Cuticle Proteins in Diverse Lines of Chickens

by

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ABSTRACT

This study investigated variability of cuticle proteins on the eggshell surface from different chicken lines, as they aged. Proteins from 10 eggs were removed from two commercial lines every 30 days from 20 - 66 weeks-of-age. In a separate study, cuticle proteins were removed from 12 eggs produced by five heritage lines and two commercial lines from the University of Alberta. Removal involved washing eggs in 1% SDS. Protein levels and molecular weight were determined using commercial protein assay and SDS-PAGE. Cuticle protein quantity harvested was highest for commercial white laying hens at 28 weeks-of-age and declined after 50 weeks for both types. Young layers produced more complex cuticles compared to older hens. Protein profiles indicated that 12, 18, 50 and 70kDa proteins were highest for Barred Plymouth Rock. This study suggests heritage lines have unique cuticle profiles compared to commercial chickens and hen age impacts cuticle protein profiles.

LIST OF ABBREVIATIONS USED

ESM	Eggshell Membrane
SE	*Salmonella enteritidis*
LL	Lohmann LSL Lite
LB	Lohmann Brown Lite
BL	Brown Leghorn
WL	White Leghorn
LS	Lite Sussex
BR	Barred Rock
BRS	Barred Rock Shaver line
ACW	Alberta Commercial White
ACB	Alberta Commercial Brown
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Poly-acrylamide Gel Electrophoresis
RCDC	Reducing Agent and Detergent Compatible
BSA	Bovine Serum Albumen
Mol. Wt.	Molecular Weight
DTT	Dithiothreitol
kDa	Kilodaltons
UV	Ultra Violet
V	Volts

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CHAPTER 1: INTRODUCTION

Chicken eggs are equipped with numerous features that act as barriers against the entrance of microbes (Chen et al., 2019a). Genetic selection has played a significant role in the improvement of egg production in commercial laying hens and is important for meeting the increasing demand for eggs. However, these genetic improvements concentrate more on increasing egg numbers, and feed efficiency, than improving the barriers against bacteria (Bain et al., 2013). While it is essential to produce adequate numbers of eggs, it is also critical that genetic selection does not adversely affect human health following consumption (Chen et al., 2019a).

Bacteria, such as *Salmonella*, are a common concern found in the food industry (Chen et al., 2019b). The food borne illness caused by *Salmonella* is known as salmonellosis and unfortunately, eggs are often reported for their role in its transmission (CDC, 2018; Chen et al., 2019b; Messens et al., 2007). While the eggshell is a natural barrier that provides protection against contamination by entering microbes, the cuticle is described as the most significant feature in the resistance of microbial invasion. The cuticle is an organic, proteinaceous layer located on the outside of the eggshell (Rodríguez-Navarro et al., 2013). This layer surrounds the outside of the shell and serves several functions in addition to protection, including control of gaseous exchange and moisture management by regulating the coverage of shell pores (Chen et al., 2019a; Wellman-Labadie et al., 2008).

Specific proteins found within the cuticle have been found to defend against microbes (Rose-Martel et al., 2012). Lysozyme C, ovotransferrin, ovocalyxin-32, and ovocleidin-17 are cuticle proteins with antimicrobial properties, meaning they are essential in the protection against bacteria

(Bain et al., 2013). The cuticle is important to maintain and improve the defense of the egg, however; commercial laying hen lines are not specifically selected for an adequate cuticle layer (Bain et al., 2013; Rose-Martel et al., 2012).

There are several other factors that influence eggshell quality in terms of shell porosity and breaking strength (Chen et al., 2019b). Hen age is a main factor that influences eggshell quality, in addition to the significant impact on the coverage and thickness of the eggshell cuticle (Messens et al., 2007; Rodríguez-Navarro, et al., 2013). Cuticle coverage refers to how much of the shell is fully encased by cuticle. While it is suggested that cuticle composition in terms of protein availability is only slightly affected by hen age, cuticle coverage, as well as cuticle thickness decreases as hens age (Muñoz et al., 2015; Roberts et al., 2013). This decline increases the permeability of the cuticle and the shell, elevating the risk of microbial penetration (Rodríguez-Navarro, et al., 2013).

Increasing shell thickness and decreasing shell porosity improve the barrier preventing microbes from passing through the eggshell (Kaur et al., 2009). Knowing that selection for shell strength in commercial lines of chickens has increased shell thickness and breaking strength, some researchers have set out to determine if non-commercial lines may have increased ability to repel microbes (Bain et al., 2013; Rathgeber et al., 2013). Some non-commercial lines of birds produce eggs with an improved resistance to microbial entrance that cannot be explained by shell thickness (Rathgeber et al., 2013). This difference may be associated with differences in cuticle protein profiles, or the thickness of the cuticle layer. Most researchers recognize that selection in

commercial laying lines is based primarily on genetics related to increasing egg production rates, rather than considering eggshell quality (Bain et al., 2013).

The focus of this research is to examine the variability of eggshell cuticle proteins among laying hens of different ages, eggshell colors, and lines. This information will be used to identify shell characteristics that could potentially enhance the barrier of the shell in these high-producing commercial poultry flocks. Minimizing the entrance and growth of bacteria within the interior of the egg may potentially lead to a decrease in the incidence of food-borne illness related to this important food product.

This research took place during the COVID-19 global pandemic. While this pandemic took a toll on the ability to collect data, at times, my committee and I are confident that this research, and thesis is full and complete.

CHAPTER 2: LITERATURE REVIEW

There is a large amount of literature related to this research which includes: eggshell structure, cuticle structure including the associated cuticle proteins; lysozyme-C, ovocleidin-17, ovocaloxin-32, and ovotransferrin, in addition to food-borne illness, with specific emphasis on *Salmonella*. The influence of hen age, breed, as well as housing location on the eggshell cuticle have also been widely studied.

2.1 Eggshell Structure

The avian eggshell is a complex structure that is essential to protect the internal contents of the egg and developing embryo (Figure 1) (Hincke et al., 2012; Rodríguez-Navarro et al., 2013). This includes protection of the growing embryo from physical damage, the shielding of the internal components from microbial contamination, as well as moisture management (Hincke et al., 2012). Although it may appear to be a simple structure, the eggshell of the domestic chicken is composed of six layers (Figure 1). These layers include the internal and external shell membranes, the mammillary cone layer, the palisade layer, the vertical crystal layer, and the outermost layer, known as the eggshell cuticle (Hincke et al., 2012; Makkar et al., 2016). Each of these layers has a role to play in the protection of the growing embryo or the egg's internal contents against microbial contamination (Hincke et al., 2012). In addition to these layers, shell pores, which extend from the mammillary layer, function in gas exchange for the growing chick. While the shell pores are essential, they are also a point of entrance for microbes (Solomon, 2010).

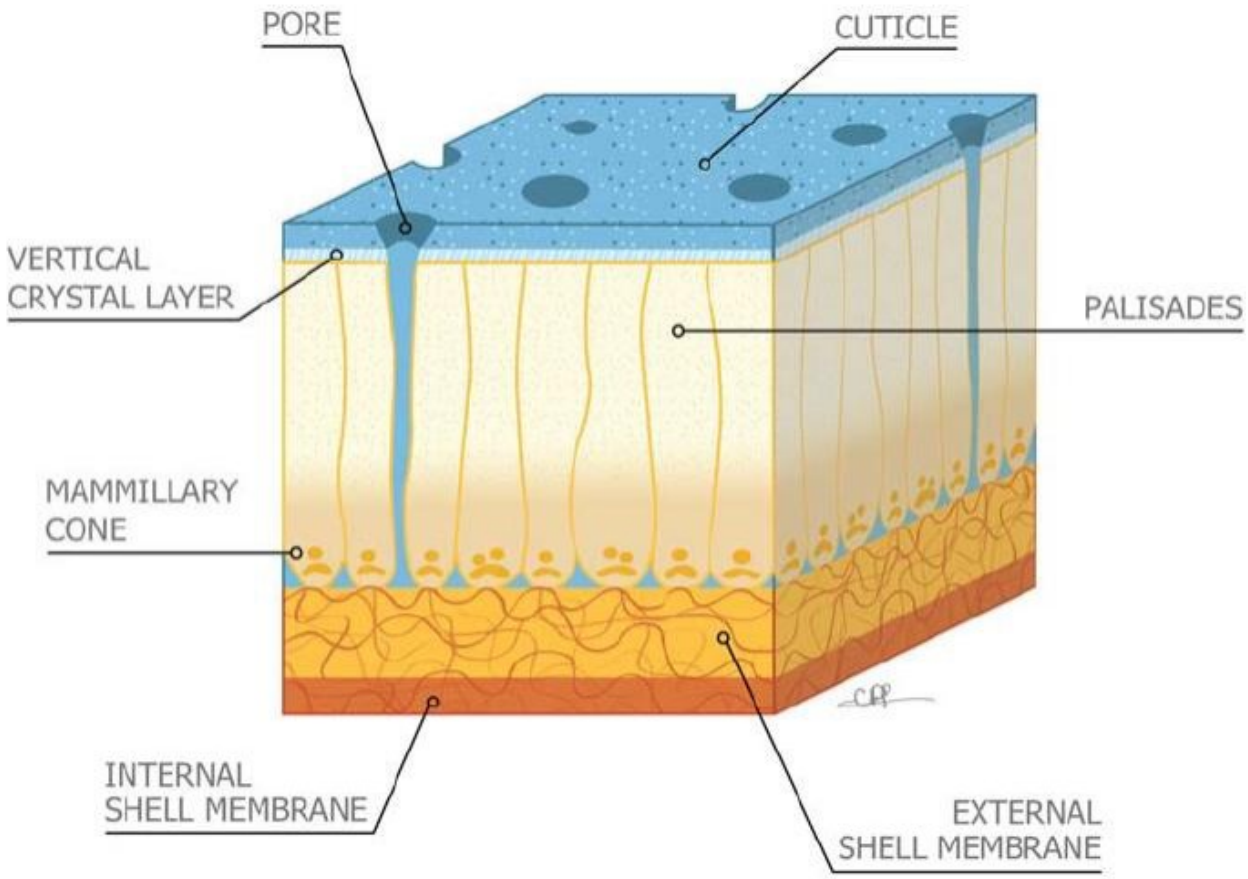

Figure 1: Cross-section of the internal structure of hen egg shell (Hincke et al., 2012)

2.2.1 Internal and External Shell Membranes

The innermost portion of the eggshell, known as the internal and external eggshell membranes (ESM) surround the albumen and are the base for the formation of the shell. They are responsible for protecting against microbial invasion, as well as supplying nutrients in the form of proteins and peptides to the developing chick (Makkar et al., 2016). ESMs are made up of insoluble proteinaceous fibres that are high in collagen (Marcet et al., 2018). Due to this structure, having antimicrobial properties, research is ongoing to create a variety of medical products using ESMs for illness or injury, such as wound healing, and nerve repair (Preda et al., 2020). In addition, the

overall availability, low expense, and biocompatibility make this a favorable future product (Marcet et al., 2018; Preda et al., 2020).

2.2.2 Mammillary Cone Layer

The innermost calcified portion of the shell is the mammillary cone layer (Rose et al., 2009). This layer is the foundation for the anchoring of calcium carbonate crystals that form the rigid portion of the eggshell. The mammillary cone layer can be identified by the arrangement of cones or knobs visualized upon microscopic examination (Rose et al., 2009). It is composed mostly of calcium carbonate however like most eggshell layers, also includes key proteins to protect the egg's internal contents. This layer acts as a calcium store to supply the growing skeleton of developing embryos. As the chick consumes this calcium, the mammillary knobs weaken, resulting in an easier hatching process (Hincke, et al., 2012). The thickness of this layer directly impacts embryonic development and hatchability. In a study completed by Liao et al. (2013), eggs with thicker mammillary layer had improved hatchability. This was due to increased calcium available for the embryo during development (Liao et al., 2013).

2.2.3 Palisade Layer

Following the mammillary cone layer is the palisade layer (Solomon, 2010). The palisade layer extends from the knobs within the mammillary layer and is the thickest portion of the eggshell, extending approximately 200μm (Solomon, 2010). The primary role of the palisade layer is to add additional strength and structural support to the shell. Similar to the mammillary layer, the palisade layer also contains proteins that create a matrix in this layer. The presence of proteins in the uterine fluid around the developing shell influences the size and shape of the calcium carbonate crystals

found in this layer (Wellman-Labadie et al., 2008). The crystal formation has the potential to impact rigidity, as well as pore size and number. Kaur et al. (2009), discovered that there is significant variation in the profile of these uterine fluid proteins. This may be related to differences observed in the structure of eggshells from heritage lines of chicken, compared to modern commercial layers. The main variation in thickness of this structure is displayed in the mammillary and palisade layers, as these layers vary in thickness between lines (Rathgeber et al., 2013).

