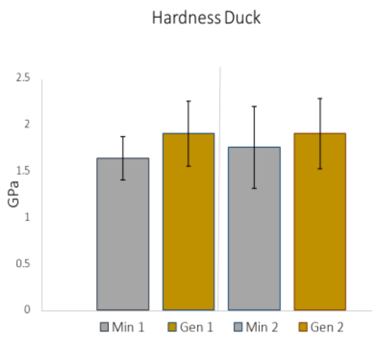


Figure 4.3.18 Fracture toughness and nanoindentation results for duck eggshell farm suppler eggs (Age & Diet Study 3).

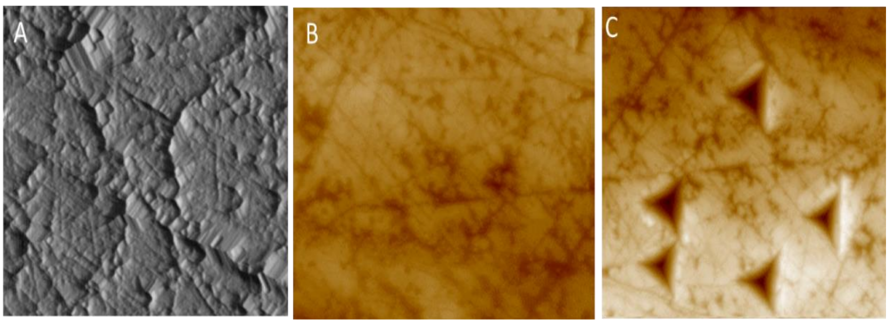


Figure 4.3.19 Images of duck eggshell surface using nanoindenter. A. Computationally composed image of untouched eggshell surface. B. In-situ image of eggshell. C. In-situ image of eggshell after indentation.

4.4 Species Comparison

The comparison of properties across species was performed. The hen data that was used was a random collection of results from Generic feed across Age & Diet Study 1. When comparing the duck and hen, it is clear that duck eggshell is larger and heavier than that of the hen (see **Figure 4.4.1**). Indentation test results did not show a great difference between the species (**Figure 4.4.2**).

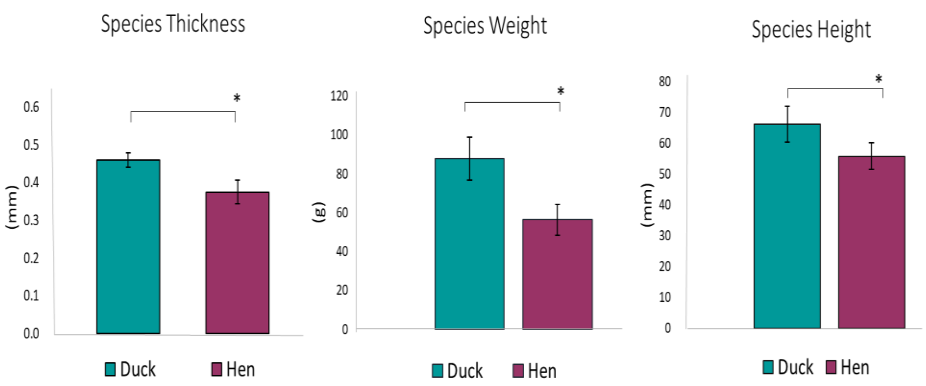


Figure 4.4.1 Thickness, egg weight and egg height (major axis) between species from farm supplier source. All of these comparisons show duck to be significantly thicker, heavier and larger than hen egg from Age & Diet Study 1.

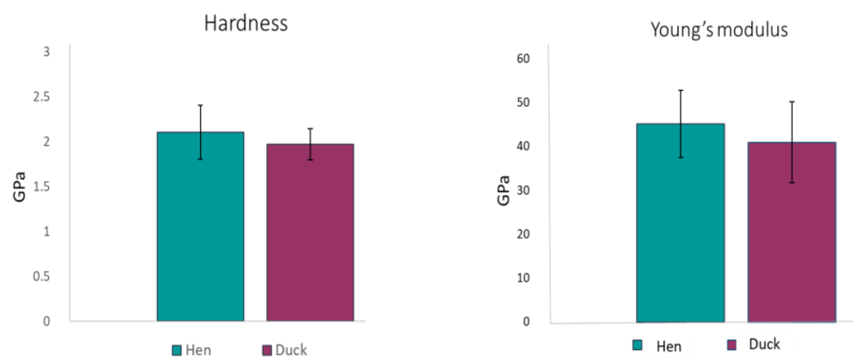


Figure 4.4.2 Nanoindentation test results for duck and Age & Diet Study 1 hen eggshell, both were farm supplier sourced eggs. No statistical differences were observed between species.

The fracture toughness results were different when comparing species. Duck eggshell had a significantly lower K_{Ic} compared with that of hen which can be seen in **Figure 4.4.3**. When compared against store purchased eggs for the Size Analysis study however there was no significant difference. The average K_{Ic} value from this study was 0.28 MPa√m.

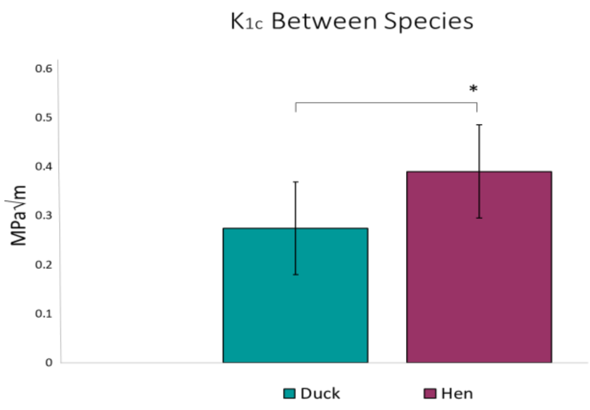


Figure 4.4.3 Fracture toughness average between hen in Age & Diet Study 1 and duck eggs. Statistical significance was seen when comparing the two.

4.5 Additional Analysis

The following results are based on Size Analysis and Age & Diet study 1. Data from each shed in the Age & Diet study 1 (*Shed A, Shed B, Shed C* and *Shed D*) was used in these examinations. Undoubtedly, pooling data together with different variables, *i.e.,* breed and diet, is not optimal. Upon reflection of the results from these previous tests it is clear diet changes did not create any clear differences between groups (generic feed and mineral supplement). The results between bird breeds (Lohmann and Hyline) was also not seen to be statistically different. The following examinations are based on general hen eggshell and so the justification for pooling results together was accepted. However, it is obvious that the differences in variables should be considered upon reflecting on the following results.
The section 4.5.2 Probability Analysis presents data from eggs in the Size Analysis which were separated by weight into medium large and very large (which were store purchased). The probability analysis of this group is compared with eggs from Age & Diet Study 1.

4.5.1 Age, weight and thickness on eggshell mechanical properties

Eggs were analysed based of the age of the parent bird. The age was measured in weeks. The effect of age on eggshell strength is apparent in **Figure 4.5.1.** From these results, the first time point (week 22-24) which was significantly different from the other weeks. The following age groups were not different from each other. This suggests that further analysis would be beneficial to assess any other changes which may occur with eggs from birds older than 43 weeks.

Egg weight is important as it is the measure in which eggs are separated out on production lines. Medium, large and very large eggs are defined by their weights. Weight analysis was considered in order to determine characteristics of these weights. **Figure 4.5.2** presents a linear regression curve of the relationship between strength and egg weight. The R^2 value does not suggest a strong interaction between failure stress and weight (0.207). However, the correlation coefficient was calculated as -0.46, which suggested a moderate correlation between the two variables. These two statistical measurements are very similar to that of the analysis performed on the same variables in store bought hen eggs (Size Analysis Study). The Size Analysis study achieved an R^2 of 0.26 and a correlation coefficient value of -0.51 when correlating weight and stress at failure. The similarities of these statistical tests from two separate studies on hen eggshell solidifies the results. It provides further confirmation that there is a link between egg weight and strength, albeit not extensive, which is often mentioned in the literature that larger eggs have less strength than smaller eggs.

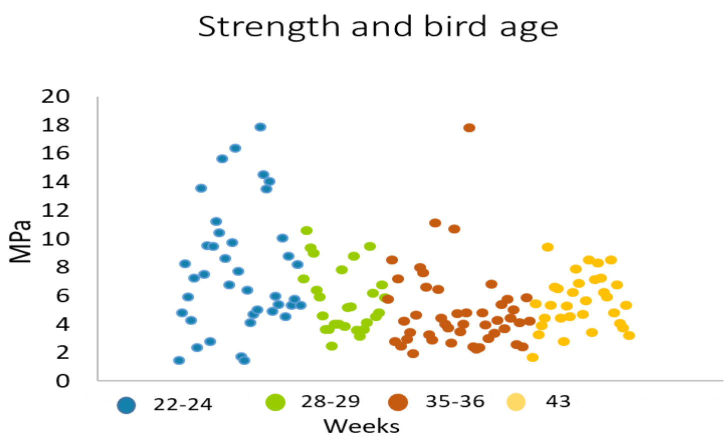


Figure 4.5.1 Age & Diet Study 1: Week 22-24 had higher stress values than following weeks. Only week 22-24 was statistically different from all other weeks. None of the subsequent weeks were different from each other.

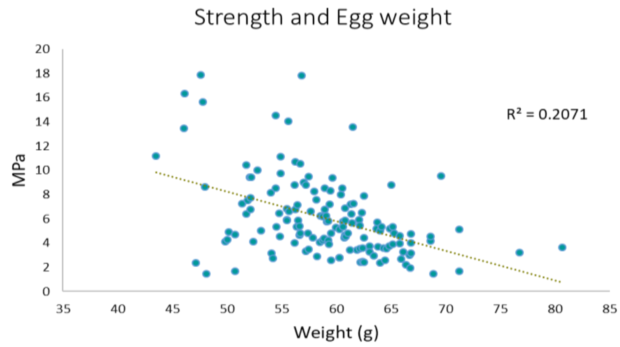


Figure 4.5.2 Relationship between strength and egg weight from eggs in Age & Diet Study 1.

Fracture toughness values were measured against bird age and egg weight. Upon evaluation of age and fracture toughness, it appears that Age and Diet 1 *Shed A* significantly impacts the result. When including this study, eggshell K_{Ic} increases significantly as the bird ages. When this study is removed from the entire group, fracture toughness increase is not significant, though there does appear to an increasing trend with K_{Ic} and age (see **Figure 4.5.3**). *Shed A* was the group which presented with a particularly prominant increase in fracture toughness over time and subsequent tests were performed in order to identify factors which influenced this result

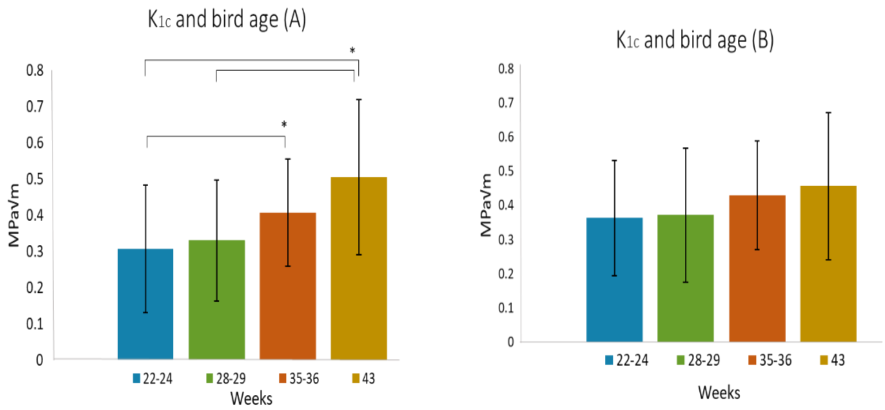


Figure 4.5.3 Fracture toughness and bird age relationship. A. Depicts results when all samples (Age &Diet Study 1) are compiled together and separated out by bird age. B. Removes Study 1 *Shed A*; as this study was the one with a significant increase in K_{Ic} over time, it is clearly influencing the results.

