THE EFFECTS OF STORAGE TIME ON VITELLINE MEMBRANE PROTEIN BANDING PATTERNS AND INTERIOR EGG QUALITY OF EGGS FROM NON-MOLTED AND MOLTED HENS

A Thesis

by

ANGELA JEAN KELLEY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2003

Major Subject: Food Science and Technology

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ABSTRACT

The Effects of Storage Time on Vitelline Membrane Protein Banding Patterns and Interior Egg Quality of Eggs from Non-Molted and Molted Hens. (December 2003)

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Vitelline membrane strength plays a role in preventing contamination of albumen by yolk during separation and is important to food safety. Two experiments were conducted to determine if a relationship exists between vitelline membrane protein banding patterns, interior egg quality, and vitelline membrane rupture strength. Eggs were gathered from commercial egg producers from pre-molt (26 wk or 72 wk) and post-molted (74 wk or 82 wk) hens. In each of two trials twenty-one eggs were gathered and stored (4°C) per experiment. Three eggs were evaluated on days 0, 7, 14, 21, 28, 35, and 42 for eggs from pre-molted hens; and 1, 7, 14, 21, 28, 35, and 42 for eggs from molted hens for changes in SDS-PAGE protein banding patterns. The yolk from each egg was isolated and rolled on a wet paper towel to remove adhering albumen. The yolk was emptied and washed. The whole membrane was placed into double deionized water and divided into two sections. The first section was the whole membrane sample and the other was separated by forceps into inner and outer membrane samples. The three sections were dissolved separately in 1% SDS/70 mM Tris/HCl, pH 6.8. Protein concentration was determined using the Lowry method and proteins separated on 4-20% gradient gel by SDS-PAGE. Protein banding

patterns were analyzed using the Bio-Rad Multi-Analyst Densitometer. Reductions of VMO I and GP II occurred along with reductions in the protein bands between 60 to 100 kDa.

In each of two trials, an additional one hundred forty eggs were gathered at the same time from the same flock and stored at 4°C. Twenty eggs were evaluated for quality on days 0, 7, 14, 21, 28, 35, and 42 for eggs from pre-molted hens; and 1, 7, 14, 21, 28, 35, and 42 for eggs from molted hens. Yolk index, albumen height, albumen pH, and yolk pH were determined. Vitelline membrane strength was determined using a compression anvil. Two different treatments were used on the yolk when evaluating rupture strength: 10 egg yolks with inner thin albumen layer, and 10 egg yolks rolled on wet paper towel to remove inner thin albumen layer. Interior egg quality and vitelline membrane strength declined during storage.

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CHAPTER I

INTRODUCTION

Although the primary purpose is for reproduction of chickens, the egg is often the nutritional standard by which other human foods are judged (Burley and Vadehra, 1989). The egg contains one-tenth the amount of proteins needed by adults (Everson and Souders, 1957). An average layer egg contains 212 mg of cholesterol (USDA, 2003). The amount of cholesterol in the egg is an important factor as to why the consumption of eggs has decreased 24% from 1970 to 1995 (McNamara, 1999). Some researchers say that people should limit their egg intake to two eggs per week and others say that one egg a day is acceptable (Assmann *et al.,* 1999; Hu *et al.,* 1999). Dawber and co-workers (1982) found no relation between egg intake and the incidence of coronary heart disease. Likewise, Hu and co-workers (1999) found that the consumption of one egg per day does not increase the risk of heart disease, but higher levels of consumption does increase risk. Even with the debate on the cholesterol content of eggs, the egg contributes other nutrients such as vitamins E, B_{12}, A and folate that help protect against heart disease (Song and Kerver, 2000). The per capita consumption of shell eggs has declined from 321 in the 1960's to 235 in the 1990's, but at the same time the consumption of egg products has increased (Madison and Harvey, 1997).

In 2002, 203 million cases of eggs were produced in the United States, and 60.3 egg cases went on to further processing (NASS, 2003). The edible liquid egg products are further processed at breaker plants into products such as refrigerated liquid egg whites, yolks; frozen egg whites, yolks; dried egg products such as flake albumen, or whole egg

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This thesis follows the style and format of Poultry Science.

solids (Cotterill, 1986). Additional value can be added by creating specialty egg products such as frozen scrambled eggs, frozen omelets, and frozen hard-cooked egg rolls or long eggs (Cotterill, 1986).

Concerns of Salmonella Enteritidis

The most important food safety issue now associated with shell eggs is foodborne illness caused by *Salmonella* Enteritidis (SE). SE, which poses a major concern for public health, has been closely associated with shell eggs (Nisbet and Ziprin, 2001). Grade A shell eggs have been the vehicle for SE outbreaks (CDC, 1988; St. Louis *et al.,* 1988). *Salmonella* Typhimurium (ST) and SE are responsible for 41% of the cases of salmonellosis which amounts to almost 15 cases for every 100,000 people (CDC, 2001). The estimated annual costs of foodborne illnesses caused by ST and SE amounts to $0.9 to 3.7 billion dollars (Frenzen *et al*., 1999). The infection of SE comes through transovarian infection or more commonly through passage of the bacteria through cracks in the egg (Nisbet and Ziprin, 2001; Smeltzer *et al.,* 1979; Timoney *et al.,* 1989). St. Louis and co-workers (1988) found that along with the incidence of SE in grade A shell eggs, the eggs were un-cracked leading them to theorize that this was transovarian infection of layers. SE is not only found in the albumen but also the yolk of eggs (Timoney *et al.,* 1989). SE grows in the yolk when it reaches the yolk or can obtain nutrients from it (Humphrey and Whitehead, 1993). Temperature is an important factor in which the egg contents permit growth of SE; SE numbers increase significantly after three weeks at 20ºC (Humphrey and Whitehead, 1993). In order to reduce the incidence of SE contamination in eggs, United States Department of Agriculture (USDA) requires testing the breeder flocks of egg producing hens that have

contributed to SE contaminated eggs (USDA, 2002).