2.2.4 Vertical Crystal Layer

The final calcified portion of the eggshell is the vertical crystal layer (Rose et al., 2009). This layer is where pigment is incorporated into the shell (Lunam & Ruiz, 2010). The pigment in brown eggs is known as protoporphyrin IX, which contains antimicrobial properties stimulated by light (Wisocki et al., 2020). When exposed to solar radiation, protoporphyrin has shown to inhibit the growth of gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus cereus* (Dearborn et al., 2017; Wisocki et al., 2020). Unfortunately, little else is known about this area, however, it is suggested that it provides an excellent setting for the last layer, the eggshell cuticle (Lunam & Ruiz, 2010).

2.2 The Cuticle Layer

The cuticle is the final layer deposited on the shell, approximately 1-2 hours before oviposition (Lunam & Ruiz, 2010). It surrounds the shell in organic material, covering and filling the pores of the outer eggshell. The cuticle is mostly composed of glycoproteins (90%), which defend against microbial contamination as the first line of defence against bacteria. The cuticle and the vertical crystal layer together contain pigments, depending on shell color, that aid in temperature regulation

as well as some forms of disguise to allow for parental recognition (Dearborn et al., 2017; Rose et al., 2009).

The cuticle layer plays a key role in the safety of the embryo as it protects the egg from sources of contamination in the surrounding environment (Mine et al., 2002). The cuticle is an organic, water-resistant layer, ranging from 0.5 – 12.8μm in thickness. It is composed mainly of protein, however, small amounts of carbohydrates (3-4%), fat (2.5-4%) and ash (3.5%) may also be present (Mine et al., 2002; Samiullah & Roberts, 2014). The cuticle's main function is to protect the egg from microbial organisms; however, one of the more important functions is water regulation. The cuticle ensures that the appropriate amount of water is lost during development and water with contaminants are not absorbed through the shell layers to affect the yolk or growing chick (Wellman-Labadie et al., 2008).

While the cuticle does an excellent job at protecting the egg, there is a small window of time in which its protection from microbes is at its highest (Muñoz et al., 2015). Eggs contain the lowest level of bacteria 6 to 72 hours after oviposition; this is due to the formation of the cuticle at this time. Immediately after the egg is laid, the cuticle is not fully mature and does not span the entire surface of the eggshell, thus allowing microbes to enter (Chen et al., 2019a; Muñoz et al., 2015). The process of cuticle maturation, while not fully known, is described as the thickening of the cuticle after the egg has been laid. Maturation is the process of stabilization due to an increase in the glycosylation of proteins, thus increasing the stability of the glycoproteins and their adherence to substrates (Muñoz et al., 2015; Rodriguez-Navarro et al., 2013). This process takes the cuticle approximately 6 hours to reach its full potential, and at this point is most effective at protecting

the egg against invading microbes. Following complete maturation, the cuticle begins to dry out and break down. This degeneration begins to occur approximately 24 h after oviposition (Muñoz et al., 2015). Research conducted by Rodriguez-Navarro et al. (2013), suggests that approximately 72 h after an egg is laid, cracks form in the cuticle due to drying. This exposes the pores in the eggshell, potentially allowing proteins from the albumen to be lost as they travel from the inside of the eggshell to the outside. While this may potentially increase the protein content found in layers of the shell as well as at the surface of the eggshell, these proteins are not necessarily specific to the eggshell cuticle (Rodríguez-Navarro et al., 2013). Roberts et al. (2013) suggest that very few eggs are completely covered by the cuticle. Cuticle cover may range from complete, meaning little to no cracks or bare patches (Figure 2A), usually in younger birds, to non-existent, meaning there is little to no cuticle present on the eggshell at all, often seen in older hens (Figure 2B) (Muñoz et al., 2015; Roberts et al., 2013). While the exact mechanisms of cuticle maturation are still uncertain, it is apparent this process is significant in the protection of the egg's internal contents from microbial contamination (Rodríguez-Navarro et al., 2013).

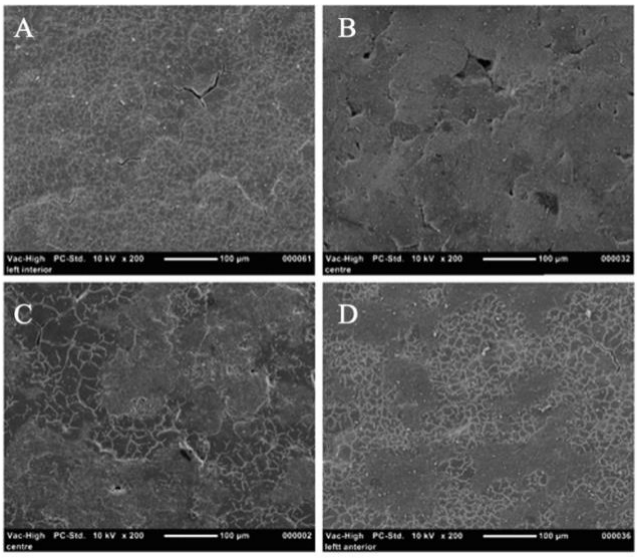

Figure 2. Variation in the Cover of the Cuticle Layer of Chicken Eggs. A has good cuticle coverage. B has no cuticle coverage. C and D show minimal or cracked cuticle (Roberts et al., 2013).

2.3 Cuticle Proteins

There are approximately 850 – 870 different proteins found within the eggshell cuticle that provide a network to protect the egg against microbial contamination (Mikšík et al., 2009; Wellman-Labadie et al., 2008). Of these proteins, only 47 have been confirmed and classified. The proteins most abundant within the cuticle are lysozyme, ovo- transferrin, ovocalyxin-32, and are known for their antimicrobial properties (Muñoz et al., 2015).

The effectiveness of the cuticle is directly dependent upon the type and quantity of these proteins (Mikšík et al., 2009). While these cuticle proteins have varying modes of action, they work together to protect the egg. These proteins collectively defend against both Gram-positive and Gram-negative bacteria, however, bacteria may still elude the eggshell cuticle and contaminate the egg (Mikšík et al., 2009).

2.3.1 Lysozyme C

One of the most key cuticle proteins is lysozyme C (Ragland & Criss, 2017). This 14.4 kDa enzymatic protein, is made up of a polypeptide chain with 129 amino acids in a tertiary, globular structure (Abeyrathne et al., 2014). It is known mostly for restricting the growth of bacteria, and is responsible for both inflammatory, and anti-inflammatory reactions (Ragland & Criss, 2017; Wellman-Labadie et al., 2008). Lysozyme C plays a significant role in the defense and protection of various organisms, including in humans, where it is found in mucous, blood and tears (Rose et al., 2009). It protects its host by invading the exterior cell wall of Gram-positive bacteria, causing cell wall break down. This protein has been deemed ineffective against *Escherichia coli* D31,

Pseudomonas aeruginosa, as well as *Staphylococcus aureus* due to the recent adaptation of these bacteria to resist this enzyme (Ragland & Criss, 2017).

2.3.2 Ovocleidin-17

Ovocleidin-17, while usually found in the membrane of the egg, is also a common cuticle protein (Mann & Siedler, 1999). It is a phosphoprotein, with a molecular weight of 15.3kDa. Currently little is known about the function of ovocleidin-17 in the cuticle, however, it is thought to aid in microbial protection (Mann & Siedler, 1999; Rose et al., 2009).

2.3.3 Ovocalyxin-32

Ovocalyxin-32, with a molecular weight of 32kDa, is found in abundance throughout the cuticle; however, it was first discovered in the eggshell (Hincke et al., 2009; Rose-Martel et al., 2012). Ovocalyxin-32 contains similarities in function to other eggshell proteins known for their part in protection from microbes, supporting the idea that it contains antimicrobial properties (Hincke et al., 2009).

2.3.4 Ovotransferrin

Ovotransferrin is a major shell, uterine fluid, egg white and cuticle protein (Gautron & Nys, 2007). It is a glyco-peptide with a molecular weight of 76kDa. The function of ovotransferrin is to transfer iron molecules throughout its host. The two forms of ovotransferrin; iron-free, and iron-bound, differ greatly in their physical and chemical characteristics. By binding to the ferric atoms, this antimicrobial protein makes iron unavailable to the bacteria found in the eggshell (Abeyrathne et al., 2014).

2.4 Key Factors affecting Cuticle Quality

There are key factors that affect cuticle quality in terms of coverage, thickness and effectiveness of the cuticle's antimicrobial properties. Some of these factors include health, genetics, as well as age of the hen (Bain et al., 2013). Additionally, stress is a key factor in cuticle formation, as it influences oviposition and cuticle formation within the uterus. Other factors that affect the cuticle include breed, housing environment, eggshell color, as well as location of the hens (Samiullah & Roberts, 2014). In addition, poor nutrition affects the outputs in terms of; egg production, egg quality, and mortality, therefore poor nutrition likely could affect the eggshell cuticle (Samiullah & Roberts, 2014; Tadelle et al., 2019).

2.4.1 Breed

Breed of hen is a factor known to affect cuticle quality, such as commercial versus heritage chickens, and brown versus white hens (Dunn et al., 2019; Samiullah & Roberts, 2014; Sirri et al., 2022). Heritage chickens are recognized as an ancestral breed, which originated before the mid-20th century. They are known to be slow growing, and less productive when compared to commercial lines, as they have not been selectively bred (Schmidt et al., 2019). Heritage birds are often used in research trials as a baseline, to show the growth of commercial lines over time. The cuticle layer of eggs from heritage breeds may contain higher amounts of protein compared to commercial layers as commercial layers are consistently bred for rate of lay, rather than considering other important qualities such as protection from microbes (Rathgeber et al., 2013; Schmidt et al., 2019).

Rathgeber et al. (2013), discovered that bacteria invade the eggshells of heritage birds differently than those of commercial chickens. Prior to this research, Kaur et al. (2009), hypothesized that this difference may be due to the variances in shell structure, and shell thickness between bird lines. A correlation between shell structure and invading bacteria, however, was not discovered at that time (Kaur et al., 2009). The results of the research by Kaur et al., (2009) suggest that the cuticle layer of the eggshell may play a larger role in bacterial invasion than shell structure, or thickness.

In Rhode Island Red hens, genetics play a large role in the cuticle quality in terms of thickness (Dunn et al., 2019). In addition to genetics, color of the egg plays a part in the deposition of the cuticle, and the cuticle layer from brown eggs are more effective at deflecting bacteria, when compared to white eggs (Samiullah & Roberts, 2014). Research suggests that breed plays a larger role on cuticle quality, compared to other factors such as housing, or location (Ketta & Tumova, 2018). Based on this information, it is important to note that cuticle quality of the hen line should be taken into consideration when planning a breeding program (Dunn et al., 2019; Kaur et al., 2009; Rathgeber et al., 2013; Sirri et al., 2022).

2.4.2 Hen age

The rate of bacterial penetration through the eggshell is directly related to age of the hen which laid the egg (Muñoz et al., 2015). Eggs from 25-week-old hens experienced a much lower rate of bacterial invasion, when compared to eggs from hens of 35 and 52 weeks-of-age (Muñoz et al., 2015). In a supporting study, eggs were collected from hens aged 16, 30, 36 and 70 weeks and results indicated that hen age played a significant role in the cuticle's composition (Rodriguez-

Navarro et al., 2013). In addition, eggs from older hens had minimal cuticle coverage, indicating potential for increased risk of microbial penetration (Rodriguez-Navarro et al., 2013).

Salmonella enteritidis (SE) contaminates eggs differently based on hen age (Jones et al., 2002). In late lay hens, SE contaminated the egg easier compared to younger hens. This rate of contamination did not change after the flock was put through a forced molting period, suggesting that cuticle quality does not improve after hens have molted (Jones et al., 2002). This information, as well as other supporting studies suggest that eggs from older hens could be at a higher risk for bacterial penetration, due to decreased cuticle coverage and/or quality (Benavides-Reyes et al., 2021; Kulshreshtha et al., 2018; Sirri et al., 2022). In two separate studies cuticle staining was used as an indication of cuticle cover (Roberts et al., 2013; Samiullah & Roberts, 2014). The results from both studies conflict with results from other research, as they indicated that hen age did not negatively affect cuticle staining, and thus cover and quality. In addition, housing systems had no effect on cuticle coverage (Roberts et al., 2013; Samiullah & Roberts, 2014).

2.4.3 Location and Housing Conditions

Habitat plays a key role in the health of the cuticle layer as birds living in challenging environments, such as extreme temperatures, contain a stronger, more intact cuticle (Bain et al., 2013). Additionally, in wild poultry, nest location influences shell parameters such as shell quality, and therefore could affect the cuticle quality as well (D'alba et al., 2016). While nest location influences the cuticle layer, body weight of the parents and nest materials are also considering factors (Kusuda et al., 2011). Eggs which were laid closer to the water have thicker cuticles

compared to eggs which were laid further inland, as a necessity, due to the fact that they are in environments which put them at higher risk for microbial penetration (Dunn et al., 2019).

Hens from poor housing conditions, such as extreme temperatures and lack of cover from environmental elements produce eggs at a lower rate (Tadelle et al., 2019). Research suggests that eggs produced from hens living in these environments had thicker shells with increased fertility, when compared to commercial laying hens (Tadelle et al., 2019). These eggs had a lower incidence of bacterial penetration, and hens showed a decreased incidence of parasites and disease, which could indicate a thicker cuticle layer, due to increased resilience (Mwambene et al., 2019). This research is not typical, as stress due to poor housing or laying conditions is a factor which contributes to poor cuticle thickness or composition (Samiullah & Roberts, 2014).