Fracture toughness and egg weight was compared. From this, it is apparent that there is very little, if any, relationship between these two variables. This weak correlation can be seen in **Figure 4.5.4.**

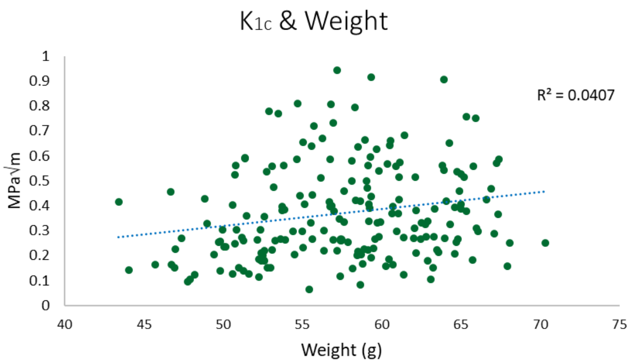


Figure 4.5.4 Fracture toughness and egg weight. No obvious correlation and R^2 value demonstrates a weak relationship between the two.

Differences between eggshell thickness and bird age were examined. From this analysis, it appeared that eggshell became slightly thinner in older bird eggs (**Figure 4.5.5**). It was only a single time-point which this decrease occurred (Week 43) and so it would be recommended that further measurements of thickness occur from subsequent bird ages.

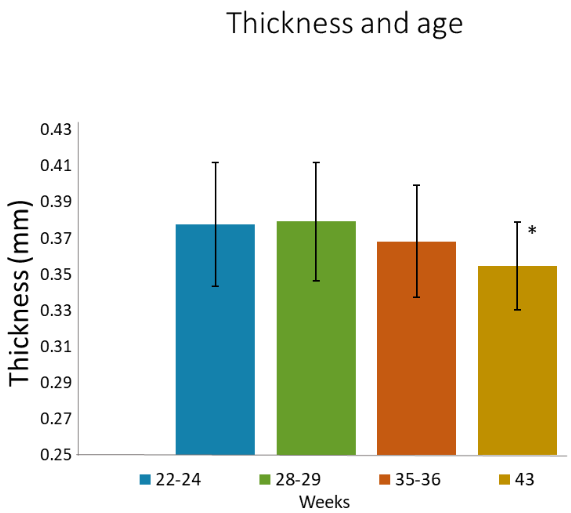


Figure 4.5.5 Thickness and bird age Age & Diet Study 1 farm supplier source. Week 43 saw a significant decrease in thickness values compared to all other previous weeks.

Eggshell weight and age was evaluated (**Figure 4.5.6**). The results from this examination displayed an increase in egg weight with eggs from older birds. Weights from all eggs from older birds (after week 22-24) remained similar. This is an expected result as suppliers routinely acknowledge eggs from older birds are larger, and more commercially desirable. Having only one time-point that is different from the others is not conclusively indicative of a trend. Therefore, it is proposed that assessment of additional time-points is recommended.

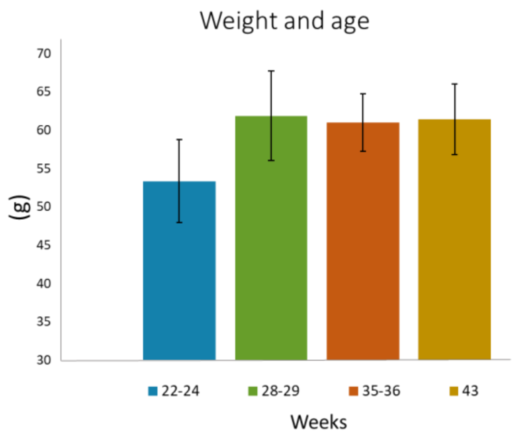


Figure 4.5.6 Egg weight and bird age Age & Diet Study 1. Eggs from birds aged 22-24 weeks displayed the lowest weight. This was statistically significantly against all other weeks.

4.5.2 Probability Analysis

From the quasi-static tests, strength values were collected and used in prediction analysis. Weibull modulus provides an indication of material consistency. Increased surface variabilities such as surface flaws, creates a broader probability range of the strength distribution curve (Lube and Danzer, 2014).

Weibull distribution and subsequent calculations were performed on the strength data from all eggs from Age & Diet Study 1 and Size Analysis study. Table 4.5.1 lists Weibull modulus data for each assessment.

Table 4.5.1 *Weibull modulus and Characteristic strength values of various egg groupings*.

Egg group	*Adjustment*	*Weibull Modulus*	*Characteristic strength (σ_0) MPa*
All eggs Age & Diet Study 1	No correction	2.43	6.68
All eggs Age & Diet Study 1	Volume correction	2.52	7.96
All eggs Age & Diet Study 1	Area Correction	2.49	7.73
Weight Age & Diet Study 1	Weight 43.5- 56g	2.24	8.71
Weight Age & Diet Study 1	Weight 56-62g	3.28	6.56
Weight Age & Diet Study 1	Weight 62-78g	3.04	4.61
Size Analysis Study Medium	-	3.72	16.7
Size Analysis Study Large	-	2.35	11.5
Size Analysis Study Very Large	-	2.42	8.57

From the initial Weibull probability assessment on all eggshell in Age & Diet Study 1, corrections for these calculation were performed taking volume and area into account. This was performed in order to examine the effect of these parameters on this probability analysis and to normalise the data in respect to these two geometric measurements. **Figure 4.5.7** depicts the relationship found between area and strength, and volume and strength. It was established that area and strength had a very low R^2 value from the curve (0.087). The correlation coefficient was found to be -0.29. Volume and strength displayed a higher R^2 value of 0.127 and a correlation coefficient of -0.357. Volume appeared to have a greater influence on eggshell strength compared to area, although it is clear that both area and volume do not have a strong correlation with strength results.

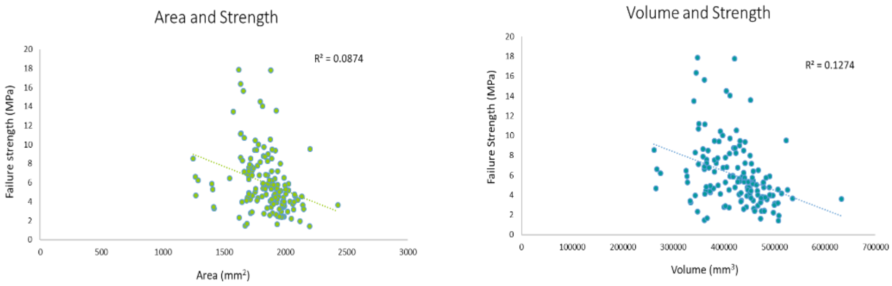


Figure 4.5.7 Relationship of area and volume with eggshell strength from store purchased eggs.

A Weibull distribution curve can be seen in **Figure 4.5.8**. Here, the eggs with no corrections, eggs with volume calculation correction and eggs with area calculation correction performed are displayed. This curve demonstrates that there is little difference observed when the calculation is altered to include volume or area corrections. The R^2 values for each of these curves are very similar and indicate that the range of scatter from the linear trend line is quite narrow.

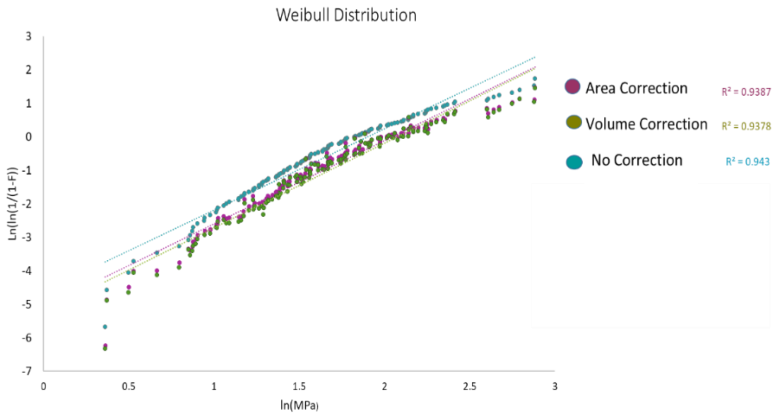


Figure 4.5.8 Weibull distribution of store purchased eggshell examining differences between calculation corrections.

The characteristic strength and Weibull modulus for all three calculations are very similar (Table 4.5.1). This suggests neither the area nor volume correction within the probability calculation is creating an extensive difference to the analysis.
Overall the Weibull modulus obtained was lower than that reported of eggshell (3.3- 5.5) (Macleod *et al*., 2006). However, it according to Meyers and Chawla (2009) traditional ceramics have a Weibull modulus of less than 3. This indicates that eggshell in this study falls within the expected range, albeit slightly lower than reported on eggshell by other

researchers. **Figure 4.5.9** shows a probability of failure curve of the hen eggshells in this project.

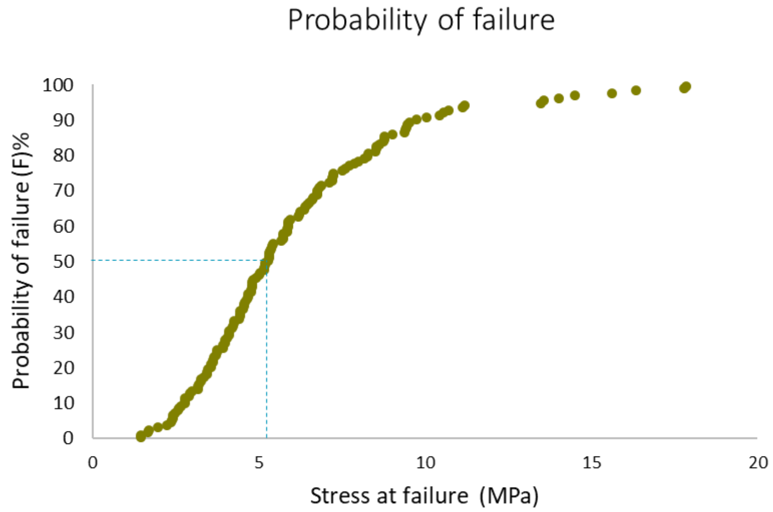


Figure 4.5.9 Probability of failure all store purchased egg samples. This graph illustrates that 50% of eggs will fail at a stress of approximately 5.1 MPa or less.

An examination of the same parameters (*i.e.,* Weibull modulus and characteristic strength) was performed on eggs from Age & Diet Study 1. The eggs from this cohort were separated out by weight. It was of interest to examine the probability of failure of these eggs against the probability of failure found with the store purchased eggs from the Size Analysis study. Specific information can be found in Table 4.5.1.