The modern commercial egg industry is undertaking a transition away from conventional caging systems, in order to improve the welfare of birds raised for egg production (Whiley & Ross, 2015). With this change, a noticeable difference was reported for the level of internal contamination of eggs from different housing systems, therefore cuticle quality needs to be considered. Eggs from hens housed in a free-range system were reported to have a 6% rate of bacterial contamination, while 16% of eggs from battery cages were contaminated (Whiley & Ross, 2015). It is suggested this variation is due to the decrease in stocking density in free-range systems (Gast et al., 2014).

2.5 Microbial Contamination

Chicken eggs are often to blame for their role in the transmission of food-borne illness (Muñoz et al., 2015). *Campylobacter, Staphylococcus,* and *E. coli* are occasionally discovered within the egg;

however, Salmonellosis is the most common illness found in eggs, and egg-related products (Singh et al., 2010). It is estimated that there are 2000 different serovars of the *Salmonella* bacterium, however, *S. enteritidis* is the most prevalent within the egg industry (Singh et al., 2010). These potentially lethal bacteria are not only a serious issue for a growing chick; it is a severe food health and safety concern, and contributes to huge economic losses (Braden, 2006).

Many procedures such as sanitation of the bird's environment, vaccination of the hens, as well as washing and refrigeration of the eggs, may be practiced to decrease the incidence of infection (Whiley & Ross, 2015). *Salmonella* vaccination of hens is a successful practice in increasing the immunity of hens and their offspring for certain strains of *Salmonella* by 62%, however it does not reduce overall *Salmonella* levels within the environment (Berghaus et al., 2011). Berghaus et al., (2011) made no mention of the contamination of eggs from the vaccinated hens. A conflicting study indicated that vaccination did not change the level of infection of the hens, however it did lower incidence of shell contamination (Arnold et al., 2014).

Method of egg collection, as well as storage may also play a part in level of microbial contamination (Whiley & Ross, 2015). Egg washing is a practice used in processing to remove bacteria from the outer shell. This may potentially decrease the defense of the egg post-wash, as it strips the water-resistant cuticle from the outer eggshell and may transfer bacteria to the interior of the egg (Whiley & Ross, 2015). A tradition of covering the eggs in substances, such as mineral oil, soy or whey proteins, was developed to overcome this problem. While this potentially kept out microbes, these materials did not contain the antimicrobials needed to inhibit bacterial growth, and therefore this practice is no longer common in modern egg processing (Rose-Martel et al., 2012).

In many countries, eggs are now washed and kept in cold storage to discourage microbial growth, however, there are some countries which no longer wash their eggs, leaving the cuticle intact (Rathgeber et al., 2013). This allows the egg to naturally inhibit microbial growth, allowing eggs to be stored at room temperature. Pasteurization, as well as irradiation, are also methods used to decrease the incidence of bacterial contamination, however, consumers are not receptive to this type of procedures due to lack of knowledge or education on the processes (Whiley & Ross, 2015). While it may not be possible to produce "bacteria-free" eggs; with many of the storage techniques, and proper food preparation, the likeliness of contamination can be greatly decreased (Rathgeber et al., 2013; Whiley & Ross, 2015).

2.5.1 Transmission

Bacteria, including *Salmonella,* can be spread through both horizontal and vertical transmission. Vertical transmission occurs when the natural microbiota from the ovaries of the hen is transferred to the egg's internal contents before the shell layer is formed (Gast et al., 2014). This process is also referred to as colonization of the reproductive organs (Gantois et al., 2009). Horizontal transmission occurs when the egg contracts bacteria from its environment, such as litter infected with feces, contaminated feed, or poor-quality air (Geveke et al., 2016). This is known as outer shell contamination (Gantois et al., 2009).

Vertical transmission is most common in environments where hens are housed in close contact with each other, such as a battery cage system (Gast et al., 2014; Singh et al., 2010). However, in a study conducted by De Vylder et al. (2011), birds housed in a free-run aviary system had a higher

incidence of bird-to-bird transmission and eggs contaminated with *Salmonella* were laid at a higher rate, compared to the battery cage system. This cage system debate is common among poultry research, and results are known to vary (Gast et al., 2014; Gast et al., 2017; De Vylder et al., 2011).

Treatments, such as vaccination of hens, may help with vertical transmission. If the bacteria are present in the environment, horizontal transmission can only be controlled by eliminating it from the environment or improving the eggs barrier to entrance (Geveke et al., 2016). While the cuticle acts as an effective barrier against horizontal transmission, as it shields the egg from external contaminants, it cannot protect against vertical transmission. Appropriate steps need to be taken to reduce the risk of hen-to-hen transmission in laying flocks (Geveke et al., 2016; De Vylder et al., 2011).

2.5.2 Salmonella

Salmonella is a zoonotic pathogen, meaning it can be passed from animals to humans (EFSA, 2009; García et al., 2011). There are many forms of this Gram-negative bacterium associated with poultry products. *Salmonella enteritidis* (SE) is the most common form found in eggs and is frequently associated with Salmonellosis diagnosed in humans (Blanc-Potard, 1999; Gast et al., 2017; García et al., 2011; Whiley & Ross, 2015). Salmonellosis is characterized by fever, nausea, abdominal pain, diarrhea, and vomiting and these symptoms usually do not last more than seven days (EFSA, 2009; Foley et al., 2011).

Salmonella is often found in the digestive tract of poultry and may be transmitted through the consumption of undercooked foods, as well as direct contact with infected livestock (EFSA, 2009;

Foley et al., 2011). Unfortunately, eggs are often a vehicle of transmission, and *Salmonella* transmission from eggs is as a global health concern (Gantois et al., 2009; Whiley & Ross, 2015). Although the cuticle is an excellent protectant, practices such as housing, feed contamination and vaccination, are exceptionally important to poultry farmers struggling with *Salmonella* outbreaks (Whiley & Ross, 2015).

2.7 Objectives

The objectives of this experiment are:

1. To investigate the effect of hen age and eggshell color (white and brown) on cuticle protein quantity and profile.
2. To investigate the differences in cuticle proteins and profile between lines of commercial and heritage chickens.

2.8 Hypothesis

The hypotheses for this experiment are:

1. Hen age and eggshell color will influence the quantity and quality of cuticle proteins.
2. Hens from different genetic lines, will have different quantities and profiles of cuticle proteins.

CHAPTER 3: EGGSHELL CUTICLE PROTEIN AND DENSITOMETRY ARE INFLUENCED BY HEN AGE AND EGGSHELL COLOR

3.1 Abstract

Eggs are often reported as a vector for the transmission of foodborne illness. This study examined the impact of hen age and eggshell color on the quantity and profile of cuticle proteins. Eighteen eggs from two commercial lines (Lohmann LSL Lite and Lohmann Brown Lite) were collected from hens beginning at 20 weeks-of-age. This was carried out every 4 weeks until hens were 28 weeks-of-age. Research resumed when hens were 50 weeks-of-age and continued until end of lay at 66 weeks-of-age. Following a 24h maturation period, samples of two eggs were washed in 1% sodium dodecyl sulphate (SDS) for 2 minutes. Protein quantity in the rinse was determined using RCDC protein assay and sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) was carried out to characterize the profile of the proteins present. Data from densitometry of key proteins was analyzed using an ANOVA to investigate differences between hen lines and age. The amount of protein harvested from Lohmann Brown eggs was highest at 20 weeks, while Lohmann White eggs had a lower initial protein content that reached the same amount as Lohmann Brown eggs at 50 weeks-of-age. The cuticle protein amounts from both commercial lines began to decline at 54 weeks of age. Young layers produced eggs with more complex mixtures of proteins in the cuticle compared to samples from older hens. The results of this trial indicate that, while color does not play a large role in variation of cuticle proteins, hen age does influence the proteins of the eggshell cuticle. The results of this research highlight the importance of using birds of the same or similar age for research involving cuticle protein investigation.

3.2 Introduction

As hens age, their eggs increase in size, thus increasing the surface area of the shell (Roberts et al., 2013). The larger the egg gets as hens age, the thinner the shell may become as hens' ability to absorb calcium also decreases (Roberts et al., 2013). This also affects cuticle coverage and composition, increasing the risk of bacterial penetration (Muñoz et al., 2015; Rodriguez-Navarro et al., 2013). This often challenges the egg industry in getting eggs from end of cycle laying hens from farm to market without breakage during transportation, washing and grading (Roberts et al., 2013).

While significant research has been completed on how the age of the hen affects eggshell quality, there is limited research confirming exactly how hen age influences cuticle thickness, composition, and cover, as well as at what age cuticle composition begins to decline (Muñoz et al., 2015). Bacterial penetration with foodborne illnesses, such as *S. enteritidis*, increases with hen age, with highest contamination in late lay hens (Benavides-Reyes et al., 2021; Jones et al., 2002; Muñoz et al., 2015; Rodriguez-Navarro et al., 2013). Age plays a significant role in cuticle composition, however, limited published work is available on details surrounding what happens to the cuticle as hens age, and how well the cuticle continues to protect against bacteria (Muñoz et al., 2015; Roberts et al., 2013; Rodriguez-Navarro et al., 2013; Samiullah & Roberts, 2014). In addition, conflicting studies have examined cuticle cover using staining techniques that indicate hen age does not negatively affect cuticle coverage, supporting the idea that further research is required to examine the effect hen age has on quantity and profile of cuticle proteins (Roberts et al., 2013; Samiullah & Roberts, 2014).

The practical applications of this research include determining at what age the cuticle begins to decline, therefore determining what could be done to avoid trans-shell contamination as this antimicrobial cover begins to deteriorate. The objective of this study was to investigate the effect of hen age and eggshell color on cuticle protein quantity and profile.

3.3 Materials and Methods

This trial began on January 22, 2020, and was carried out every 4 weeks until March 17, 2020, when research was paused due to the COVID-19 Global Pandemic. Research resumed on August 18th and was carried out every 4 weeks until December 8, 2020. Before this study began, method development was conducted to determine the most appropriate cuticle wash solution (see appendix 1). In addition, logistics associated with transporting eggs over borders was investigated to ensure this did not alter the results.

3.3.1 Birds and Housing

Eggs were collected from two commercial lines of laying hens during this trial: Lohmann LSL Brown, which lay brown eggs, and Lohmann LSL Lite, which lay white eggs, to obtain samples from both brown and white eggs. These birds were located at the Atlantic Poultry Research Institute (APRC), on the Dalhousie University, Faculty of Agriculture Campus in Truro, Nova Scotia, Canada. Sampling began on January 22nd, 2020, when hens were 20 weeks-of-age and was carried out every four weeks until March 17th, 2020, when hens were 28 weeks-of-age. Research resumed on August 18th, 2020, when hens were 50 weeks-of-age and was carried out every 4 weeks until end of lay on December 8th, 2020, when hens were 66 weeks-of-age.

3.3.2 Sample Collection and Preparation

Eighteen eggs from each bird line were randomly selected and collected when hens were 20, 24, 28, 50, 54, 58, 62 and 66 weeks-of-age. Eggs were laid under supervision between the hours of 9am and 11am. Once dry, the eggs were collected and placed into a sterile plastic bag. Eggs were candled to ensure no abnormalities or micro-cracks were present. The target was to retain ten eggs from each bird line after screening. The samples were left at room temperature for 24 hours, to ensure the cuticle was fully mature before completing the protein assay. The eggs were placed in a Whirl-Pak bag containing an extraction solution of 2ml of 1% SDS, and manually massaged one at a time, for 2 minutes per egg, for a total of 2 eggs per sample, for a total of 5 replicates per bird line. Once the cuticle was removed, each sample was placed into 100uL aliquots for storage at -80^{o}C, in preparation for further analysis.

3.3.3 RCDC Protein Assay

Reducing Agent and Detergent Compatible (RCDC) Protein Assay (BIO-RAD) was used to determine protein concentration (mg/ml) using bovine serum albumin as a standard. The 1.5ml microcentrifuge tube assay procedure was followed, and absorbance was read at 650nm using BIOTEK Micro-plate Reader, and a standard curve was created using the absorbance values. Protein quantity was calculated using these values and dilutions using 1% SDS were carried out to ensure all samples were of the same protein concentration before SDS-PAGE was carried out.

3.3.4 Sodium Dodecyl Sulphate-Poly-acrylamide Gel Electrophoresis (SDS-PAGE)

Samples were placed into a sample buffer containing 8M urea, 2M Thiourea, 3% SDS, 0.7M 2-mercaptoethanol, 25mM Tris-HCl with a pH of 6.8 in a 30/70 mixture. Samples were heated to

90ºC for 5 minutes and centrifuged before loading onto SDS-PAGE gels. SDS-PAGE was carried out using 4–15% polyacrylamide Mini-PROTEAN TGX Stain-Free Precast Gels purchased from BIO-RAD. Electrophoresis was performed at 60V for 20 minutes, increased to 100V for approximately 10 minutes, then completed at 250V for the time remaining (95–105 minutes). Protein bands were visualized by using ultraviolet (UV) activation for 2.5 minutes. The BIO-RAD gel imaging system was used for UV activation and documenting the gels. Once the image was captured, it was sent to the photo editing and analysis program, ChemiDoc. Proteins were then characterized by their molecular weight in relation to molecular weight standards. Densitometry was completed on the images using the same software.