Looking at the Age & Diet Study 1 firstly, the separation of eggs was performed based on egg weight. Ideally, the eggs would be separated out by weight in order to follow the size categorisation of eggs *i.e.,* medium, large and very large (British Lion Eggs, 2021). This could not occur due to the distribution of weight and sample number into these categories. Instead it was separated out so that there could be an even sample number in three weight groups. The groups were separated out as follows: group A: 43-56 g; group B: 57-62g; group C: 62-76g. **Figure 4.5.10** displays the Weibull distribution and failure probability curve. Group A, the smallest weight group, has the highest R^2 value. The characteristic strength (found in Table 4.5.1) is higher in the smallest weight group A (8.71 MPa) and decreased with increasing egg weight (B: 6.51 MPa and C: 4.61 MPa). This indicates that the eggs which weigh more have a higher probability of failure. This can be visualised in **Figure 4.5.10** from the probability of failure curve, group A displays lower probability of failure. This is followed by group B (middle weights) and group C (heaviest weights).

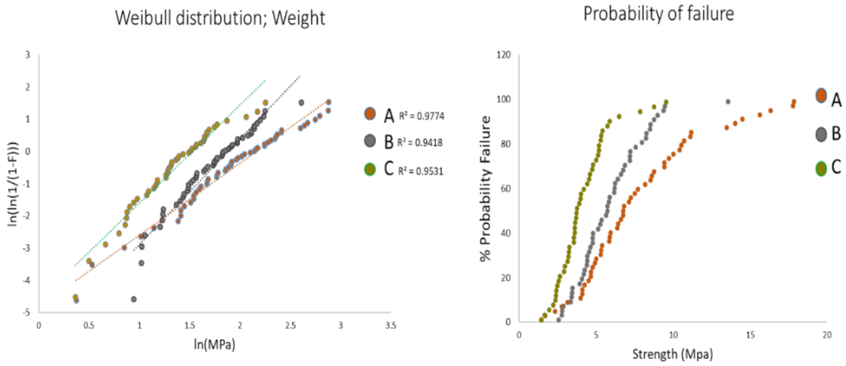


Figure 4.5.10 Weibull distribution and probability of failure curve of eggshell from farm supplier source Age & Diet Study 1 eggs. A: 43-56 g; B: 57-62g; C: 62-76g.

The Weibull modulus of these three weight groups (A, B & C) is 2.24, 3.28 and 3.04, respectively. The smaller eggs had a lower Weibull modulus value compared to the larger groups. A low Weibull modulus indicates that there is more variability within the sample (Roylance, 1985). However, the characteristic strength values decreased with larger eggs, but the distribution of results was narrower, hence a higher Weibull modulus.

Weibull probability analysis was performed on eggs from the Size Analysis study. Information can be found in Table 4.5.1. This group was composed of store purchased eggs separated out by commercial 'size' or weight (medium, large and very large). **Figure 4.5.11** displays the Weibull distribution and the probability of failure curve for the Size Analysis data. The R^2 value on the Weibull distribution plot was highest for very large eggs and lowest for large eggs. The Weibull modulus for was highest for medium eggs (3.72) and lowest for large eggs (2.35). This indicated that there is higher variability in large eggs compared to the other two groups. Medium eggs had the highest Weibull modulus and so this group had the least amount of variance. The characteristic stress increased with decreasing egg weight. This can be observed in the probability of failure curve in **Figure 4.5.11** where medium eggs appear to have a lower probability of failure and very large eggs have the highest probability of failure in the test group.

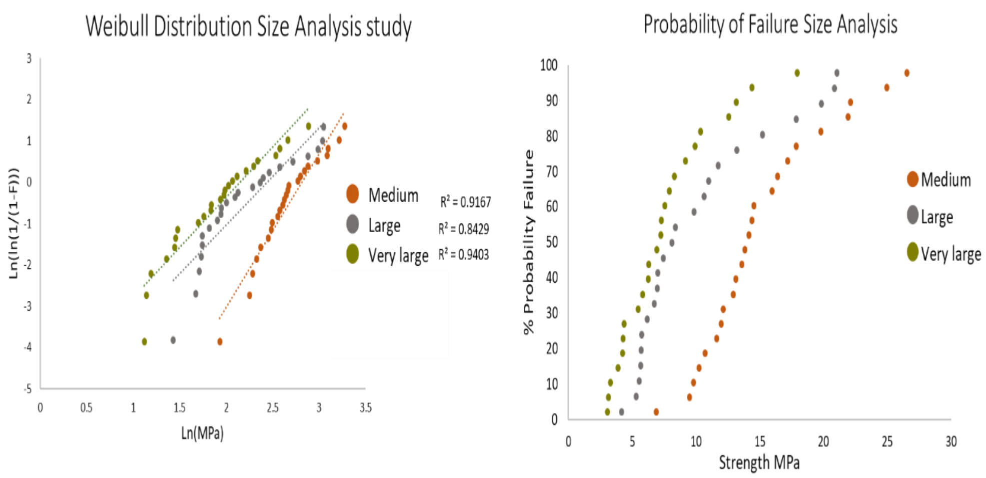


Figure 4.5.11 Weibull distribution and probability of failure curve on store bought eggs.

Both Age & Diet Study, when separated out by weight, and Size Analysis display a similar trend; heavier eggs are more likely to fail. It is interesting to see in Age & Diet study, the Weibull modulus was lower for the smaller group and increased with the larger group. The opposite occurred in the store bought Size Analysis study.

The Size Analysis study presented with greater characteristic strength values compared to the Age & Diet study. As previously mentioned, it is unclear why strength values in farm supplied eggs were lower than store bought eggs.

In addition to Weibull probability analysis, other probability methods were explored in an attempt to identify the impact that defects have on eggshell. In their 2016 paper, Taylor *et al*. attempted to address defect tolerance of eggshell by using various approaches. Modelled from that paper, the theory of critical distances (TCD) was used to predict failure in eggshell containing defects of varying sizes. **Figure 4.5.12** presents the prediction of eggshell failure as a result of flaws. Here, the TCD is measured, along with combining the TCD and Weibull modulus and finally the LEFM (linear elastic fracture mechanics), which incorporates the calculation used throughout this project (see Eq.3.1 and Eq.3.2 in Methods). For these predictions, certain factors needed to be given. The Weibull modulus used was 2.7, this was the average value found in eggshell from this project. The parameter which posed some difficulty in these predictions was σ_0. σ_0 is the tensile strength of the material containing no defects. As all eggshells contain defects, it was not possible to achieve a perfect value for this. However, based on the test results from the project, the average strength of eggshell from quasi-static tests with no 'pre-formed flaw' was 11 MPa. Thus, this value was used. In order to calculate the TCD prediction, the following equations were used which were obtained from

Taylor *et al.,* (2016). The critical distance was first calculated in order to obtain the TCD prediction. This was achieved by the following equation:

Eq. 4.1.

$$L=\frac{1}{\pi}\left(\frac{K_{Ic}}{\sigma_0}\right)$$

Where L is the critical distance and σ_0 is the stress. Once the critical stress is determined, the TCD prediction can be calculated:

Eq. 4.2.

$$\sigma_{tf}=\frac{\sigma_0}{1+\frac{a}{2L}\left(1-\frac{1}{(1+\frac{2L}{a})^3}\right)}$$

Where σ_{tf} is the TCD prediction factor, L is the critical distance, σ_0 is the stress and a is the crack length. The Weibull stress reduction factor is calculated using the following equation:

Eq. 4.3.

$$m_R = V_s^{1/m}$$

Where m_R is the Weibull stress reduction factor, V_s is the volume of material under the stress and m is the Weibull modulus of eggshell.

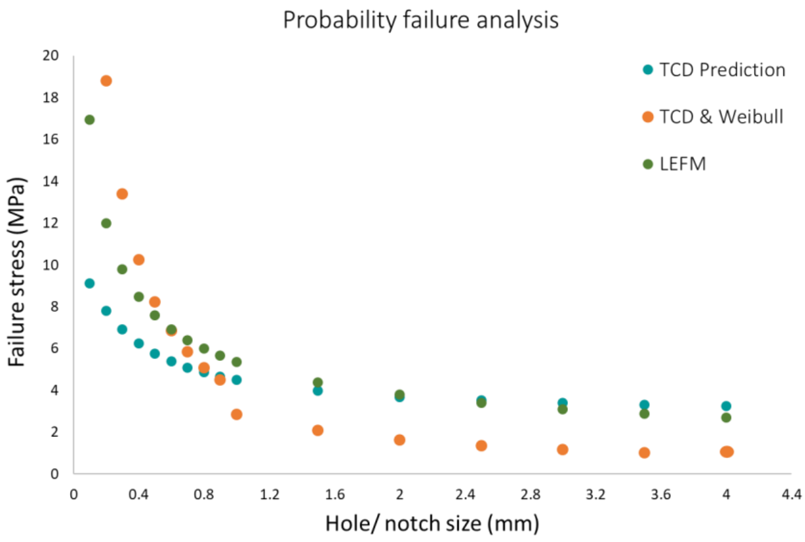


Figure 4.5.12 Probability failure stress as a function of the size of notch in the eggshell. TCD is the theory of critical distance approach. LEFM is the linear elastic fracture mechanics approach which was the one used in this project to measure stress and K_{Ic}.

In the calculations, when measuring failure predictions below a notch/hole size of 0.1mm, the results began to become unreasonable. This occurred for TCD & Weibull and LEFM. However, it did not occur for TCD method alone. For example, in the TCD & Weibull predicted failure of eggs, when the notch was 0.01mm, it gave a predicted failure stress of 500 MPa. Similarly, with the LEFM predictions, a notch size below 0.001mm predicted a failure strength of eggs to be 169 MPa. Based on practical understanding of the eggshell, it seems unlikely that it could ever display these strength values. This obviously displays that there is a limitation to examining the size of flaws and their impact on predicting failure. It is interesting that the TCD approach alone was able to predict a reasonable failure stress when the notch size was as low as 0.00001mm. Perhaps this method is more useful in prediction of failure as a result of flaw compared to the other two measures.

4.5.3 Nanoindentation layers

For Age & Diet Study 1, which were farm sourced eggs, nanoindentation was performed on various parts of the shell. Specifically, the specific areas of focus were the mammillary layer, the palisade layer and the upper palisade layer. From this, a collection of all layer data was compared to identify any differences overall in hen eggshell layers. Table 4.5.2 provides results from nanoindentation from these layers. The palisade layer has the lowest hardness in comparison to the inner mammillary layer and the outer palisade. This was seen to be statistically significant. **Figure 4.5.13** and **Figure 4.5.14** illustrates hardness and Young's modulus results.

Table 4.5.2 *Mechanical properties at various eggshell layers.*

	Hardness (GPa)		*Young's modulus (GPa)*	
Region	Mean	Sd	Mean	Sd
Mammillary	2.60*a	0.53	48.58*b	9.39
Palisade	1.95	0.34	41.82	7.59
Upper palisade	2.68*a	0.36	50.289*b	6.88

Hardness*a was significantly lower in the palisade layer compared to the inner mammillary and the upper palisade (p value <0.05). Likewise, Young's modulus*b was lower in palisade layer compare to the inner and outer layer.

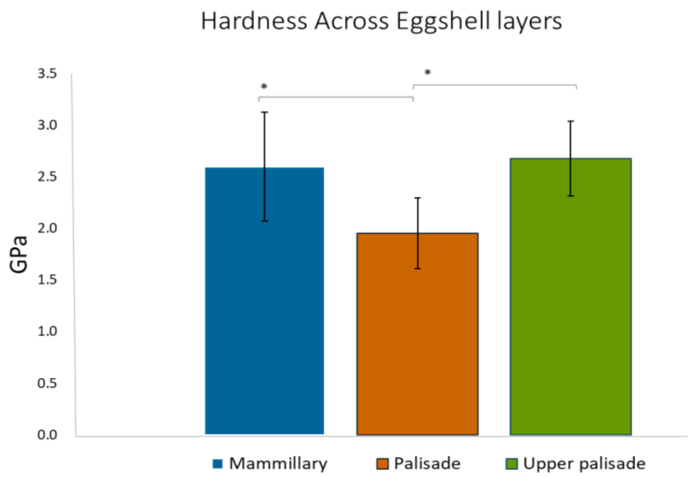


Figure 4.5.13 Hardness (GPa) results of eggshell across various layers. Mammillary and upper palisade were significantly harder than the inner palisade layer of Age & Diet Study 1 farm sourced eggs.