3.3.5 Statistical Analysis

This experiment was a completely randomized design with each pair of sampled eggs as the experimental unit, with 5 replicates per treatment. The Proc Mixed procedure of SAS was used to run a two-way ANOVA to investigate the interactions between line of hen (Lohmann White and Lohmann Brown) and hen age (weeks) (SAS, 2018). The total protein extracted, and the individual proteins separated on SDS-PAGE were the response variables analysed. The assumptions of normal distribution equal variance were tested. The quantity of the individual protein bands is expressed as percent of sample (%), based on the individual proteins bands compared to the total amount of stain from the banding pattern of the sample. Percent was then used to estimate the amount of protein (mg/ml) in each band based on the total protein in the sample. Results were deemed significant if the P-value was <0.05 (to two decimal places). Standard error was reported with the mean.

3.4 Results and Discussion

3.4.1 Results

The results obtained are from evaluation of the eggshell cuticle change with hen age and shell color. Both Lohmann White (LW) and Lohmann Brown (LB) eggs follow a similar trend (Table 1). There is a significant interaction apparent between the two main affects: line of bird (Lohmann White vs Lohmann Brown) and age of bird. Amount of cuticle proteins harvested stayed constant for Lohmann White eggs up to 50 weeks-of-age and Lohmann Brown eggs remained constant until 54 weeks-of-age, after which the amount of protein harvested decreased for both lines through to the end of lay at 66 weeks-of-age.

Table 1. Quantity of cuticle protein harvested and standard error from the eggs of two lines of commercial laying hens from 20 to 66 weeks-of-age ($P<0.006$).

Age (weeks)	LB (mg/ml of protein)	LW (mg/ml of protein)
20	$2.16^{ABCDE}\pm0.28$	$1.55^{BCDEFG}\pm0.28$
24	$1.90^{BCDE}\pm0.25$	$2.46^{ABC}\pm0.25$
28	$2.26^{ABCD}\pm0.25$	$3.39^{A}\pm0.25$
50	$2.82^{AB}\pm0.25$	$2.74^{AB}\pm0.25$
54	$1.89^{BCDE}\pm0.27$	$1.02^{DEFG}\pm0.27$
58	$0.67^{FG}\pm0.25$	$1.27^{CDEFG}\pm0.25$
62	$0.19^{G}\pm0.33$	$0.83^{EFG}\pm0.33$
66	$0.83^{EFG}\pm0.25$	$1.06^{DEFG}\pm0.25$

LB= Lohmann Brown (Commercial laying hen with brown-shelled eggs)
LW= Lohmann White (Commercial laying hen with white-shelled eggs)
A-G = means with different superscripts are significantly different $\alpha \leq 0.05$
Mg/ml = milligrams of protein per millilitre of extraction solution

Proteins with an estimated molecular weight of 10, 12, 18, 27, 30, 50, 64 and 70 kDa were examined and compared between Lohmann White and Lohmann Brown laying hens of 20 and 24

weeks-of-age, respectively (Figure 3). These banding patterns are an example of an SDS-PAGE gel which depict which proteins are present or absent depending on age and/or line of laying hen. Differences in diversity of protein profiles are present between Lohmann Brown and Lohmann White samples. Lohmann White samples from 24 week-old hens had proteins present at 12, 18, and 64kDa that were not present in Lohmann Brown samples from 20 week-old-hens.

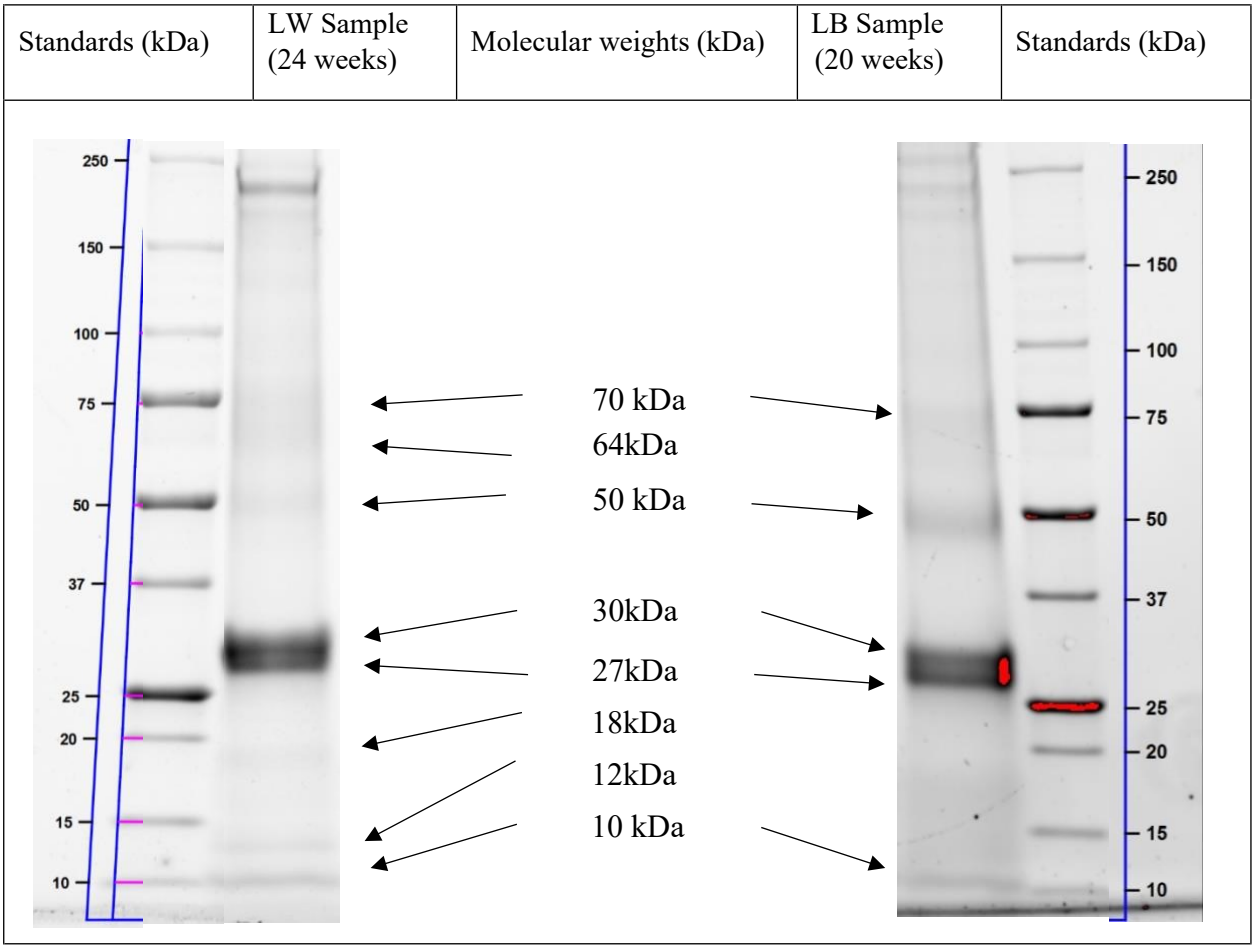

Figure 3. SDS-PAGE Molecular Weight Image of Protein Harvested from Commercial Lohmann LSL Lite (24 weeks) and Commercial Lohmann LSL Brown Laying Hens (20 weeks).

Tables 2 and 3 provide the protein profiles from each section of the trial. The percent protein in the band at 10kDa was significantly affected by the line of bird and the age of the bird ($P<0.05$) (Table 2). An interaction was present for 10kDa between age and type of bird for percent of sample ($P<0.05$). Lohmann Brown eggs from 62 week-old hens had a significantly higher percentage of cuticle protein at 10kDa (29.42%) than at other ages, and were significantly higher than Lohmann White birds at 62 weeks-of-age (3.26%). The protein band at 12kDa is significantly affected by an interaction of age and line ($P<0.05$). This 12kDa protein is only present for Lohmann White hens at 24 weeks-of-age (4.48%) and Lohmann Brown hens at 24 (0.49%) and 28 weeks-of-age (1.66%). The protein found at 18kDa was significantly affected by age, and bird line ($P<0.05$). This 18kDa protein was infrequent in appearance and was found in higher amounts in Lohmann Brown eggs (1.25%), compared to Lohmann White (0.20%) ($P<0.05$). The band at 27kDa was the most prominent for percent protein compared to other protein bands discovered. Means for line of bird were statistically different with higher levels of percent of sample for Lohmann White eggs (45.49%), compared to Lohmann Brown eggs (26.48%) ($P<0.05$). Changes in bird age were subtle, as eggs from Lohmann White hens at 66 weeks-of-age were highest for this protein (77.50%) and means from the oldest age group, 66-week-old hens, had the highest proportion of this protein (69.88%) ($P<0.05$). The protein band estimated to be at 30kDa was impacted only by bird age ($P<0.05$). Mean values are highest for eggs from 50-week-old hens (47.76%), and lowest for 66-week-old hens (9.00%) ($P<0.05$). Percentage of cuticle protein present, estimated to be at 50kDa was present in all except samples from Lohmann White eggs at 54-weeks-of-age. The mean value for Lohman Brown samples was significantly higher (9.34%), when compared to Lohman White samples (1.67%) ($P<0.05$). The protein bands present at 64kDa was not significant for percent protein ($P>0.05$), irrespective of breed or age, therefore no data was included for this sample set.

Protein typically found at 70kDa was consistent low throughout the lay cycle. There was no relationship with age of bird, or color of eggshell established for the 70kDa protein ($P>0.05$), therefore data for percent protein was not included.

The absolute amount of protein found at 10kDa was not significantly different compared to other treatments ($P>0.05$) (Table 3). Type of bird was significantly different with LB having a higher amount of 10kDa protein (0.15mg/ml) than Lohmann White birds (0.09mg/ml) ($P<0.05$). As birds aged, the amount of protein decreased, with the last two collections having lower levels compared to samples from younger birds. At 12kDa there was an interaction present between line and age of bird ($P<0.05$) (Table3). The amount of protein found at 12kDa was significant for age of bird ($P<0.05$). This 12kDa protein is only present in minimal amounts and was not present for the samples collected at 20 weeks-of-age or from any birds older than 28 weeks-of-age. An interaction was present between type of bird and age of bird for amount of protein in each 18kDa band. The band was most evident for Lohmann Brown eggs from hens that were 28 weeks-of age (0.13 mg/ml) ($P<0.05$). At 27kDa, means for egg color were statistically different with higher levels of protein for Lohman White eggs (0.83mg/ml), compared to Lohman Brown eggs (0.40mg/ml) ($P<0.05$). Eggs collected from 62-week-old hens had the lowest amount of protein for 27kDa (0.11mg/ml) compared to samples collected from 28-week-old hens (1.07mg/ml), which had the highest ($P<0.05$). The amount of protein estimated to be at 30kDa was impacted only by bird age ($P<0.05$). Mean value is highest for samples from 50-week-old hens (1.33mg/ml) ($P<0.05$). An interaction was present between age of bird and amount of protein present at for protein percentage at 50kDa, as this band significantly decreases as birds age ($P<0.05$). Samples from Lohman Brown hens taken at 20-weeks-of-age (0.53mg/ml) had the highest value compared to samples taken from

Lohmann White birds of the same age (0.10mg/ml), and 58 weeks-of-age (0.03mg/ml), 62 weeks-of-age (0.01mg.ml) and 66-week-old Lohmann Brown layers (0.03mg/ml) ($P<0.05$). The protein discovered at 64kDa is only present in Lohmann White eggs, therefore a significant difference between Lohamnn White and Lohmann Brown is present ($P<0.05$). No distinct change with bird age is clear in these results for the 64kDa protein ($P>0.05$). The protein band present at 70kDa was consistent low throughout the lay cycle. There was no relationship with age of bird, or color of eggshell established for the 70kDa protein, therefore data for amount of protein was not included.

Table 2. Densitometry value and standard error of protein bands from SDS-PAGE gels expressed as a percentage of cuticle protein samples harvested from the eggs of two lines of commercial laying hens from 20 to 66 weeks-of-age.

		Age in weeks									Overall Mean Values	
Mol. Weight	**Bird Line**	**20**	**24**	**28**	**50**	**54**	**58**	**62**	**66**	**P-Value**	**Mean**	**P- Value**
10kDa	**LB**	10.95^{B} ±3.66	10.36^{B} ±3.27	11.44^{B} ±3.27	5.40^{B} ±3.27	11.96^{B} ±3.27	17.98^{AB}± 3.27	29.42^{A} ±3.27	7.08^{B} ±3.27	0.013	13.07^{A} ±1.17	<0.001
	LW	3.27^{B} ±4.22	6.96^{B} ±3.66	4.64^{B} ±3.27	2.86^{B} ±3.27	7.55^{B} ±3.27	7.30^{B} ±3.27	3.26^{B} ±3.27	4.10^{B} ±3.27		4.99^{B} ±1.22	
12kDa	**LB**	0.00^{B} ±0.79	4.48^{A} ±0.71	0.00^{B} ±0.71	0.00^{B} ±0.71	0.00^{B} ±0.71	0.00^{B} ±0.71	0.00^{B} ±0.71	0.00^{B} ±0.71	0.035	0.560^{A} ±0.25	0.429
	LW	0.00^{B} ±0.91	0.49^{B} ±0.79	1.66^{AB} ±0.71	0.00^{B} ±0.71	0.00^{B} ±0.71	0.00^{B} ±0.71	0.00^{B} ±0.71	0.00^{B} ±0.71		0.268^{A} ±0.26	
18kDa	**LB**	0.00^{AB} ±1.13	2.54^{AB} ±1.01	5.44^{A} ±1.01	0.00^{AB} ±1.01	0.00^{B} ±1.01	0.00^{B} ±1.01	2.02^{AB} ±1.01	0.00^{AB} ±1.01	0.097	1.25^{A} ±0.36	0.049
	LW	0.00^{AB} ±1.30	1.32^{AB} ±1.12	0.00^{AB} ±1.01	0.30^{AB} ±1.01	0.00^{AB} ±1.01	0.00^{AB} ±1.01	0.00^{AB} ±1.01	0.00^{B} ±1.01		0.20^{B} ±0.38	
27kDa	**LB**	22.78^{AB} ±12.84	24.6^{AB} ±11.48	14.64^{B} ±11.48	16.50^{B} ±11.48	40.88^{AB} ±11.48	12.20^{B} ±11.48	17.96^{B} ±11.48	62.26^{AB} ±11.48	0.531	26.48^{B} ±4.12	0.002
	LW	62.27^{AB} ±14.82	31.27^{AB} ±12.84	59.24^{AB} ±11.48	32.02^{AB} ±11.48	37.50^{AB} ±11.48	30.88^{AB} ±11.48	33.28^{AB} ±11.48	77.50^{A} ±11.48		45.49^{A} ±4.29	
	Mean	42.52^{AB} ±9.80	27.93^{B} ±8.61	36.94^{AB} ±8.12	24.26^{B} ±8.12	39.19^{AB} ±8.12	21.54^{B} ±8.12	25.62^{B} ±8.12	69.88^{A} ±8.12	0.002	-	
30kDa	**LB**	17.80^{A} ±13.32	40.50^{A} ±11.92	36.74^{A} ±11.92	43.98^{A} ±11.92	16.4^{A} ±11.92	45.96^{A} ±11.92	40.5^{A} ±11.92	18.00^{A} ±11.92	0.822	32.49^{A} ±4.28	0.625
	LW	16.57^{A} ±15.38	45.62^{A} ±13.32	16.50^{A} ±11.92	51.54^{A} ±11.92	26.04^{A} ±11.92	33.34^{A} ±11.92	46.02^{A} ±11.92	0.00^{A} ±11.92		29.45^{A} ±4.45	
	Mean	17.18^{AB} ±10.18	43.06^{AB}± 8.94	26.62^{AB} ±8.43	47.76^{A} ±8.43	21.22^{AB} ±8.43	39.65^{AB} ±8.43	43.26^{AB} ±8.43	9.00^{B} ±8.43	0.015	-	
50kDa	**LB**	23.58^{A} ±2.80	7.76^{BC} ±2.50	15.20^{AB} ±2.50	6.56^{BC} ±2.50	9.02^{BC} ±2.50	5.98^{BC} ±2.50	3.8^{BC} ±2.50	2.86^{BC} ±2.50	0.155	9.34^{A} ±0.90	<0.0001
	LW	6.50^{BC} ±3.23	0.08^{C} ±2.80	3.44^{BC} ±2.50	0.46^{C} ±2.50	0.00^{C} ±2.50	1.89^{C} ±2.50	0.96^{C} ±2.50	0.00^{C} ±2.50		1.67^{B} ±0.93	

LB= Lohmann Brown (Commercial laying hen with brown-shelled eggs) LW= Lohmann White (Commercial laying hen with white-shelled eggs)
A-B = means with different superscripts are significantly different $\alpha \leq 0.05$

Table 3. Densitometry value and standard error of proteins band from SDS-PAGE gels expressed as milligrams of protein per millilitre of cuticle protein samples harvested from the eggs of two lines of commercial laying hens from 20 to 66 weeks-of-age.

		Age in Weeks									Overall Mean Values	
Mol.Weight	**Bird Line**	**20**	**24**	**28**	**50**	**54**	**58**	**62**	**66**	**P-Value**	**Mean**	**P-Value**
10kDa	LB	0.22^{AB} ±0.05	0.21^{AB} ±0.04	0.24^{A} ±0.04	0.16^{AB} ±0.04	0.21^{AB} ±0.04	0.11^{AB} ±0.04	0.05^{AB} ±0.04	0.05^{AB} ±0.04	0.517	0.15^{A} ±0.01	0.03
	LW	0.05^{AB} ±0.05	0.169^{AB} ±0.05	0.18^{AB} ±0.04	0.08^{AB} ±0.04	0.07^{AB} ±0.04	0.1^{AB} ±0.04	0.03^{B} ±0.04	0.04^{AB} ±0.04		0.09^{B} ±0.02	
12kDa	LB	0.00^{B} ±0.02	0.10^{A} ±0.01	0.00^{B} ±0.01	0.00^{B} ±0.01	0.00^{B} ±0.01	0.00^{B} ±0.01	0.00^{B} ±0.01	0.00^{B} ±0.01	0.035	0.012^{A} ±0.01	0.403
	LW	0.00^{B} ±0.02	0.01^{B} ±0.02	0.03^{AB} ±0.01	0.00^{B} ±0.01	0.00^{B} ±0.01	0.00^{B} ±0.01	0.00^{B} ±0.01	0.00^{B} ±0.01		0.006^{A} ±0.01	
18kDa	LB	0.00^{B} ±0.02	0.05^{AB} ±0.02	0.13^{A} ±0.02	0.00^{B} ±0.02	0.00^{B} ±0.02	0.00^{B} ±0.02	0.01^{B} ±0.02	0.00^{B} ±0.02	0.039	0.024^{A} ±0.01	0.083
	LW	0.00^{B} ±0.03	0.03^{AB} ±0.02	0.00^{B} ±0.02	0.01^{B} ±0.02	0.00^{B} ±0.02	0.00^{B} ±0.02	0.00^{B} ±0.02	0.00^{B} ±0.02		0.004^{A} ±0.01	
27 kDa	LB	0.49^{AB} ±0.31	0.46^{AB} ±0.28	0.31^{B} ±0.28	0.45^{AB} ±0.28	0.83^{AB} ±0.28	0.08^{B} ±0.28	0.03^{B} ±0.28	0.58^{AB} ±0.28	0.102	0.40^{B} ±0.10	0.005
	LW	1.01^{AB} ±0.36	1.01^{AB} ±0.31	1.83^{A} ±0.28	0.84^{AB} ±0.28	0.47^{AB} ±0.28	0.42^{B} ±0.28	0.18^{B} ±0.28	0.83^{AB} ±0.28		0.83^{A} ±0.10	
	Mean	0.75^{AB} ±0.24	0.74^{AB} ±0.21	1.07^{A} ±0.20	0.65^{AB} ±0.20	0.65^{AB} ±0.20	0.25^{AB} ±0.20	0.11^{B} ±0.20	0.70^{AB} ±0.20		-	
30kDa	LB	0.37^{AB} ±0.30	0.74^{AB} ±0.27	0.80^{AB} ±0.27	1.21^{AB} ±0.27	0.28^{AB} ±0.27	0.33^{AB} ±0.27	0.08^{B} ±0.27	0.09^{B} ±0.27	0.97	0.49^{A} ±0.10	0.766
	LW	0.21^{AB} ±0.35	0.85^{AB} ±0.30	0.69^{AB} ±0.27	1.45^{A} ±0.27	0.19^{AB} ±0.27	0.37^{AB} ±0.27	0.47^{AB} ±0.27	0.00^{B} ±0.27		0.53^{A} ±0.10	
	Mean	0.29^{B} ±0.23	0.79^{AB} ±0.20	0.74^{AB} ±0.19	1.33^{A} ±0.19	0.23^{B} ±0.19	0.35^{B} ±0.19	0.28^{B} ±0.19	0.04^{B} ±0.19	0.0002	-	
50kDa	LB	0.53^{A} ±0.06	0.15^{BC} ±0.06	0.38^{AB} ±0.06	0.20^{BC} ±0.06	0.16^{BC} ±0.06	0.03^{C} ±0.06	0.01^{C} ±0.06	0.03^{C} ±0.06	0.014	0.19^{A} ±0.02	<0.0001
	LW	0.10^{BC} ±0.07	0.002^{C} ±0.06	0.12^{BC} ±0.06	0.01^{C} ±0.06	0.00^{C} ±0.06	0.02^{C} ±0.06	0.01^{C} ±0.06	0.00^{C} ±0.06		0.03^{B} ±0.02	
64kDa	LB	0.00^{A} ±0.01	0.00^{A} ±0.01	0.00^{A} ±0.01	0.00^{A} ±0.01	0.00^{A} ±0.01	0.00^{A} ±0.01	0.00^{A} ±0.01	0.00^{A} ±0.01	0.213	0.000^{B} ±0.00	0.04
	LW	0.02^{A} ±0.01	0.00^{A} ±0.01	0.00^{A} ±0.01	0.01^{A} ±0.01	0.05^{A} ±0.01	0.01^{A} ±0.01	0.004^{A} ±0.01	0.00^{A} ±0.01		0.012^{A} ±0.00	

LB= Lohmann Brown (Commercial laying hen with brown-shelled eggs) LW= Lohmann White (Commercial laying hen with white-shelled eggs)
A-B = means with different superscripts are significantly different $\alpha \leq 0.05$

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3.4.2 Discussion

The total amount of cuticle protein harvested from the eggs decreased from both lines after birds reached 50 weeks-of-age (Table 1). The protein concentration of both Lohmann Brown and Lohmann White eggs remained high between 28-50 weeks-of-age and 28-54 weeks-of-age respectively, followed by a gradual decline in protein concentration, which is consistent with previous research (Muñoz et al., 2015). Muñoz et al., (2015) examined the variability of the cuticle's properties with hen age, such as cuticle thickness, coverage, composition, and bacterial penetration. Surface microbial analyses and penetration assays were used to determine how *Salmonella* penetrated the egg at various ages. Bacterial penetration has lower incidences with eggs from hens that were 25 weeks-of-age. This suggests that the cuticle is most effective with younger hens, supporting the findings of this research (Muñoz et al., 2015). Bacterial penetration began increasing at 35 weeks, suggesting that cuticle composition or coverage begins to decline at this age (Muñoz et al., 2015). Future research should include data at these time points, to compare to published findings. Given that protein amounts stayed elevated until 50 – 54 weeks-of-age for both commercial laying lines, it may be assumed that cuticle protection may stay high until 50 – 54 weeks in these commercial chickens. This does, however, conflict with Muñoz et al. (2015) who saw an increase in bacterial penetration of eggs from 35-week-old hens. Additional research is required to fully determine why the decrease in bacterial protection is at 35 weeks-of-age, as higher penetration may not mean lower overall cuticle proteins (Muñoz et al., 2015). This reduction in cuticle protein concentration following the 50 weeks-of-age may be due to a decrease in available uterine proteins as hens age (al-Batshan et al., 1994; Kaur et al., 2013).

Limited research is available on measuring specific cuticle protein changes with bird age. A study conducted by Rose-Martel et al. (2012), used a similar cuticle wash method to examine cuticle proteins from commercial Lohmann LSL Lite chickens. Dominant protein bands at 10 and 32kDa, and additional bands at 8, 14, 20 and 27kDa were discovered (Rose-Martel et al., 2012). These results are comparable to the findings from this research with dominant bands at 10, 27 and 30kDa, however, the age of hens was not examined in the research conducted by Rose-Martel et al., (2012). In another study conducted by Rodriguez-Navarro et al. (2013), changes in cuticle proteins over a lay cycle were measured in commercial, white-shelled layers at 16, 30, 36 and 70 weeks-of-age. The methods used in the study were transmission and scanning electron microscopy, energy dispersive x-rays and total reflection-Fourier transform infrared spectroscopy, to measure the morphology and composition of the cuticle layer. It was discovered that as birds age, cuticle thickness decreased and glycosylation of proteins also decreased as permeability increased (Rodriguez-Navarro et al., 2013). One may surmise that eggs in this present study have increased permeability with age due to the decrease in protein amounts and profiles.