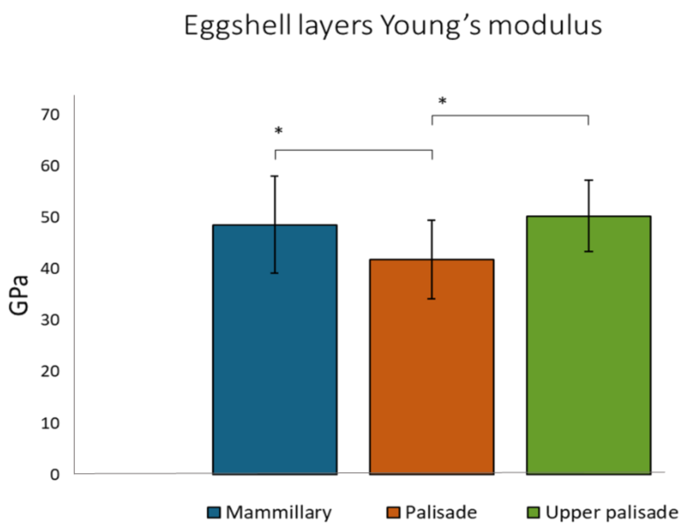


Figure 4.5.14 Young's modulus results at different eggshell layers of Age & Diet Study 1 farm sourced eggs. Mammillary and upper palisade layers had significantly higher Young's modulus than the palisade.

Stiffness and hardness both appear to be lower in the middle palisade region. The increase in stiffness and hardness in both the inner and outer portions of the shell is of interest and corresponds with other research which performed the same analysis on eggshell (Athanasiadou *et al.*, 2018).

Chapter 5 Discussion

5.1 External influences

External factors which impact eggshell were assessed. Bird age and diet were the two external factors assessed in this project.

A factor in this project which should be considered before examining the results in detail is the age of the birds used. Specifically referring to Age & Diet study 1, the most in-depth and longest time frame studied was birds aged 22 weeks to 42 weeks. The lay span of birds, hens specifically, is much longer than that. This age group would be representative of 'early' to the start of the 'mid' stage of hen lay (Roberts *et al*., 2013). Considering this, the eggs used are not representative of 'older' birds. The later or 'older' time frames used in these studies (Age & Diet study 1) are in fact not old birds at all and they are just approaching the 'mid' lay phase. This should be taken into consideration when assessing differences between age groups.

5.1.1 Diet

The dietary assessment of eggshell was carried out. Organic trace mineral supplement and generic avian feed was provided to birds. The mechanical analysis revealed that the mineral supplement had no clear influence on the eggshell. There is large degree of variation in the present Age & Diet studies, which is expected from a naturally formed product. There are two studies which present significant differences between diets. In the Diet & Age study 1 *Shed B*; the stiffness results indicated that mineral supplement diet eggs increased in stiffness over time and decreased over time in the generic diet. This suggests that the mineral supplement diet correlated to an increase in stiffness of these eggs whereas the generic feed presented with a decrease in stiffness. However, in *Shed C*, Young's modulus results depicted the opposite occurrence; eggshell from mineral supplement diet decreased in stiffness and eggshell from generic feed diet increased over time.

These results indicate that there is no conclusive difference in effects from either diet. The organic mineral supplement did not appear to alter the eggshell mechanical properties in anyway. Yet, there was some evidence from the literature which suggest there could be a difference had more time points been examined to conclude the benefit of organic trace mineral supplement in older birds. As this study was stopped at age 43 weeks, birds remain in a laying phase for a considerable time after this age. Assessing eggshell from young birds until the end of their lay cycle after receiving both diets all could increase the likelihood of observing a significant difference.

From the literature there is reason to believe that altering mineral content could influence eggshell mechanical properties (Mabe *et al*., 2003) (Zamani, Rahmani and Pourreza, 2005) (Stefanello *et al.*, 2014). However, there has been variation in suggestion that the source (*i.e.,* inorganic bound or organic bound) of this mineral supplementation has a profound impact on the eggshell. Stefannello *et al*. (2014) and Zamani *et al*. (2005) both proposed that organic bound mineral supplementation causes increase breaking strength (measured incorrectly as force). Mabe *et al*. (2003) presented results which were contrary to this and illustrated that there was no real difference in strength values obtained from different sources of mineral supplement. The time frame used for this test was significantly longer than the previous two tests mentioned. Mabe *et al*., (2003) used eggs from birds of 32 weeks to 82 weeks, a long study. Stefannello *et al*. (2014) and Zamani *et al*. (2005) used birds aged 47 weeks to 62 weeks and 28 weeks to 40 weeks, respectively. Mabe and team (2003) clearly performed a study over a longer period using a wider range of bird ages and perhaps the results from this study are more representative.

5.1.2 Age

Changes in eggshell over time were observed. Some studies provided more definitive results when assessing changes over time compared to others.

In the Results Chapter (4.5 Additional Analysis) the effect of age on eggshell was examined. Eggshell strength from aging birds was assessed (**Figure 4.5.1** in Results) and the results showed that there was a decrease in strength with the aging birds. The highest strength value was apparent in the eggs from the first time point (aged 22-24 weeks) and the decrease in strength occurred in the subsequent weeks. However, the strength for the next three time points remained consistent and unchanging. This means that only one time-point had significantly higher strength than the rest. Despite the decrease in strength occurring from one time-point, there is evidence to suggest that the decrease with bird age is present. This aligns with other research which observed a decrease in breaking strength with aging birds. Tůmová and team (2014) observed a significant decrease in breaking strength of eggshell from aged 22 weeks and 83 weeks.

The fracture toughness results displayed some change with bird age. It was apparent that *Shed A* from the Age & Diet Study 1 which displayed significant differences in K_{Ic} as the birds aged (see **Figure 4.3.1** in Results). When assessing eggs from the Age & Diet study 1, whilst omitting *Shed A*, there appeared to be an increase over time but it was not significant (**Figure 4.5.3** in Results). When combining all eggs, including *Shed A*, there was a statistically significant with increase in K_{Ic} with bird age. From this, it can be proposed that even though *Shed A* was the only group to display obvious changes in K_{Ic} over time, that an increase in K_{Ic} with bird age was still apparent, even with the omission of eggs from *Shed A*. Thus, indicating an increase in fracture toughness with eggs from hens between age 22 weeks to 43 weeks.

The intriguing result of increased fracture toughness with bird age provoked a need for further investigation to deduce the reason for this change in material property. Subsequent structural and elemental tests were performed in order to identify the cause of these mechanical changes. These were performed on eggs from *Shed A*. The statistically significant difference in K_{Ic} over time occurred for eggs from both feed diets, which dismissed the suggestion that the variation in feed was responsible. In contrast to the fracture toughness of the eggshells from *Shed A*, the strength results did not appear to be following a similar trend (**Figure 4.3.2** in Results). Instead what was seen was an increase in time-point 2 only to decrease again for the following time-points. It is clear from these eggshells that the ability to resist crack propagation increased over time, whereas the strength results remained relatively unchanging, perhaps slightly decreased.

It was unclear as to why this change was occurring and a number of arguments were derived. Eggshell structure and elemental composition were assessed. Structural analysis was completed to assess crystallography and porosity. The change in K_{Ic} needed to be clarified and external factors such as diet was ruled out. Intrinsic changes in the eggshell itself needed to be assessed to aid in the identification of mechanical changes.

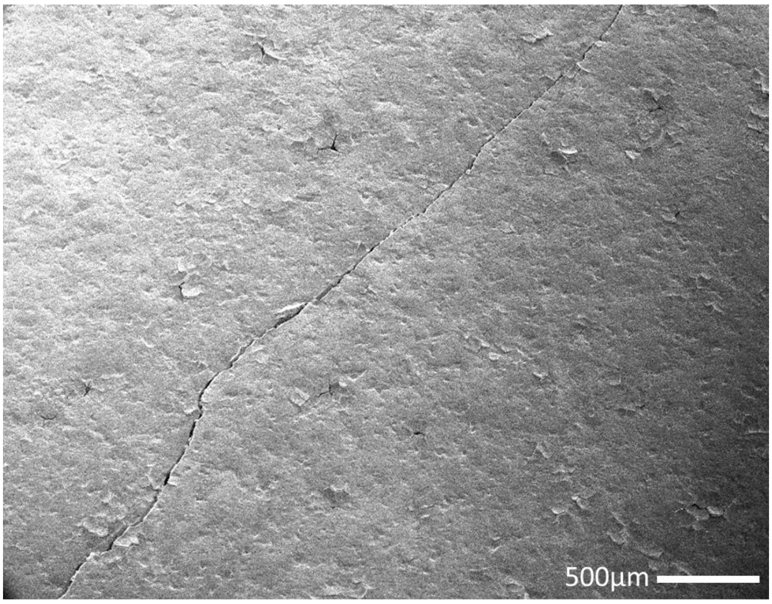


Figure 5.1.1. Crack on hen eggshell surface from fracture toughness test (project image).

Crystallographic assessment was performed using 2D-XRD. Crystal orientation was discussed previously as a potential driving factor for changes in eggshell mechanical properties. It has been proposed that smaller and less oriented crystals in the eggshell cause a crack tip to change direction more frequently which requires more energy, resulting in increased crack resistance (Rodriguez-Navarro *et al.*, 2007) (Rodriguez Navarro *et al.*, 2002) (Ahmed *et al.*, 2005). Because of this theory, an assessment of the crystal size and orientation within the shell was made. Crystal size, of which intensity is an indicator ({104} intensity peak), displayed variation over time but did not present with any consistent trend over time (see Table 4.3.3). Intensity decreased in the second and third time point and increased again with the final time point. The peak intensity ratio of the *hkl* planes {110} and {104} is said to increase with increased preferential orientation of the crystal (Rodriguez-Navarro *et al*., 2007). What was apparent from the intensity ratio results was that intensity increased in the second time point and decreased again at the final time point.

The FWHM angular breadth is also thought to be an indicator of crystal orientation. The higher the FWHM, the larger degree of misorientation of crystals (Rodriguez-Navarro *et al*., 2007). From the results, the FWHM were similar to the intensity ratio in that there was an increase in the second time point and a decrease in the final time point.

These results obtained from the 2D-XRD technique present with inconsistent changes in the shell crystal which does not align with the fracture toughness results. It can be implied that the crystal orientation and size within the shell may not be the reason for the change in K_{Ic}

results over time. Crystal changes in the aging bird does not appear to be drawing any trend *i.e.,* increase or decrease in crystal alignment over time. Perhaps bird age does influence the crystallography of the eggshell or the time frame used was not adequate to see definite changes. As the birds in this study were from early to mid-lay phase, looking at shells from birds in the older phase may be beneficial in seeing differences. Nevertheless, it is clear that from this test, crystallography does not appear to influence the fracture toughness results in this study.

Porosity analysis was performed in an attempt to understand the fracture toughness results in *Shed A*. It was clear that pores travel directly through the shell (Figure 4.3.10 in Results). The results displayed no significant differences in the pores from the data. However, when identifying the volume of the largest pore present, an increasing trend and increase in variation over time was apparent (Figure 4.3.8 in Results). The variation in largest pore volume from the eggs from the older birds may be indicative of greater pore size differences in these eggshells. In addition, pore diameter of the largest pore was observed to increase over time (Figure 4.3.8 in Results). The mechanical data demonstrates that the material increases in toughness, but the strength remains relatively unchanging, perhaps with a slight decrease with bird age. If the material itself is in fact getting tougher, strength would presumably increase too. This was not the case. Therefore, perhaps the increase in pore size is having an effect on this. Overtime, the material itself is becoming tougher, but larger pores are present, which means that the material requires less stress to fail as there are large defects present. From the data at hand, it may explain why K_{Ic} is increasing with time but strength is not. The sections of the egg used for pore analysis were taken from the equator, as load compression tests displayed that failure occurred under axial loading at the equator (Hahn *et al*., 2017) the presence of these large pores at this location may be significant in how the egg fails.

How or why pore volume is changing in ageing shells poses a question. It has been observed that increased porosity correlated with decreased incubation time for a developing bird (Zimmermann, Hipfner and Burger, 2007). Although pore frequency was not seen to increase over time in the current study, pore volume did. Perhaps the birds aged in early to mid-lay phase are producing eggs with changing pores volume to ensure quicker incubation for their offspring. In addition, perhaps the increased fracture toughness is also a factor of the birds reaching a 'peak' lay health and eggshell health in their lay life cycle.

From the data an additional theory can be drawn. As eggs increase in size as the bird ages and the eggs showed that there was only a very slight change in thickness, perhaps the bird's reproductive system becomes less 'compact' and therefore the eggs become larger, but the actual content of material for shell is unchanging. To make up for the growing egg size, the shell develops larger pores, which takes up more space to allow for the shell material to successfully cover the shell appropriately. This is simply a theory and not based on studies, therefore should only be taken as a suggestion. In a future study, muscular action during reproduction could be assessed with birds to identify if the stress applied to the developing egg during development changes over time.

The surface elemental analysis technique XPS showed some differences in results over the 4 time-points. The results are visible from Table 4.3.5 in Results. The first time point, birds aged 22 weeks, appeared to display differences in the elemental composition of the outer portion of the shell compared to the other 3 time points (see Figure 4.3.13 in Results). Calcium and carbon were found in lower concentrations in week 22 compared to other time points. This indicates that there was a relative lower concentration of calcium carbonate in the outer portion of eggs from the younger birds.

There is an indication that calcium carbonate concentration in hen eggshell can equates to a good quality shell (>2.2 grams) (Butcher and Miles, 2019). There is no indication from the literature that concentrations of calcium decreasing in the outer portion correlates to a decrease in mechanical properties. Furthermore, it can be theorised that from the current results that the lower concentration of calcium carbonate in the outer shell may be related to the low fracture toughness values in the younger birds' eggshell.

The mechanism of this is unclear. Perhaps the lower level of calcium carbonate impacts the crystalline structure. However, the differences in crystal from week 22 was not apparent from the 2D-XRD analysis. In addition, with the 2D-XRD analysis the inner and outer portion of the shell were not assessed separately. Examining crystallography across various locations within the shell may offer a better insight into what was observed from the XPS results. However, the correlation between lower calcium carbonate and fracture toughness cannot be confirmed.

Sodium and chlorine (usually found as sodium chloride or NaCl) were observed to be higher in the outer portion of eggshell from birds aged 22 weeks. In the shell gland during egg formation, NaCl has been seen to effect the enzyme calcium anhydrase. This enzyme has a functional role in the formation of calcium carbonate (Gutowska and Mitchell, 1945). Studies have found that NaCl has been seen to inhibit this enzyme, which can ultimately reduce the production of calcium carbonate (Yoselewitz and Balnave, 1989), (Balnave and Zhang, 1993),(Chen and Balnave, 2001). Although these studies measured NaCl in the shell gland and not in the shell itself, it could be suggested that the increase in NaCl in the shell could be linked to the decrease in calcium carbonate observed.

Belnave and Zhang (1993) reported a decrease in breaking strength in the eggshell which corresponded with an increase in NaCl in bird diet. It should be noted that, like many other papers which assess avian eggshell, ‘breaking strength’ as reported was measured in force only with no geometric parameters included and therefore incorrectly characterised as strength. However, what can be suggested was that less force was required to break the shells when an increase in NaCl was given to the birds. The authors suggested that there could be a link with NaCl having an influence on the mechanical properties of the shell.

With this theory, it is interesting to see this happening at the eggshell from the earlier time point, week 22, which saw the lowest fracture toughness results in the mechanical tests performed. Perhaps NaCl levels have an impact on eggshell mechanical properties by means of reducing the content of calcium carbonate which could represent what was seen in the fracture toughness tests. However, the NaCl and calcium carbonate concentrations did not correspond with this theory for the other time points *i.e.,* NaCl was not seen to decline over time and calcium carbonate was not seen to increase over time. Essentially it was only observed in week 22 and therefore more studies would be needed to prove this theory. Nevertheless, it is an intriguing result and proves that elemental analysis should be considered when assessing eggshell mechanical properties.

5.1.3 Supplier Source

Hen eggs were obtained from two sources: store purchased and direct farm supplied eggs. The mechanical tests that were performed on store purchased eggs were quasi-static compression and fracture toughness tests. These results were compared with results from farm supplier eggs from the same mechanical tests mentioned. What was observed was that the strength results from the quasi-static was higher in the store bought when compared to farm supplier eggs. Fracture toughness results demonstrated the reverse, where results were lower in the store purchased eggs when compared to the farm supplied eggs.

The range of K_{Ic} values is much wider than expected in the farm supplier eggs from Age & Diet Study 1. The K_{Ic} result for eggshell from the store purchased eggs in Size Analysis Study and other external reports (Taylor *et al*., (2016) and Tanaka *et al*., (2020)) was approximately 0.3 MPa√m. The results from the farm supplier eggs from Age & Diet Study 1 reach beyond this value (up to 0.72 MPa√m) suggesting the fracture toughness of these eggs have a broader range than the commercial eggs used in the other aforementioned studies. Perhaps the higher K_{Ic} values from the farm supplier were as a result of greater variation in supply. Quality checks for shell damage or flaws usually occurs onsite before being commercially sold. Perhaps the reason for variation in K_{Ic} values was just as a result of natural variation which could occur normally in, but with quality checks, only a particularly small standard of shell is obtained commercially. Or, perhaps the material quality of the eggs in the farm supplier study were just in fact superior to the commercial eggs used, but the lack of quality checks from these eggs meant that defects or flaws were more likely and therefore the eggs failed more easily *i.e.,* lower strength results.

It could be suggested that the lower strength values in commercial group eggs is a reflection on the fact that these eggs were tested straight from farm; whereas the commercial eggs used for the other studies were tested after they were processed through an on-site quality check. It is feasible that the farm supplier eggs did not go through the typical quality check and therefore eggs which may be discarded during the selection process were not. If a crack was present in an egg from the store purchased eggs, the egg may have been removed. With the eggs from the farm supplier, this may not have happened. Pre-existing flaws or defects may have been present in the farm supplier eggs and reduced the overall strength of the tested group. Perhaps the eggs which were used in the Age & Diet Study 1 had a mixture of quality.

It is not fully understood why the strength range is much lower than other reports when fracture toughness values range higher than other reports. The average strength value from the farm supplied Age & Diet Study 1 was 6 MPa. The store purchased eggs from the Size Analysis study reported the average strength of eggs to be 11 MPa. External sources using similar load techniques obtained values which range from 14.8 - 20.9MPa (Macleod, 2006) (Hahn *et al*., 2017). Macleod (2006) tested eggs directly from lay against commercially sourced retail eggs and discovered lower strength values for the latter. This result is not what was displayed by the eggs in the current farm suppler study.

The differences observed in mechanical properties from differently sourced eggs was not expected. Specifically, the strength results from the farm supplied eggs were lower than store purchased. The difference here is difficult to account for. Perhaps the breed of bird used is different, although two breeds were assessed in the Age & Diet study and there were no differences seen between them. The suggestion that quality standard checks onsite could play a role as to why there was such differences between supply.

5.2 Physical properties

This project assessed multiple egg measurements from multiple sources of eggs. From this analysis, some results coincided with what was already published in the literature, some of the results did not.

5.2.1 The Thickness effect

Eggshell thickness would be considered important in egg quality. It is suggested that eggs are larger from older birds and that the shells become thinner. According to Roberts and team (2013) hen eggshell becomes thicker in mid lay (aged week 40- 55) and gradually decreases as the bird ages.

For all of the eggs used in this project, conflicting results were observed. In store purchased eggs which were categorised into medium, large and very large, no difference in thickness was observed. There was no correlation with egg size and thickness as an R^2 value of 0.02 was achieved.

For the Age & Diet Study 1 (all sheds) it was significant that at week 43, eggshell was thinner than the previous weeks (for thickness results *Shed A, B, C* and *D* see Table 4.2.1, Table 6.2.1, Table 6.2.3 and Table 6.2.5 in Appendix, respectively). This drop in thickness was seen only at week 43.

In Age & Diet Study 2, which was also examining hen eggshell, albeit from a different farm source, a decrease in thickness was not observed (see Table 6.2.7 in Appendix). The age of the birds from this study were 68 weeks to 77 weeks. This age group would be considered 'old' in the hen lay lifespan. Interestingly, the results in this study were similar to Age & Diet Study 1, and not significantly lower. This suggests that eggshell thickness from these older hens may not be significantly reduced as previously thought. Of course to decidedly answer if eggshell thickness reduces with age, eggs would need to be obtained from the same birds at the start of their lay life span to the end of their lay life span.

The reduction in thickness observed in week 43 of Age & Diet study 1 poses a question. If these eggs are from birds in the mid lay cycle, why are their eggs reducing in thickness? Perhaps an external factor is at play such as an environmental change which could impact eggshell and alter the results from this time point.

One of the questions in this project was in relation to shell thickness and mechanical properties. If the eggs simply got thinner and therefore weaker, or if there were characteristic

changes based on factors such as age, size and diet. From the results, it appears that the changes in thickness over time are not as obvious as initially expected, if at all. For the different sized eggs, thickness was not seen to change. Referring this result back to the Size Analysis study, which used store purchased eggs, eggs reduced in strength as they got larger but also increase in fracture toughness as they got larger. There was no change in thickness. Therefore, it can be proposed that the difference in these mechanical properties was not a factor of thickness and that there is another mechanism at play. Perhaps the calcite crystal structure impacted these results.
Farm supplier eggs similarly presented with no relationship between weight and thickness (R^2 of 0.001). This demonstrates further that there was no association with egg size and eggshell thickness. These results further confirm that any mechanical differences observed from varying sized eggs is independent of thickness.