There are differences in cuticle proteins in samples from younger hens, however, not all proteins were reduced with age, such as bands at 10, 27 and 30kDa. It is, therefore, essential to compare birds of the same age during cuticle protein comparison or analysis, as there may be cuticle protein changes not necessarily related to type of bird. Chen et al., (2019a) conducted a comparison between the opacity of the cuticle of 20-week-old Rhode Island Red hens and White Leghorns versus 45-week-old Dwarf layers. The Dwarf Layers had the thickest, most resistant cuticle. However, as demonstrated from this present research, a direct comparison of these lines may have been impacted by age, rather than type of bird. Mean overall protein is lower at 20 weeks compared

to 50 weeks-of-age (Table 1) and samples from 24 and 28-week-old hens had proteins present that were not shown in samples from 20 weeks-of-age (Tables 2-3). This indicates that age has a significant effect on cuticle protein profile, and samples should be collected and compared from birds of the same age.

All ages had a very clear protein band at 10kDa (Tables 2-3). This protein began to increase as Lohmann Brown birds aged, however it did not increase as an absolute amount, suggesting that the total amount of protein goes down with age, although 10kDa protein does not decrease (Tables 2-3). Unfortunately, this protein could not be identified based on literature and further analysis is required to identify. The protein band at 12kDa was present for eggs from both Lohmann White and Lohmann Brown eggs at 24 weeks-of-age and from Lohmann White eggs at 28 weeks-of-age although was not present in either commercial line before 20 weeks-of-age or after 28 weeks-of-age (Tables 2-3). The protein band at 12kDa is thought to be apovitellenin-1 (Mikšík et al., 2014). Apovitellenin-1 is a protein, which is often found in the reproductive tract of female birds. This protein is produced in the liver and released into the blood stream during estrogen production (MacLachlan et al., 1996; Mikšík et al., 2014). It is unclear at this time the function that Apovitellenin-1 would play on the eggshell cuticle, as it is typically not a cuticle protein. It is also possible that this protein is lysozyme C, however this is a protein that should be found in abundance, rather than select samples and is typically found between 14 and 16kDa, depending on SDS-PAGE running conditions (Wellman-Labadie et al., 2008).

The protein at 18kDa is limited, appearing in minimal amounts in both Lohmann White and Lohmann Brown eggs throughout the laying cycle (Figure 3; Table 2 – 3). Proteins at 18kDa were

found in higher amounts for Lohmann Brown eggs when compared to Lohmann White. This protein could be lysozyme C, an effective anti-microbial protein that is commonly found in the eggshell cuticle and eggshell matrix, however it is usually found in abundance in the eggshell cuticle at 14–16kDa (Mikšík et al., 2014; Wellman-Labadie et al., 2008). It seems likely that this protein is ovocleidin-17, which is often found at 17kDA in the eggshell matrix however it could have leaked through to the eggshell cuticle (Wellman-Labadie et al., 2008).

The 27 and 30kDa proteins were the most consistent and prominent bands identified in this research (Figure 3; Tables 2–3). At the beginning of this study, these bands were often viewed as one single, large protein band, at 29 – 30kDa. Through the use of varying electrophoresis methodology, these two bands were eventually pulled apart to form two distinct 27 and 30kDa protein bands. At 27kDa, changes with age of bird were subtle as 66-week-old hens had the highest proportion of this protein, however, this is because this protein was constant throughout lay period compared to other protein bands which diminished as birds aged (Tables 2-3). Lohmann White eggs had higher 27kDa protein amounts compared to Lohmann Brown eggs, and there was an increase in this protein at 28 weeks-of-age in Lohmann White eggs. The amount of the protein at 58 and 62 weeks-of-age for Lohmann Brown hens were significantly lower compared to Lohmann White hens of the same age. This 27kDa protein band is likely ovocalyxin-32 (Bain et al., 2013; Hinke et al., 2009; Rose-Martel et al., 2012). Ovocalyxin-32 is an antimicrobial protein that is found in abundance throughout the eggshell cuticle (Hinke et al., 2009; Rose-Martel et al., 2012). Though ovocalyxin-32 has a molecular weight of 32kDa, it is often found between 29 and 32kDa (Figure 3) (Bain et al., 2013; Mikšík et al., 2014; Wellman-Labadie et al., 2008). The 30kDa was the second most prominent band (Figure 3; Tables 2-3). Higher values were found for both

Lohmann White and Lohmann Brown eggs from 50-week-old hens. This 30kDa band is thought to be similar to Kunitz-like protease inhibitor, which is often found around 36kDa (Bain et al., 2019; Mikšík et al., 2014). Kunitz-like protease inhibitor are small proteins often found in a variety of organisms, which are very important in halting the replication of viruses (Hernández-Goenaga et al., 2019).

The band displayed at 50kDa was more prominent in Lohmann Brown eggs, than in Lohmann White eggs (Tables 2-3). It appears this band was also more substantial early in the lay cycle and decreased as the birds aged. There are two possibilities for the identification of this 50kDa protein; ovocalyxin-36 or clusterin (Bain et al., 2013; Mikšík et al., 2014; Wellman-Labadie et al., 2008). Clusterin is a 51kDa glycoprotein often found in the eggshell and egg white of birds (Mann et al., 2003). Clusterin is typically not a cuticle protein and is discovered in the eggshell matrix and/or the egg white (Bain et al., 2013). Ovocalyxin-36 is discovered in the uterine fluid of laying hens, and in the eggshell cuticle at 48kDa, however, ovocalyxin-36 has a molecular weight of 36kDa (Bain et al., 2013; Gautron et al., 2007; Wellman-Labadie et al., 2008).

The final two bands, at 64kDa and 70kDa were inconsistent in presence in the eggshell cuticle. The 64kDa protein was only present in samples collected from Lohmann White eggs (Tables 2-3). No distinct change with bird age was clear in these results. A study conducted by Mikšík et al., (2014) also discovered a 64kDa protein that was identified as serum albumin; however, serum albumin is a 70kDa protein most often discovered in blood and body tissues (Jiang et al., 2022). The protein band estimated to be at 70kDa was consistently low throughout the production cycle for both bird lines. It is likely the protein estimated to be at 70kDa is ovotransferrin (Mann et al., 2003). Ovotransferrin is a major antimicrobial protein often found in the eggshell, egg white,

uterine fluid, and cuticle protein (Abeyrathne et al., 2014; Gautron & Nys, 2007; Mann et al., 2003). While the molecular weight of ovotransferrin is typically 76kDa, previous studies have discovered it, both below and above this molecular weight (Mikšík et al., 2014; Wellman-Labadie et al., 2008). Ovotransferrin as a very prominent cuticle protein, however the 70kDa protein found was sporadic throughout the present research. Ovotransferrin has also been found mostly in the eggshell matrix and was shown to double in amount as birds age (Panheleux et al., 2000). This increase with age in the eggshell matrix could compensate for the reduction seen in the cuticle layer of this research (Panheleux et al., 2000). More research is required for the exact identification of the proteins discovered in the eggshell cuticle.

3.5 Conclusion

It is clear the cuticle layer of the eggshell is variable in the quantity and presence of proteins. The bands at 10kDa, 27kDa and 30kDa were the most prominent and consistent throughout each sampling time in the laying cycle. These results indicate that the proteins at 10, 12, 18, 50, 64 and 70kDa tended to decrease as birds aged, however proteins at 27kDa and 30kDa tended to remain more consistent when compared to other proteins, with protein amounts actually increasing, as the other proteins decreased. In general, protein banding patterns were more complex from birds early on in their production cycle. As the total amount of protein decreased with age, the most prominent proteins remained at the same level, while less prominent proteins diminished. Overall, the types of proteins remained consistent between Lohmann White and Lohmann Brown eggs, and some key proteins could be related to differences between these shell colors. The results of this trial indicate that age plays a significant role in the amount, distribution and type of proteins found in the eggshell cuticle, therefore highlighting the importance of using birds of the same or similar age for research involving cuticle protein investigation.

CHAPTER 4: EGGSHELL CUTICLE PROTEIN AND DENSITOMETRY ARE INFLUENCED BY HEN LINE

4.1 Abstract

This study investigated the variability of antimicrobial cuticle proteins in commercial and heritage laying hens. Twelve eggs from two commercial lines (Alberta Commercial White and Alberta Commercial Brown) and 5 heritage lines of laying hens (Brown Leghorn, White Leghorn, Light Sussex, Barred Plymouth Rock, Shaver line of Barred Plymouth Rock) were collected from University of Alberta, and samples of two eggs were washed in 1% SDS for three minutes per egg at Dalhousie University. Protein quantity in the rinse was determined using RCDC protein assay and SDS PAGE was carried out to determine protein profile. Data from densitometry of key proteins was analyzed by one-way ANOVA to investigate differences between lines. The proteins at 12, 18, 50 and 70kDa were generally highest for both Barred Plymouth Rock lines. Bands at 27kDa were consistent between bird lines and 30kDa proteins were highest for Brown Leghorn. This study provides evidence that some heritage lines have unique cuticle protein profiles compared to commercial chickens.

4.2 Introduction

Breed, genetics, housing, as well as egg color are some of the many factors that influence the quality of the eggshell including the eggshell cuticle (Sirri et al., 2022). Commercial lines of laying hens are known to be selectively bred for high production rates, and often eggshell quality, however, rate of microbial penetration is not considered during breeding programs. In comparison, heritage lines are often used in research as a baseline, as they have not been selectively bred (Schmidt et al., 2019; Sirri et al., 2022). Variation was found in cuticle thickness in Rhode Island

Red hens compared to commercial layers (Dunn et al., 2019). Additional research on heritage lines have shown increased resistance to microbial penetration that is not necessarily related to differences in shell layers and may be due to variation in the eggshell cuticle layer (Kaur et al., 2009; Rathgeber et al., 2013). Previous research indicates that more investigation is required in the area, as the cuticle layer may play a larger role in microbial invasion when compared to shell structure, quality, or thickness.

Laying hen genetics, as well as eggshell color are both known to also play a role in the cuticle quality in terms of thickness and effectiveness (Samiullah & Roberts, 2014). The cuticle from brown eggs have been shown to be more effective when compared to white eggs, however the reasoning behind this has not yet been determined (Dunn et al., 2019; Samiullah & Roberts, 2014). Additional research is required to better explain these differences, as it may be due to cuticle protein profiles. Since breed and genetics play such a large role on cuticle quality, especially compared to other factors, such as housing or location, it is important to note that cuticle quality should be considered when preparing a breeding program (Ketta & Tumova, 2018).

The objective of this study was, therefore, to investigate the difference in cuticle protein quantity and profile between lines of commercial and heritage chickens.

4.3 Materials and Methods

The samples from this trial were collected on May 10, 2021, from the Poultry Research Centre, Edmonton at the University of Alberta, Canada, and shipped via FedEx to Dalhousie University

Agricultural Campus. The samples arrived on May 12, 2021, and procedures were immediately carried out.

4.3.1 Birds and Housing

Eggs were collected from two commercial lines of laying hens during this trial, Lohmann LSL Lite and a Lohmann Brown Lite; and 5 lines of heritage chickens: White and Brown Leghorn, Light Sussex, Barred Plymouth Rock, and a Shaver Line of the Barred Plymouth Rock. These birds were located at the University of Alberta in Edmonton, Alberta. The heritage birds were housed separated by breed, in a free run environment, while the commercial laying hens were housed in a battery cage system. The commercial lines were 66 weeks-of-age while the heritage lines were 44 weeks-of-age.

4.3.2 Sample Collection and Preparation

Twelve eggs from each line of bird were randomly selected and collected by a staff member at the University of Alberta. Eggs laid overnight were collected and placed into an egg carton for shipping. The eggs were shipped via FedEx to Dalhousie University Agricultural Campus.

Eggs arrived at Dalhousie University within 48 hours and were candled to ensure no abnormalities or micro-cracks were present. Eggs which had broken during transport were discarded. A total of 50 samples remained after candling and transport breakage. Ten Commercial Lohmann White, eight Commercial Lohmann Brown, 10 Light Sussex, 10 Barred Plymouth Rock, 10 Shaver line of Barred Plymouth Rock, 10 White Leghorn, and unfortunately, only two Brown Leghorn endured the trip due to breakage and exterior yolk contamination. The eggs were placed in a Whirl-Pak bag containing the extraction solution (see appendix 1) of 2ml of 1% Sodium dodecyl

sulfate (SDS), and manually massaged one at a time, for three minutes per egg, for a total of two eggs per sample, with the exception of the Brown Leghorn samples which were washed 1 egg per sample, as limited amounts were available. Once the egg was washed, each sample was placed into 100uL aliquots for storage at -80ºC, in preparation for further analysis.

4.3.3 RCDC (Reducing Agent and Detergent Compatible) Protein Assay

RCDC Protein Assay (BIO-RAD) was used to determine protein concentration using bovine serum albumin (BIO-RAD) as a standard. The 1.5ml microfuge tube assay procedure was followed. Absorbance was read at 650nm using BIOTEK Micro-plate Reader, and a standard curve was created using the absorbance values. Protein content was calculated using these values and dilutions using 1% SDS were carried out to ensure all samples were of the same protein concentration before sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE) was carried out.