5.2.2 The Size effect

Egg size is an important parameter in the commercialisation of eggs as larger eggs are sought after by consumers. As previously mentioned, the plight of the poultry industry is to produce larger eggs that can withstand handling and transportation. Consequently, assessing changes in properties of varying egg sizes was an essential part of this project.
Initially focusing on the Size Analysis study, which was performed on store purchased eggs, egg size was decided by weight. Heavier eggs are advertised as being larger (British Lion Eggs, 2021). There was a positive correlation found between egg height (major axis length) and egg weight. This demonstrates that weight is an adequate marker for egg size. Furthermore, it was necessary to identify differences in eggshell properties based on this size measurement. It was discovered that eggshell decreased in strength when the size (weight) of the egg increased (see Figure 4.2.2 in Results). As revealed with this study, thickness never changed, therefore it cannot be related to the decrease in strength.
It could be proposed that with more material, *i.e.,* more eggshell, there is greater chance of failure due to pre-existing defects. However, this idea is conflicted when the fracture toughness results are taken into account. The fracture toughness appeared to have the opposite outcome to the strength results. The lowest K_{Ic} was obtained for medium eggs, which significantly increased in large and to a lesser extent, very large eggs (Figure 4.2.1 in Results).
An increase in fracture toughness suggests an increased amount of energy required to propagate a crack of a given length. The results suggest that with the larger sized eggs, more

energy is required for crack propagation. What is changing in the material for it to become less likely to fracture as it gets larger? But overall the material appeared to increase in size with a decrease in strength. Perhaps it is related to the crystal orientation and size. A decrease in crystal size and increase in crystal misalignment may increase the energy required for crack propagation. This theory is suggested by Ahmed *et al*., (2005) and Rodriguez-Navarro *et al*. (2007). However, this theory was not confirmed in the results from the 2D-XRD analysis (Table 4.3.3 and **Figure 4.3.6** in Results). Or, perhaps changes in porosity lead to the strength of the material decreasing because larger flaws are present, but the increase in intrinsic material property (fracture toughness) is increasing.

In Age & Diet Study 1 across all sheds, egg size was also assessed. Weight was seen to increase with aging birds, however only the first time point had a significantly lower weight; the subsequent time points had a similar weight to each other (**Figure 4.5.6** in Results). This outcome demonstrates that there was an increase in weight observed over time, even if it was slight. Looking further into the weight of the egg and the relationship between it and egg length, there was a positive correlation observed; with an R^2 value of 0.5 and a coefficient of correlation value of 0.81. This illustrates that egg weight is associated with other egg size measurements. Therefore, weight can be used as an indicator of egg size.

Egg size and mechanical properties was examined and there are varied results found to the Size Analysis study. There was a weak relationship between decreased strength and increasing egg weight (**Figure 4.5.2** in Results). Although the correlation was not very high, there was still a relationship between the two. This corresponds with the results from the store bought eggs in Size Analysis study previously mentioned. Thus suggesting that larger eggs are weaker.

The fracture toughness results from these eggs were not as clear as in the Size Analysis study. In the regression analysis of fracture toughness and weight, an R^2 and coefficient of correlation of 0.04 and 0.2 was achieved, respectively. Thus, indicating a very weak relationship between the two. Moreover, it is difficult to link egg size and fracture toughness when studies show conflicting results. It was not proved that fracture toughness changes based on the size of the egg. Although, it can be suggested that there is a possibility that there may be a relationship and further studies should be done to confirm the interaction between size and fracture toughness.

Probability analysis was able to provide a better explanation to eggshell failure and egg size. A Weibull probability analysis was performed on eggs from both Size Analysis and Age & Diet Study 1 (see Table 4.5.1). Eggs were separated out into three weight groups in the Age & Diet Study 1 from lighter to heavier; A: 43-56 g; B: 57-62g; C: 62-76g. The smaller eggs (A) were less likely to fail at higher strengths than heavier eggs (**Figure 4.5.10** in Results). The characteristic strength of the smaller eggs was highest, and decreased with increasing egg weight. The Weibull modulus however was lower for the smaller eggs (A: *m*=2.24) than the higher eggs (B: *m*=3.28 and C: *m*=3.04). Thus, proposing that there is greater variation observed in the smaller eggs.

The same probability assessment was carried out for store purchased eggs from the Size Analysis study. Similarly, it is evident that the smaller eggs (medium) were less likely to fail at higher loads than the larger eggs (**Figure 4.5.11** in Results). Similar to the Age & Diet Study 1, characteristic strength decreased with increasing egg weight. The Weibull modulus values for the differing weights was not consistent with Age & Diet study 1. A higher Weibull modulus was obtained for medium eggs (*m*=3.72) and the larger eggs presented with lower values (large: *m*=2.35 and very large: *m*=2.42). This suggests that the smaller eggs had less variation in failure strength results compared to the larger eggs. The opposite was seen for the Age & Diet Study 1.

These probability results indicate that lower weighted eggs, or smaller eggs, are less likely to fail compared to larger eggs. This probabilistic approach provides a valuable insight into failure mechanics of a brittle material. The results suggest larger eggs are more likely to fail and less stress is required for failure to occur. The difference in Weibull modulus between both studies is not completely clear. Weibull modulus is indicative of variance and requires a very large sample size (>30 samples) (Quinn and Quinn, 2010) and although this minimum sample size was achieved, a higher sample number may offer more accuracy.

5.2.3 Eggshell layers

Several tests performed in this project looked at different parts of the eggshell. Specifically, nanoindentation and XPS were performed on various locations and presented with differences observed across the shell.

Nanoindentation was performed on the cross section of the shell, the mammillary layer, the palisade layer and the upper palisade layer. Hardness and stiffness results were obtained from the test. Table 4.5.1 in Results contains the information regarding these tests. **Figure 4.5.13** and **Figure 4.5.14** in Results also presents these results. Stiffness between the layers behaved in a similar manner to the hardness results; the outer and inner layer were stiffer than the middle of the shell. The low stiffness and hardness in the palisade layer corresponds with other studies which performed a similar examination. The location on the eggshell and the corresponding hardness can be visualised below in **Figure 5.2.1**.

Athanasiadou *et al*. (2018) achieved a similar set of results on hen eggshell whereby the palisade was less hard and stiff than the mammillary and upper palisade. The authors of this paper interpret the higher stiffness and hardness in the outer portion of the shell to be linked to the increase in nanostructure in this region. The Hall-Petch theory is thought to be a factor in this increased hardness as the authors discovered that the outer portion of the shell has decreased subunit sized particles. However, this theory does not explain why the inner portion of the shell, the mammillary layer, has higher stiffness and hardness too. It is thought that perhaps this increase in property values is as a result of less structural homogeneity.

It poses a question of why there is a difference in hardness and stiffness across the eggshell. In metals, a process called 'case hardening' is used to produce a product which has a harder exterior than the inside of the material. The reason for this is because fully harder materials are often more brittle and more likely to fracture. The hard exterior is needed to avoid surface abrasion, and a softer inside can absorb more stress without cracking. The combination of the properties provides a more durable material (Bryson, 2015). Perhaps the results observed for eggshell is a natural adaptation of this 'case hardening' process. The middle palisade layer being softer in order to absorb stresses and minimize cracks occurring.

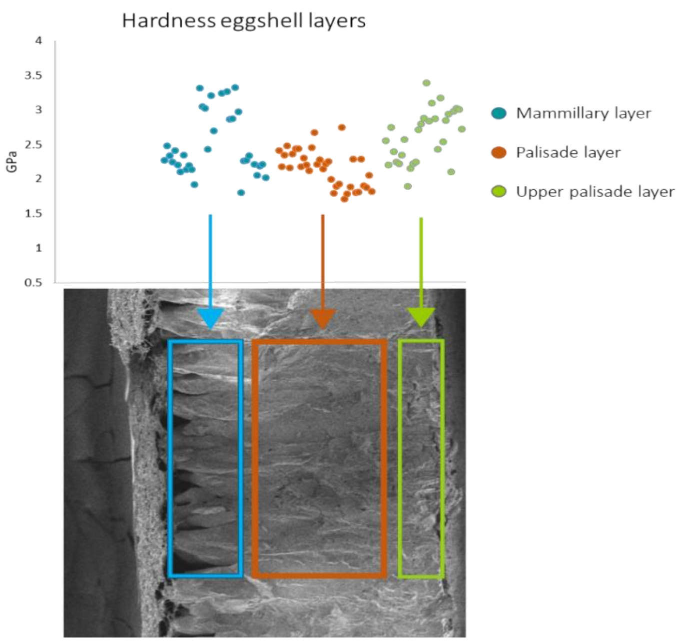


Figure 5.2.1. Hardness results and corresponding location on the eggshell (project image).

XPS surface analysis was performed on the inner and outer portion of the shell. This would equate to the mammillary layer and the outer vertical crystal layer. This elemental analysis produced results which indicated that elemental composition on the inner and outer portion of the hen eggshell is different. There appeared to be an increase in phosphorus from all time points in the outer shell (**Figure 4.3.12** in Results). Cusack *et al*. (2003) suggested that the reason for a rise in phosphorus in the outer portion of the shell is that phosphorus has an active part in the termination process of eggshell formation and actively inhibits calcium carbonate aggregation. It was also seen in that study that there was a higher concentration of phosphorus in younger bird eggs compared to older bird eggs. This was not seen in the XPS results here as the phosphorus concentration appeared relatively unchanging across the 4 time-points.

From assessing the inner and outer shell, what was discovered was that there was carbon species present on the inner eggshell, but not in the outer shell (**Figure 4.3.11** in Results). The results indicate that there is a peak on which indicate an amide, acid or ester present in the inner shell. The presence of these carbon groups suggest that there may be a functional role of these in the inner portion of the shell; or as a results of the organic membrane. Amides have been seen to inhibit calcite crystal growth (de Leeuw and Cooper, 2004). Perhaps this functional group is found on the surface of the inner shell as a result of inhibiting calcite crystals growing inwards. Little is known of esters and calcium carbonate crystal growth.

The results indicate that there are differences along the eggshell surface. Elemental and mechanical differences are evident. It is clear that there is scope for further analysis assessing the eggshell layers and perhaps greater understanding of the structure and composition could be achieved.

5.3 Probability analysis

Further probability analysis was performed in addition to Weibull modulus and characteristic strength. The LEFM and TCD approached modelled from Taylor and team (2016) was performed (see **Figure 4.5.12** in Results and Eq.3.14 and Eq.3.15 in Methods). The methods in question were used to identify the probability of failure in relation to flaw or pre-formed notch size. The results showed that LEFM and TCD were very similar once a notch size of 0.8mm was present. With lower notch size, the LEFM predicted higher strength than the TCD. The TCD and Weibull combination, which was performed to assess the TCD results using a Weibull correction factor (Eq.3.15. in Methods), was different to the other groups as it predicted higher eggshell strength at low notch sizes and lower eggshell strength at larger notch sizes compared to the other two curves. The notch size used in fracture toughness experimentation was approximately 4mm and the results from that indicated that the average strength achieved was 3.1 MPa. **Figure 5.3.1** demonstrates the probability results along with the results from the fracture toughness experimentation. It is apparent that from the experimental results that with a notch size of 4mm, the results fall alongside the LEFM test.

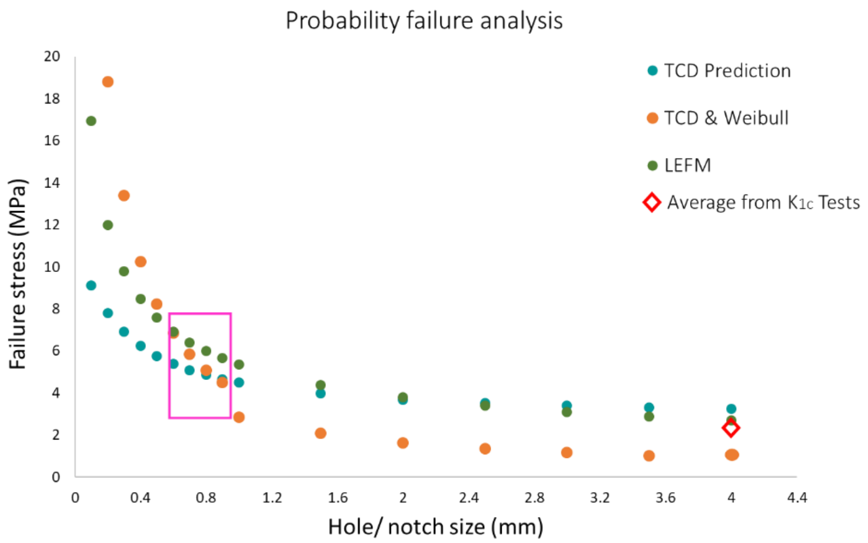


Figure 5.3.1 Probability failure stress as a function of notch size. The average notch size and failure strength is presented. The three types of analysis start to intercept each other from about 0.8mm.

The LEFM probability results align mostly with the results from the experimentation. This is expected as the calculation for LEFM in this probability analysis is the same calculation used for the experimental methods. The TCD predication, after the LEFM, is closest to the actual experimental result. The TCD & Weibull is the furthest from the actual results at notch length of 4mm. These results may indicate that the TCD approach offers an acceptable indication of failure prediction in relation to notch size.

As mentioned in the Results section, the predictions seem unreliable below a certain notch size. With the LEFM and the TCD & Weibull, the failure stress at notch lengths below that of 0.1mm displayed failure strength results far greater than expected, or realistic, for eggshell. The TCD approach alone did not display this unrealistic failure strength when notch length was below 0.1mm. This, along with the relatively similar result to the experimentation values, indicate that the TCD approach may be a useful method for eggshell failure probability analysis.

Despite the varying data, researchers have agreed that defects within the eggshell of certain size will reduce the strength of the eggshell. The agreement also confirms that theoretical analysis complements experimental data. Both Entwistle and Reddy (1996) and Taylor's (2016) teams proposed that a defect of more than 0.5mm will reduce strength of the eggshell. MacLeod and team (2006) declare that microdefects in the area of load result in irreversible failure. From the probability analysis in this project, it appears that from approximately 0.6mm the analysis starts to intercept each other and some agreement occurs between the three types. Perhaps this demonstrates that defects from this size start to dramatically impact the overall eggshell strength.

Very small defect size in probability analysis resulted in unreliable test results using the prediction methods. Therefore, it could be suggested that the probability analysis and experimental tests indicate that larger flaws in eggshell *i.e.,* 0.6mm, can definitely reduce the strength of the material.

5.4 Species Comparison

Species comparison was assessed between hen and duck eggshell. Duck eggshell was larger, heavier and thicker than hen eggshell (see **Figure 4.4.1** in Results). Duck eggshell appeared to be harder and stiffer than hen eggshell (**Figure 4.4.2** in Results). **Figure 5.4.1** depicts fracture toughness results across a variety of natural materials. Despite the superior size of duck eggshell, fracture toughness tests demonstrated that the shell were less resistant to crack propagation when compared to hen eggshell (**Figure 4.4.3** in Results). Perhaps the porosity or crystal grain orientation/size is significantly different in duck eggshell compared to hen eggshell.

There is little fracture toughness data on duck eggshell reported. A study by (Zhang *et al.*, 2022) reported achieving a K_{Ic} of duck shell, using the same calculation from Mabe *et al*. (2003), as being 512 N/mm$^{3/2}$. When converted, this equated to 16.2MPa√m. This would demonstrate that the K_{Ic} of duck eggshell from that study ranged beyond that of a ceramic (~10 MPa√m) (Vavro and Soucek, 2013). As previously discussed, the calculation provided by Mabe *et al*. (2003) does not demonstrate a correct measurement of K_{Ic} of shell.

It would be advantageous to have a more robust cross-species fracture toughness study to identify differences between shell species. Particularly, assessing shell structure (*e.g.* porosity and crystallinity) could provide better understanding of duck eggshell and perhaps develop an understanding of shell across various species.

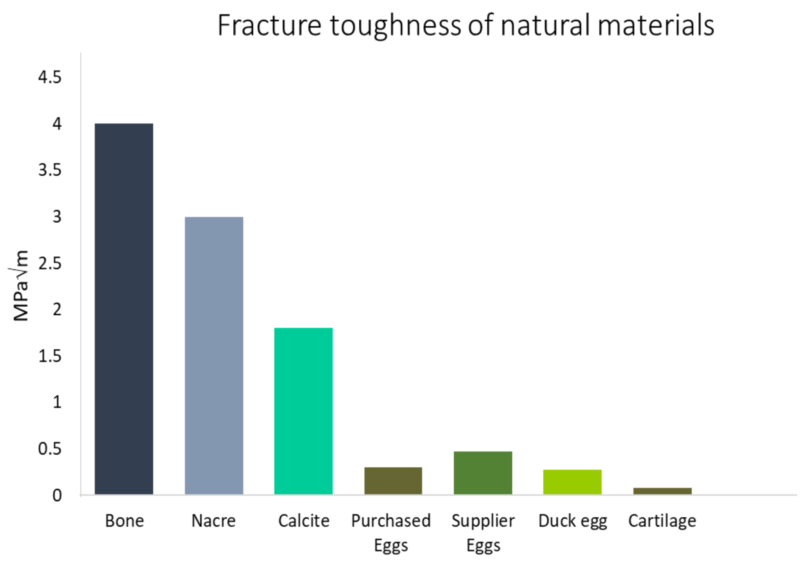


Figure 5.4.1 Fracture toughness of some natural materials. Bone from Ashby and Jones (1991); nacre from Currey *et al*. (2001); calcite from Vavro and Soucek (2013) and Lv *et al*. (2015), cartilage from Chin-Purcell and Lewis (1996); purchased eggs from Size Analysis study, supplier eggs from Age & Diet Study 1 and duck egg from Age & Diet Study 3.

5.5 Experimental Limitations and Future Analysis

There were several limiting factors to this project. Namely the age of the birds used and the number of time points assessed. As mentioned, in the most extensive study (Age & Diet Study 1) eggs were obtained from birds at the early to start of mid phase of lay cycle. This does not provide a comprehensive indication of eggshell behaviour from aging birds. The birds were not yet entering into an older phase and therefore it was difficult to conclude the changes over time or indeed compare to external research which looked at early and late lay phase. Examining eggs continuously for a longer period would be optimal.

In mechanical tests, which used egg cup holders, only axial loading was performed for fracture toughness and quasi-static compression tests. Loading at the minor axis or egg equator is suggested to obtain a better indication of failure upon different points of the eggshell. From these mechanical tests, it is also important to note that this experimental set up assumed full tensile stress along the egg equator. This may not be fully representative. Although the cup holders are thought to disperse the loading stress, compressive forces may still be occurring at the cup holder and egg contact point. Using FEA, Hahn *et al*., (2017) demonstrated that the maximum stress was located just below the cup and not directly on the egg equator (**Figure 5.5.1**). Whereas Taylor *et al*., (2016) discussed that the tensile stress is along the equator of the eggshell.

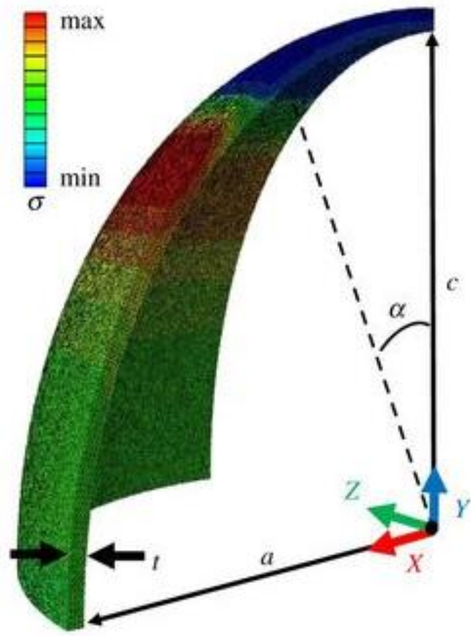


Figure 5.5.1 Hahn *et al*., (2017) demonstrated that the highest tensile stress is just below the loading area.

The location of the maximum stress across the egg when axial loading is applied is therefore not fully concluded. Including incorporating measurements of the cup in the equations may be useful in deriving a more realistic stress result. In this project, calculation of the failure stress of eggshell assumed a spherical shape. Eggshell is more similar to that of a prolate spheroid. Assuming the geometric shape of the egg to be fully spherical could alter the results as it is not a true representation of the egg shape. However, one suspects that the assumption of a sphere may not alter the results significantly.

Failure to identify the reason for increasing fracture toughness in older and larger eggs limits this project. Further analysis such as crystallography, porosity and elemental analysis was completed but did not definitively explain the change in material property observed. Perhaps the eggs were from birds reaching their peak health and therefore the production of their eggshell was also improved. Assessing eggs from these birds for a longer period would really aid in understanding this. The overall increase in pore volume over time perhaps is what inhibited the strength results from increasing. The presence of larger pores potentially allowed for lower stresses to propagate a crack, but the material itself was tougher and so inhibited failure from occurring quite as easily. Essentially the increase in fracture toughness mitigated against the strength from decreasing in the material despite the larger pores being present. This is a not conclusive but the results indicate that there is a likelihood of this being the reason for the mechanical results occurring in such a way *i.e.,* higher fracture toughness with unchanging strength. Fracture toughness tests in the future could be performed without the contents removed from both poles, therefore a more accurate representation of eggshell failure could be conducted.

It could also be proposed that the quasi-static compression test set up may not be very sensitive to material changes as the fracture toughness test. Therefore, the determination of strength data may not be as sensitive as required.

Assessing structure in more detail may help uncover the mechanical differences observed. For example, measuring the shape, size and number of mammillary cones amongst differing aged birds could be beneficial. There have been suggestions relating to mammillary cones density and mechanical properties from external sources (Park and Sohn, 2018). Assessing crystal size using cross-polarized light microscopy as seen from Rodriguez-Navarro *et al*., (2007) may act as an additional study to help identify material changes over time and with varying diets.

5.6 Conclusion

The project successfully tested the effect of organic mineral supplement diet on eggshell. It confirmed that there was no mechanical change observed in Lohmann classic and Hyline breed when the supplement was introduced to the bird diet in birds aged from 22 to 43 weeks. In the Age & Diet Study 1 study, fracture toughness increased as the bird aged. In addition to this, volume of the largest pores also increased with bird age. A suggestion for the increase in fracture toughness could be that the birds were reaching the peak health of their lay cycle and therefore the eggshell material was produced more efficiently. The pore changes could also be determined by the birds reaching peak lay term, and increased porosity was seen to correlate with decreased incubation period for developing chicks (Zimmermann, Hipfner and Burger, 2007). Another theory regarding this pore change observed is the reproductive tract of the bird becomes less compact with age and therefore the eggs become larger to fill the space. In order to successfully cover the content of the egg with shell larger pores are created to decrease the amount of material needed (this is not based on any study and is simply a theory. It would be interesting to assess egg porosity in aging birds and deduce the reasons for any changes).

Weight of the eggs was concluded to be a good indicator of overall egg size. What was discovered in the Size Analysis study was that strength decreased with increasing size and fracture toughness increased with increasing size. Age and Diet Study did not display a clear relationship between weight and the mechanical properties. Upon statistical evaluation, it was observed that both Age and Diet Study and Size Analysis did indicate that larger eggs resulted in a higher likelihood of failure. The characteristic failure strength decreased with increased egg weight for both study groups. Thus, suggesting that larger eggs are more likely to fail. The suggestions for this could be related to pore size increasing or changes in crystal size, this is not confirmed and would need to be proven in future. In addition, it was observed that thickness off the eggshell is in fact relatively unchanging.