4.3.4 Sodium Dodecyl Sulphate-Poly-acrylamide Gel Electrophoresis (SDS-PAGE)

Samples were placed into a sample buffer containing 8M urea, 2M Thiourea, 3% SDS, 0.7M 2-mercaptoethanol, 25mM Tris-HCl with a pH of 6.8 in a 30/70 mixture. Samples were heated to 90ºC for 5 minutes and centrifuged before loading onto sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was conducted using 4 – 15% polyacrylamide Mini-PROTEAN TGX Stain-Free Precast Gels (BIO-RAD). Electrophoresis was performed at 60 volts (V) for 20 minutes, then increased to 100V for approximately 10 minutes then completed at 250V for the time remaining. Protein bands were visualized by using UV activation for 2.5 minutes. The BIO-RAD gel imaging system was used for UV activation and documenting the gels. Once the

image was taken, it was sent to the photo editing and analysis program known as ChemiDoc, where it was analyzed and proteins were characterized by their molecular weight in relation to molecular weight standards. Densitometry was then carried out on the images using the same software.

4.3.5 Statistical Analysis

This experiment is a completely randomized design with each pair of sampled eggs as the experimental unit, with five replicates per treatment. The Proc Mixed Procedure of SAS was used to run a one-way ANOVA to investigate the interactions between bird lines (White and Brown Leghorn, Light Sussex, Barred Plymouth Rock, and a Shaver Line of the Barred Plymouth Rock) (SAS, 2018). The total protein extracted, and the individual proteins separated on SDS-PAGE were the response variables analysed. The assumptions of normal distribution equal variance were tested. The quantity of the individual protein bands is expressed as percent of sample (%), based on the individual proteins bands compared to the total amount of stain from the banding pattern of the sample. This percentage was then used to estimate the amount of protein (mg/ml) in each band based on the total protein in the sample. The results were deemed significant if the P-value was <0.05 (to two decimal places). Standard error was reported with the mean.

4.4 Results and Discussion

4.4.1 Results

The results obtained are from evaluation of the eggshell cuticle composition change with hen line. The level of protein harvested was not statistically different between commercial and heritage lines, despite the fact that the two commercial layers were 68 weeks-of-age, and the five heritage lines were 44 weeks-of-age at the time of collection ($P>0.05$) (Table 4).

Table 4. Quantity of cuticle protein harvested and standard error from commercial (68 weeks-of-age) and heritage lines (44 weeks-of-age) of chickens from the University of Alberta.

Breed	Age (weeks)	Total Protein (mg/ml)	P-Value
Commercial Brown	68	4.99^{A} ±0.46	0.068
Commercial White	68	3.80^{A} ±0.36	-
Barred Rock Heritage	44	4.96^{A} ±0.57	-
Barred Rock Shaver	44	4.86^{A} ±0.36	-
Light Sussex	44	4.05^{A} ±0.36	-
White Leghorn	44	4.72^{A} ±0.36	-
Brown Leghorn	44	3.29^{A} ±0.36	-

Mg/ml = milligrams of protein per millilitre of extraction solution
A-B = means with different superscripts are significantly different $\alpha \leq 0.05$

Tables 5 and 6 provide the protein profiles from each section of the trial. Mean percent sample, and mean amount of protein was included when significant to the results.

The 10kDa protein band was present in all samples (Tables 5). Percent of sample for this band was significantly impacted by breed of bird ($P<0.05$). Eggs from Brown Leghorns had the lowest levels of this protein (12.92%), especially compared to the Shaver line of Barred Plymouth Rock (20.65%) and White Leghorn (21.70%) ($P<0.05$). The Heritage line of Barred Rock was the only breed to have 12kDa and 18kDa proteins present. The protein band at 27kDa was the most prominent bands throughout this study for all bird lines (Figure 3). However, this band was not statistically different for percent of protein between lines ($P>0.05$). The percent of 30kDa protein band had a significant difference between lines ($P<0.05$). The highest percent sample was from the Brown Leghorn (57.08%), which were only statistically different from the Heritage line of Barred Plymouth Rock (42.73%) ($P<0.05$). All the other samples were not significantly different from Brown Leghorn ($P>0.05$). The 50kDa band was statistically different for percent protein between bird lines ($P<0.05$). The Shaver line of Barred Plymouth Rock

samples had the highest percent for this protein (4.23%), while the lowest percentage was found in Brown Leghorn (0.70%), and Commercial White (0.76%) ($P<0.05$). The 70kDa protein band was present in all samples, except the Commercial White eggs. This band had the highest percent sample for the two Barred Plymouth Rock samples (2.84% and 2.80%), which were statistically higher than the others, except Commercial Brown eggs (2.20%) ($P<0.05$).

The amount of protein in the 10kDa protein band was significantly different between bird lines ($P<0.05$) (Table 6). Eggs from Brown Leghorns had significantly lower amounts (0.42mg/ml), compared to the Shaver line of Barred Plymouth Rock (1.00mg/ml) and White Leghorn (1.02mg/ml) ($P<0.05$). The Heritage line of Barred Rock was the only breed to have 12kDa and 18kDa proteins present. The protein band found to be at 27kDa was the most prominent bands throughout this study for all bird lines (Figure 3). This band was not statistically different for the breed of hen ($P>0.05$). The band at 30kDa was consistent throughout each breed, and there was no significant difference between lines ($P>0.05$). The amount of protein in the 50kDa band was statistically different between lines ($P<0.05$). The Shaver Barred Plymouth Rock samples had the highest amount of protein (0.21mg/ml), while the Commercial White had the lowest value (0.03mg/ml). A significant difference was found in the amount of protein in the 70kDa band between lines ($P<0.05$). This protein was found in all samples, with the exception of Commercial White eggs. Commercial Brown (0.11mg/ml), and both Barred Rock samples (0.14mg/ml) were statistically higher to all other bird lines ($P<0.05$).

Table 5. Densitometry value and standard error of protein bands from SDS-PAGE gels expressed as a percentage of cuticle protein samples harvested from the eggs of two lines of commercial laying hens (68 weeks-of-age) and five lines of heritage hens (44 weeks-of-age) from the University of Alberta.

Molecular Weight	Commercial Brown	Commercial White	Barred Plymouth Rock Heritage	Barred Plymouth Rock Shaver	Light Sussex	White Leghorn	Brown Leghorn	P-Value
10kDa	15.94^{AB} ±1.46	19.09^{AB} ±1.13	16.02^{AB} ±1.13	20.65^{A} ±1.13	16.99^{AB} ±1.13	21.70^{A} ±1.13	12.92^{B} ±1.79	0.002
27 kDa	19.01^{A} ±2.3	15.24^{A} ±1.79	19.36^{A} ±1.79	19.01^{A} ±1.79	12.29^{A} ±1.79	16.43^{A} ±1.79	20.28^{A} ±2.83	0.082
30kDa	53.14^{AB} ±2.80	52.12^{AB} ±2.16	42.73^{B} ±2.16	47.52^{AB} ±2.16	50.89^{AB} ±2.16	49.22^{AB} ±2.16	57.08^{A} ±3.42	0.02
50kDa	3.03^{A} ±0.81	0.76^{A} ±0.63	2.86^{A} ±0.63	4.23^{A} ±0.63	1.08^{A} ±0.63	1.75^{A} ±0.63	0.7^{A} ±1.00	0.008
70kDa	2.20^{AB} ±0.46	0.00^{C} ±0.36	2.84^{A} ±0.36	2.80^{A} ±0.36	0.79^{BC} ±0.36	0.20^{C} ±0.36	0.62^{BC} ±0.57	<0.0001

A-B = means with different superscripts are significantly different $\alpha \leq 0.05$
Mg/ml = milligrams of protein per millilitre of extraction solution

Table 6. Densitometry value and standard error of protein bands from SDS-PAGE gels expressed as milligrams of protein from cuticle protein samples harvested from the eggs of two lines of commercial laying hens (68 weeks-of-age) and five lines of heritage hens (44 weeks-of-age) from the University of Alberta.

Molecular Weight	Commercial Brown	Commercial White	Barred Plymouth Rock Heritage	Barred Plymouth Rock Shaver	Light Sussex	White Leghorn	Brown Leghorn	P-Value
10kDa	0.79^{AB}	0.72^{AB}	0.80^{AB}	1.00^{A}	0.70^{AB}	1.02^{A}	0.42^{B}	
	±0.10	±0.10	±0.10	±0.10	±0.10	±0.10	±0.12	0.004
27 kDa	0.96^{A}	0.58^{A}	0.96^{A}	0.92^{A}	0.53^{A}	0.77^{A}	0.73^{A}	
	±0.15	±0.11	±0.11	±0.11	±0.11	±0.11	±0.18	0.07
30kDa	2.64^{A}	1.97^{A}	2.12^{A}	2.31^{A}	2.07^{A}	2.31^{A}	1.82^{A}	
	±0.26	±0.20	±0.20	±0.20	±0.20	±0.20	±0.32	0.366
50kDa	0.15^{B}	0.03^{AB}	0.14^{AB}	0.21^{A}	0.04^{AB}	0.08^{AB}	0.04^{AB}	
	±0.04	±0.03	±0.03	±0.03	±0.03	±0.03	±0.05	0.004
70kDa	0.11^{A}	0.00^{B}	0.14^{A}	0.14^{A}	0.03^{B}	0.01^{B}	0.01^{B}	
	±0.02	±0.01	±0.01	±0.01	±0.01	±0.01	±0.02	<0.0001

A-B = means with different superscripts are significantly different $\alpha \leq 0.05$
Mg/ml = milligrams of protein per millilitre of extraction solution

4.4.2 Discussion

The total level of protein harvested was not statistically different between the seven lines of laying hen, despite the fact that the two commercial layers were 68 weeks-of-age, and the five heritage lines were 44 weeks-of-age at the time of collection (Table 4). This result was surprising, as it has been shown that age played a large role in the amount of protein found in the cuticle (Chapter 3). In addition, when comparing data from commercial layers from Dalhousie Agricultural Campus at 66 weeks-of-age (Chapter 3; Table 2) with commercial layers from the University of Alberta at 68 weeks-of-age (Table 4), there is a clear variation, as the amount of protein extracted from Commercial eggs from the University of Alberta were substantially higher. This could be due to the fact that eggs from the University of Alberta were shipped via air and changes in cabin pressure could have altered the protein on the outside of the eggshell. Next steps for this research should include a study examining how cabin pressure could alter cuticle and eggshell proteins.

The difference between local lines and those from the University of Alberta could be due to housing differences or location. The five heritage lines from the University of Alberta were housed in a floor pen system, while the Alberta commercial lines and two local commercial lines were housed in a conventional cage system. However, this is disputed in research conducted by Ketta and Tumova, (2018) who studied the difference between three commercial lines of laying hens, and the effect of different housing conditions on cuticle amounts. It was discovered that there was a difference between lines as Lohmann Brown had the highest cuticle deposition, followed by Isa Brown and Hy-Line Silver-Brown laying hens. They also discovered that there was no significant difference in cuticle amounts based on different housing conditions (Ketta & Tumova, 2018).

The present study shows that protein amounts do not differ among lines, however, protein profiles do differ. Previous research supports this finding as it indicates that breed and shell color play a large role in cuticle thickness and effectiveness of the cuticle layer (Dunn et al., 2019; Kaur et al., 2009; Ketta & Tumova, 2018; Rathgeber et al., 2013; Samiullah & Roberts, 2014; Sirri et al., 2022). Dunn et al., (2019) used cuticle staining methods were used to determine cuticle differences between five lines of commercial and heritage chickens. It was determined that line of bird played a role in cuticle deposition and should be taken into consideration in poultry breeding to reduce the incidence of foodborne illness in eggs. However, the five lines used in that study were of different ages, therefore age may have played a factor (Dunn et al., 2019). Sirri et al., (2022) also investigated the effects of chicken breed and age on cuticle deposition using cuticle staining methods. Their research examined three Commercial laying hen lines at various age sequences (20, 30, 40, 50, 60 and 70 weeks-of-age) (Sirri et al., 2022). Sirri et al., (2022) determined that line of bird influenced cuticle profile and age played a large role in the degree of cuticle coverage.

The band discovered at 10kDa, had highest values for both amount and percentage of protein in the White Leghorn and Shaver line of Barred Plymouth Rock, and lowest values were found in Brown Leghorn, with all other bird lines falling between (Tables 5-6). Though this protein band has appeared in previous research, the protein could not be confirmed based on literature values (Rose-Martel et al., 2012). This 10kDa protein is consistently found on the eggs of all bird lines, and, therefore, may play a key role in the protection from microbes. Additional research is required to identify this protein and determine its significance.