Mechanical changes were observed across the various layers within the shell using nanoindentation. Hardness and stiffness was high in the inner (mammillary) and outer portions (upper palisade), but decreased in the middle (palisade layer). What was suggestion for this was that potentially this is similar to the metal process of case hardening where the outer portion of the material is harder to resist abrasion but the inner portion of the material is softer to absorb shock (Bryson, 2015). It is possible that the eggshell acts in a similar manner to this process. It is noted that eggshell does not behave in a manner of metals (*e.g.* plastic deformation) but the processes mentioned could be linked to a certain degree.

TCD offered an alternative failure probability approach and it was discovered that the results from the TCD data aligned reasonably well with the experimental data. It was observed that below a notch size of 0.4 mm, failure results began to appear unreliable. It was suggested that the probability analysis determined that a notch length from 0.6 mm reduced the strength of the material.

Although there are differences among values obtained in tests, more studies will need to produce results which we can fully confirm factors which impact shell mechanics. Greater knowledge of the material properties of eggshell will benefit the poultry industry as it can lead to innovative ways of changing the production line to produce less waste. This project demonstrated that there is scope for future work in assessing eggshell failure and the intrinsic and extrinsic factors that could influence it.

Chapter 6 Appendix

6.1. Supplementary information from Methods

Table 6.1.1 *Testing Equipment information and location of access. Note XPS and XMT tests were completed externally by other parties.*

Equipment	*Product Identification*	*Access location*	*Company*
Loading machine	Z5 5kN Universal Test Machine	DCU	Zwick Roell Ltd. Herfordshire, UK
Light microscope	3D digital Microscope	NRF, DCU	Keyence UK Ltd. Milton Keynes, UK
SEM	JSM-IT 100 InTouchScope	NRF, DCU	Jeol Ltd. Herfordshire, UK
Nanoindentation	HYSITRON TI-Premier	NRF, DCU	Bruker, Karlsruhe, Germany
Surface analyser	Contour GT 3D optical profiler	NRF, DCU	Bruker, Karlsruhe, Germany
2D-XRD	APEX-DUO	School of Chemistry, TCD	Bruker, Coventry, UK
XPS	Axis 165 Spectrometer	Bernal Institute, UL	Kratos Analytical Ltd. Manchester, UK
X-ray µCT	Nanotom	Waterford IT	Waygate Technologies, Germany
Surface gold coater	Scancoat Six Sputter Coater	DCU	HHV Ltd. Sussex, UK
Scales	AT261 Delta range	DCU	Meller, Leicester, UK
Callipers	Digital callipers	DCU	Hilka Tools Ltd. Surrey, UK

Table 6.1.2 *Information regarding consumables used.*

Product	*Product Information*	*Company*
Epoxy resin	AKA-Cure Slow & AKA-Resin liquid epoxy	Akasel, Roskilde, Denmark
Sanding paper	P800, P1200 and P2400 grit size	Rhaco Grit, Akasel
Diamond suspension	9µm, 6 µm, 3 µm and 1 µm particle size	MetPrep Ltd. Coventry, UK
Colloidal silica	0.06 µm particle size	MetPrep Ltd. Coventry, UK
Polishing clothes	Trounoir polishing clothes	MetPrep Ltd. Coventry, UK
Sponge	Absorbent cellulose	Tesco
Sodium Hypochlorite	Sodium Hypochlorite	Emplura®
Store purchased eggs	Medium, large and very large	Ballyfree (Tesco)
Hen eggs	Age and Diet studies	Supplier, Northern Ireland
Duck eggs	Age and Diet study	Supplier, Northern Ireland

Age & Diet Study Results

Table 6.2.1 *Age & Diet Study Shed B; Quasi-static and fracture toughness test results.*

		Weight (g)		*Thickness (mm)*		*Stress (MPa)*		*K_{Ic} (MPa√m)*	
Week	Diet	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
22	Mineral Supplement	51.9	5.3	0.39	0.05	11.47*b	2.97	0.56	0.2
22	Generic feed	56.67	3.3	0.40	0.02	10.13*c	4.32	0.5	0.1
28	Mineral Supplement	62.28	5.1	0.39	0.02	4.29	1.82	0.49	0.2
28	Generic feed	62.39	4.2	0.39	0.02	4.90	2.08	0.32*d	0.1
36	Mineral Supplement	59.21	3.7	0.36*a	0.04	6.31	3.04	0.52	0.1
36	Generic feed	62.04	3.6	0.36*a	0.02	5.05	2.86	0.38	0.2

Sd: Standard deviation; Thickness in Week 36 decreased in both diet groups from previous weeks (p value <0.05). Stress*bc values in the first time point were significantly higher than the following two time-points. This was seen in both diets. K_{Ic} *d of generic feed was lowest in Week 28 but increased again in the final week.

Table 6.2.2 *Nanoindentation results from Age & Diet study 1 Shed B. Young's modulus generic feed significantly decreased in the final time point*

		Hardness (GPa)		*Young's Modulus (GPa)*	
Week	Diet	Mean	Sd	Mean	Sd
22	Mineral Supplement	2.31	0.4	37.49*b	4.6
22	Generic Feed	2.46	0.5	53.47*c	7.5
28	Mineral Supplement	2.48*a	0.5	45.12	5.9
28	Generic Feed	2.08	0.6	50.06	6.8
36	Mineral Supplement	2.41	0.60	45.96	8.2
36	Generic Feed	2.33	0.22	35.70	3.4

Sd: Standard deviation. Hardness*a Mineral supplement hardness in week 28 was significantly higher than the previous week. Modulus*b Mineral supplement increases over 3 time points. Modulus*c Generic feed sees eggs decrease in Young's modulus results over time.

Results *Shed C*

Table 6.2.3 *Age & Diet Study 1: Shed C; Fracture toughness and quasi-static test results.*

		Weight (g)		*Thickness (mm)*		*Stress (MPa)*		*K_{Ic} (MPa√m)*	
Week	Diet	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
22	Mineral Supplement	51.67	4.4	0.40	0.03	3.88	1.9	0.30	0.10
22	Generic Feed	51.39	3.47	0.40	0.03	12.96*b	4.8	0.24	0.07
29	Mineral Supplement	55.9	6.52	0.39	0.01	6.07	2.4	0.24	0.06
29	Generic Feed	59.53	4.54	0.38	0.03	5.79	0.9	0.45	0.23
36	Mineral Supplement	61.46	3.64	0.39	0.02	5.77	5.9	0.45	0.20
36	Generic Feed	59.95	4.74	0.40	0.02	4.03	1.6	0.33	0.18
43	Mineral Supplement	59.66*d	2.72	0.33*a	0.04	6.03	1.4	0.47	0.30
43	Generic Feed	60.20	4.70	0.35	0.02	6.18	2.2	0.57*c	0.20

Sd: Standard deviation; Thickness*a significantly decreases in Week 43 In both diet groups. In the first time point, Generic diet sample has a significantly large force and Stress*b. In K_{Ic} there is a significant difference between generic feed diet week 22 and generic feed diet Week 43.

Table 6.2.4 *Nanoindentation results for Age & Diet Study 1 Shed C.*

		Hardness (GPa)		Young's Modulus (GPa)	
Week	Diet	Mean	Sd	Mean	Sd
22	Mineral Supplement	2.27	0.43	51.26*c	6.38
22	Generic Feed	2.06	0.28	43.94	4.34
29	Mineral Supplement	2.48*a	0.33	52.37	3.39
29	Generic Feed	2.36	0.32	40.71*d	2.68
36	Mineral Supplement	1.92	0.20	47.75	5.21
36	Generic Feed	2.04	0.32	47.79	2.99
43	Mineral Supplement	1.85	0.23	34.18*c	1.90
43	Generic Feed	2.49*b	0.54	49.69*d	4.10

Hardness results for Mineral Supplement*a: Significant decrease in hardness is apparent. Hardness and Young's modulus values for Mineral Supplement*ac diet group increases over time. Hardness and Young's modulus values for Generic feed diet*bd: increase in GPa results over time which was seen to be statistically significant in both stiffness and hardness values.

Mechanical test results Shed D

Table 6.2.5 *Age & Diet Study 1; Shed D; Fracture toughness and quasi-static tests*

		Weight (g)		Thickness (mm)		Stress (MPa)		K_{Ic} (MPa√m)	
Week	Diet	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
24	Mineral Supplement	54.65	2.81	0.41	0.03	6.47	2.44	0.41	0.19
24	Generic Feed	56.54	4.06	0.41	0.03	7.00*a	1.72	0.35	0.17
35	Mineral Supplement	62.17	3.89	0.37	0.03	4.80	0.79	0.43	0.17
35	Generic Feed	61.70	3.45	0.40	0.03	4.03*b	1.35	0.47	0.13
43	Mineral Supplement	59.33	1.85	0.37	0.04	6.83	1.44	0.46	0.15
43	Generic Feed	63.95	6.07	0.36	0.03	4.60	1.43	0.30	0.26

Sd: Standard deviation. Stress Generic feed *a week 24 was significantly higher than the 2 subsequent weeks. Stress Mineral supplement*b week 43 was significantly higher than week 35.

Table 6.2.6 *Age & Diet Study 1.4 nanoindentation results.*

		Hardness (GPa)		*Young's Modulus (GPa)*	
Week	Diet	Mean	Sd	Mean	Sd
24	Mineral Supplement	2.55	0.83	50.36*b	8.95
24	Generic Feed	2.39	0.41	49.72	8.42
35	Mineral Supplement	2.35	0.58	42.34	5.59
35	Generic Feed	2.09	0.15	29.63*c	6.31
43	Mineral Supplement	2.57	0.49	49.98	3.61
43	Generic Feed	2.63*a	0.54	51.93	4.64

Sd: Standard deviation. Hardness Generic Feed*a week 43 displayed increased hardness compared to that of hardness from week 35. Young's modulus Mineral Supplement*b was significantly higher than week 35. Modulus in Generic Feed*c saw a significant decrease in results in week 35, which increased again in week 43.

Age & Diet Study 2

Table 6.2.7 *Nanoindentation results Age & Diet Study 2.*

		Weight (g)		*Thickness (mm)*		*Hardness (GPa)*		*Young's Modulus (GPa)*	
Week	Feed	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
68	Mineral Supplement	66.29	4.3	0.38	0.02	1.16	0.5	36.06	17.2
68	Generic feed	63.65	4.9	0.37	0.03	1.58	0.8	43.76	17.7
71	Mineral Supplement	63.53	4.9	0.40	0.02	1.27	0.5	42.34	12.8
71	Generic feed	63.68	4.5	0.39	0.03	1.67	0.6	35.72	10.6
74	Mineral Supplement	66.69	4.3	0.38	0.04	2.17	0.6	44.49	8.2
74	Generic feed	65.12	5.5	0.38	0.02	2.47*	0.6	53.71	9.5
77	Mineral Supplement	65.14	4.3	0.38	0.01	1.56	0.7	41.03	12.5
77	Generic feed	66.34	5.1	0.39	0.02	1.48	0.9	36.06	12.1

Hardness tests presented with an increase in hardness for both mineral supplement and generic feed in Week 74. This increase was significant in generic feed sample.

Chapter 7 Bibliography

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