The protein band discovered at approximately 12kDa, previously speculated to be apovitellenin-1 (Chapter 3), was only present for the Heritage line of Barred Plymouth Rock samples, therefore

was not included in the data tables (Mikšík et al., 2014). All other egg samples did not have this protein in the cuticle. Only egg samples from hen lines at 28 weeks-of-age or younger were positive for this protein in Chapter 3, therefore it was not surprising for it to be largely absent (Chapter 3; Tables 2-3). This may suggest that this protein is only available in birds with increased resistance to microbes, as the Barred Rock birds had higher levels of protein and more bands, compared to other lines. This same heritage Barred Plymouth Rock sample was the only sample to have a protein band at approximately 18 kDa, also not shown in this data set. This band was most evident for brown eggs from hens that were 28 weeks-of-age and at low levels for either colored egg at 24 weeks-of-age in the first trial (Chapter 3; Tables 2-3). This protein was speculated to be either lysozyme C, which is typically seen between 14 and 16kDa, or ovocleidin-17, often found at 17kDa, in the eggshell matrix (Mikšík et al., 2014; Wellman-Labadie et al., 2008).

Both lines of Barred Plymouth Rock had higher levels of protein in each band when compared to the other heritage lines, which is supported by previous research carried out by Rathgeber et al., 2013. Rathgeber et al., (2013) examined how *Salmonella* penetrated the eggshell differently in five heritage lines of chickens, and one commercial laying line. Using an eggshell penetration assay, it was determined that Barred Plymouth Rock eggs had the lowest bacterial penetration, while Light Sussex and Brown Leghorn had the highest, and all other lines were intermediate. The results of the research conducted by Rathgeber et al. (2013) concluded that these differences between bird lines could not be explained by eggshell structure, and may be related to other factors, such as cuticle composition, supporting the findings of the present study.

Bands discovered at 27 and 30kDa were the most prominent throughout this study, in addition to the previous age trial (Chapter 3). These proteins were a very similar in molecular weight and had a substantial proportion of the total protein affiliated with them (Tables 5-6). The protein estimated to be at 27kDa was not statistically different for type of bird for either protein amount or percentage of protein in the bands. The protein discovered at 30kDa had a higher percent of protein in the Brown Leghorn, and lowest in the Barred Rock Heritage breed, while all other samples were intermediate between these. Based on literature values, the band discovered at 27kDa was previously estimated to be ovocalyxin-32, which is often found between 29 and 32kDa, and the 30kDa protein is similar to Kunitz-like protease inhibitor, which is often found around 36kDa, however little is known about these specific protein bands (Bain et al., 2013; Mikšík et al., 2014).

The protein band estimated to be at 50kDa was different between treatments, with the Shaver line of Barred Plymouth Rock being the highest for this protein (Tables 5-6). As previously stated, there are two possibilities for the identification of this protein; ovocalyxin-36 or Clusterin (Bain et al., 2013; Mikšík et al., 2014; Wellman-Labadie et al., 2008). Typically, ovocalyxin-36 is discovered in the uterine fluid, which could indicate that this protein came from uterine fluid proteins deposited on the eggshell cuticle during eggshell formation (Kaur et al., 2013). Previous research, conducted by Kaur et al., (2013), studied the variation in uterine proteins in two heritage lines and two commercial layers. The study collected uterine fluid and examined proteins using SDS-PAGE. Both heritage lines; Fayoumi and Light Sussex, in addition to the Commercial Brown layers, had higher protein concentrations when compared to Commercial White hens. Proteins were found at similar molecular weight to this study; however, these proteins were not identified (Kaur et al., 2013).

The protein discovered at 70kDa, previously estimated to be ovotransferrin, was found in all samples, apart from the Commercial White hens (Table 5-6) (Mann et al., 2003). This band was found in highest amounts in the Barred Rock lines. This seems to show a similar trend throughout this study, that Barred Rock lines have increased protein profiles, and thus better protection from microbes, compared to other bird lines (Rathgeber et al., 2013). Additional research is required to correctly identify these proteins.

4.5 Conclusion

In conclusion, it is clear there is variation among cuticle protein profiles due to genetic variability. The amount of overall protein from each sample did not differ between the seven lines included in this trial, even between those of different ages. However, amount and percentage of protein in each band, did vary between bird lines. In general, protein banding patterns were more complex with the Heritage Line of Barred Plymouth Rock which had protein bands at 12 and 18 which were not present in the other samples. Protein bands estimated to be at 10, 50 and 70kDa were also highest for both lines of Barred Plymouth Rock. Bands at 27kDa were consistent between lines, while the protein band at 30kDa showed in increased percent of sample for Brown Leghorn and lowest for Barred Plymouth Rock. Results from this research provide evidence that some heritage lines of birds have unique profiles of proteins compared to commercial chickens, that may relate to improved protection against entry by foodborne illness. This study provides a basis for further investigation into what proteins in the cuticle layer are necessary to optimize protection against entrance by microbes into chicken eggs through the shell.

CHAPTER 5: CONCLUSION

5.1 Summary and Conclusion

More proteins were present in the samples from younger hens and banding patterns appeared more complex. There was a significant decrease in proteins estimated to be at 10, 12, 18, 50, 64 and 70 kDa as birds aged, and the amount of protein harvested from the eggshell cuticle was reduced after the birds reached 50 weeks-of-age. This overall reduction of proteins as birds got older was clear, however SDS-PAGE revealed that while these proteins seemed to diminish, other proteins at 27 and 30kDa increased. It may be speculated that these are the key cuticle proteins required for protection of the egg's internal contents from microbes. Overall, it appeared that proteins remained consistent between Lohmann White and Lohmann Brown eggs, however some key differences were apparent, such as a protein band at 64kDa which only appeared in white eggs. This indicates that age plays a significant role in the proteins of the eggshell cuticle, emphasizing the importance of using birds of similar ages in research investigating cuticle proteins. Variation in cuticle proteins by breed is also a key consideration when completing research involving different lines of chickens. While the amount of protein did not seem to differ between lines, proteins, and specific amounts in each protein band, did vary between lines. Banding patterns appeared more complex with the Heritage line of Barred Plymouth Rock hens, which had protein bands at 12kDa, and 18kDa, that did not appear in any other breed. Both Barred Plymouth Rock lines also showed increased amounts in 10, 50 and 70kDa proteins when compared to other lines, although showed lower amounts for the key protein at 30kDa, when compared to other lines. The constant band discovered at 27kDa remained consistent for each breed, suggesting this is a key cuticle protein. The results from this study indicate that heritage lines of birds have distinct cuticle protein profiles when compared to commercial hens, which could indicate an increased resistance to microbial

penetration and decreased incidence of food borne illness. Further investigation is required to identify these key proteins.

5.2 Future Prospective of Research

It is essential to ensure the anti-microbial proteins are abundant in the eggshell cuticle and are working to protect the shell against microbes. This research was intended to bridge the gap in research in relation to cuticle proteins and how they differ among bird line, and age. The protein amount available in the cuticle as well as protein profile differs with age and breed. The results of this research can be applied when considering the genetics of commercial laying hens, as it indicates that heritage lines have higher values of protein, therefore it is presumed that they have a greater defense against bacteria.

5.3 Next Steps

This research indicates that age, and breed affect the eggshell cuticle. The next steps in terms of further research would be to identify these proteins and evaluate how the results of this research compare to the protein amount of profile from indigenous chickens. In addition, research should also focus on how the results of this study apply to hatching eggs, however care would need to be given to avoid negatively impacting the profile during incubation. Additional research surrounding how differences in cuticle proteins relate to proteins embedded in the eggshell matrix and eggshell membranes would be beneficial. Next steps for this research should also include a study examining how cabin pressure could alter cuticle and eggshell proteins. Another recommendation would be to collect eggs every two weeks for the full production cycle of laying hens to examine cuticle changes in more detail.

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APPENDIX

Appendix 1. Preliminary Trial

A1.1 Introduction

Due to restrictions of transporting chemicals and biological material across international borders, a preliminary trial was conducted to determine the best cuticle extraction method of harvesting cuticle proteins and transporting them safely. The objective of this preliminary study was to determine the most effective and consistent method for removing the eggshell cuticle while still being able to transport these chemicals and biological materials safely.

A1.2 Materials and Methods

This trial was conducted prior to the start of the primary research. Three extraction solutions were used to determine which solution would be the best choice for this study. 1% SDS, 1% SDS 2mM DTT, and 1N HCl.

A1.2.1 Birds and Housing

Eggs were collected from two commercial lines of laying hens during this trial: Lohmann LSL Lite and Lohmann Brown Lite. These birds were located at the Atlantic Poultry Research Institute, on the Dalhousie University Faculty of Agriculture Campus in Truro, Nova Scotia. Sampling took place when hens were 60 weeks-of-age

A.1.2.2 Sample Collection and Preparation

Thirty-six eggs were randomly selected and collected. Eggs were laid under supervision between the hours of 9am and 11am. Once dry, the eggs were collected and placed into a sterile plastic bag.

Eggs were candled to ensure no abnormalities or micro-cracks were present. The samples were left at room temperature for 24 hours, to ensure the cuticle was fully mature before completing the protein assay. The eggs were placed in a Whirl-Pak bag containing an extraction solution of 2ml of either 1% SDS, 1% SDS 2mM DTT, or 1N HCl and manually massaged one at a time, for 1 minutes per egg, for a total of 3 eggs per sample. Once the cuticle was removed, each sample was placed into 100uL aliquots for storage at -80^{o}C, in preparation for further analysis.

A1.2.3 Reducing Agent and Detergent Compatible (RCDC) Protein Assay

Reducing Agent Detergent Compatible (RCDC) Protein Assay (BIO-RAD) was used to determine protein concentration using bovine serum albumin (BIO-RAD) as a standard. The 1.5ml Microfuge tube assay procedure was followed, and absorbance was read at 650nm using BIOTEK Micro-plate Reader, and a standard curve was created using the absorbance values. Protein content was calculated using these values and dilutions using 1% SDS were carried out to ensure all samples were of the same protein concentration before SDS-PAGE was carried out.

A1.2.4 Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were placed into a sample buffer containing 8M urea, 2M Thiourea, 3% SDS, 0.7M 2-mercaptoethanol, 25mM Tris-HCl with a pH of 6.8 in a 30/70 mixture. Samples were heated to 90^{o}C for 5 minutes and centrifuged before loading onto sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was conducted using 4 – 15% polyacrylamide Mini-PROTEAN TGX Stain-Free Precast Gels purchased from BIO-RAD. Electrophoresis was performed at 60 volts (V) for 20 minutes, then increased to 100V for approximately 10 minutes then completed at 250v for the time remaining. Protein bands were visualized by using UV

activation for 2.5 minutes. The BIO-RAD gel imaging system was used for UV activation and documenting the gels. Once the image was taken, it was sent to the photo editing and analysis program known as ChemiDoc, where it was analyzed and proteins were characterized by their molecular weight in relation to molecular weight standards. Densitometry was then carried out on the images using the same software.

A1.2.5 Statistical Analysis

This experiment is a completely randomized design with a sample of three eggs as the experimental unit. PROC MIXED of SAS was used to run ANOVA to investigate the significant differences between extraction solutions on amount of protein harvested. The total protein extracted was the response variables considered ($P<0.05$).

A1.3 Results and Discussion

Table 7. Cuticle Protein Harvested from Three Wash Solutions

Extraction Solution	Protein (mg/ml)	P-Value
1% SDS	2.57 B	<0.0001
1% SDS 2mM DTT	2.88 B	
1N HCl	5.13 A	

The amount of protein harvested was variable, depending on the cuticle extraction solutions (Table7). While the use of 1N HCl increased the protein content compared to SDS alone, or SDS and DTT, it was determined that this extraction solution may also increase the risk of removing shell proteins and alter the results. There was no significant difference between 1% SDS and 1% SDS with DTT.

A1.4 Conclusion

1% SDS was the most suitable wash solution as it was the most consistent and provided the least number of issues as DTT increases risk during handling and transport.

Appendix 2. Cuticle Identification

Table 8. Identification Estimates of Cuticle Proteins Discovered (Bain et al., 2013; Mikšík et al., 2014; Rose-Martel et al., 2012)

kDa	Suspected protein	kDa of Suspected protein
10	Unidentified	-
12	Apovitellenin-1	12 (Mikšík et al., 2014)
12	Lysozyme C	14 – 16 (Mikšík et al., 2014; Wellman-Labadie et al., 2008)
18	Lysozyme C	14 – 16 (Mikšík et al., 2014; Wellman-Labadie et al., 2008)
18	OC-17 ovocleidin-17	15 - 17 (Bain et al., 2013; Mikšík et al., 2014)
27	Ovocalyxin-32	30 – 32 (Bain et al., 2013; Mikšík et al., 2014; Wellman-Labadie et al., 2008)
30	Ovocalyxin-32	30-32 (Bain et al., 2013; Mikšík et al., 2014; Wellman-Labadie et al., 2008)
30	LOC771972 similar to Kunitz-like protease inhibitor	36 (Bain et al., 2013; Mikšík et al., 2014)
50	Ovocalyxin – 36	48 (Bain et al., 2013; Wellman-Labadie et al., 2008)
50	clusterin	51 (Bain et al., 2013; Mikšík et al., 2014)
64	ALB-serum albumin	64 (Mikšík et al., 2014)
70	Ovotransferrin	77.8 – 78 (Mikšík et al., 2014; Wellman-Labadie et al., 2008)