Effect of Yolk on Albumen Proteins

The vitelline membrane (VM) keeps the yolk and the albumen separated (Trziszka and Smolinska, 1982). Rupturing the VM and allowing yolk to contaminate the albumen is not necessarily a food illness concern, but is an industry concern because it negatively affects the functionality of the albumen proteins (St. John and Flor, 1931). When egg white foam is contaminated with as little as one drop of yolk, the foam volume drops from 140 mL to 40 mL (St. John and Flor, 1931). Foaming failure for angel food cakes can result with as little as 0.275% yolk lipid contamination (Smith, 1959). When the yolk is mixed with the albumen, the low density fraction of the yolk changes from an insoluble form into a soluble form (Parkinson, 1972). There are two explanations as to why yolk reduces the functionality of the albumen proteins. One is that the fat in the yolk retards the foam formation due to a reduced surface tension (St. John and Flor, 1931). The second explanation of this damaging effect of yolk on foams is the formation of a yolk ovomucin complex, or "yolk bullets" (Cunningham and Cotterill, 1964).

Yolk proteins and functionality. The ratio of protein to lipids in the yolk is 1:2 (McIndoe, 1971). The yolk components originate from the liver and are transferred to the yolk by plasma (Vieira *et al.,* 1995). The lipids needed in the yolk also pass through the plasma, and the amount of lipids in the ovarian follicles is associated with the rate of egg production or yolk deposition (Shivaprasad and Jaap, 1977). The yolk is made up of a base of lipoproteins, very low density protein, and vitellogenin (Vierira *et al.,* 1995). High density lipoprotein, another component of yolk, contains two apolipoproteins, and although the function is not known, it might be a source of lipids needed in the yolk (Vierira *et al.,*

1995). One of the base components, vitellogenin is an important part of the yolk because it is the precursor for phosphoproteins, lipovitellin and phosphovitin (Deeley *et al.,* 1975). Lipovitellin is a high density lipoprotein, and is flooded into a globular arrangement with lipids attached to its surface (Evans *et al.,* 1968). Lipovitellin is also what makes up the core of yolk granules (Willems and Stockx, 1973). Yolk granules are made of a core of the lipovitellines surrounded by phosphovitin; its outer layer is composed of low density lipoproteins (Willems and Stockx, 1973). As for the characteristics and functionality of the yolk, emulsion stability, an important functional property, is affected by the breed of the layer along with the age of the bird (Varadarajulu and Cunningham, 1972). Brown leghorns produce yolks that have double the amount of emulsion stability of white leghorns (Varadarajulu and Cunningham, 1972).

Albumen proteins and functionality. The albumen consists of about 40 proteins (Gilbert, 1971). Table I-1 summarizes the major proteins, their amounts, type of protein, isoelectric point, and function if known. Ovalbumin is a glycoprotein that accounts for the majority of the proteins in egg white (Stevens, 1991). The function of ovalbumnin is a heat stabilizer (MacDonnell *et al.,* 1955). Ovotransferrin functions as an iron transporter (Stevens, 1991). Ovomucin does not contribute to the rigidity of the albumen, but a network of fibers of an ovomucin-lysozyme complex is a factor that contributes to rigidity (Brooks and Hale, 1959; Brooks and Hale, 1961). Ovomucin does contribute to foaming and emulsion properties (Kato *et al.,* 1985). Viscosity of the white affects the foaming properties of ovomucin, and emulsion stability is dependent on surface hydrophobicity (Kato *et al.,* 1985). The proteinase inhibitors in the albumen are ovomucoid, ovoimhibitor, cystatin, and ovostatin (Stevens, 1991). Cystatin has two forms with pI values of 6.5 and

5.6, respectively (Anastasi *et al*.,1983). This protein is not a glycoprotein and is found in the serum; therefore it is not synthesized in the egg (Anastasi *et al*.,1983). Cystatin inhibits cysteine proteinases but the mechanism is unknown (Anastasi *et al*.,1983). Another protein is ovostatin which originates in the oviduct (Nagase *et al.*, 1983). Lysozyme is another important protein in the albumen, which can bind with ovomucin, ovalbumin, and ovotransferrin (Stevens, 1991). Lysozyme is the only bactericidal protein in the albumen. It exerts its bactericidal activity by cleaving the cell walls of gram positive bacteria (Stevens, 1991). The vitamin binding proteins are avidin, riboflavin binding protein, and thiamin binding protein (Stevens, 1991). Avidin functions as a biotin binding protein (Bush and White, 1989). As biotin diffuses into the albumen from the yolk, it is free biotin, avidin traps it (Bush and White, 1989), thereby making it unavailable to support bacterial growth. Riboflavin binding protein in the egg white captures flavin in the white and binds it as a flavoprotein (Rhodes *et al.*, 1959). Thiamin binding protein is not a glycoprotein and is similar to riboflavin binding protein in molecular weight and binding abilities (Muniyappa and Adiga, 1979).

TABLE I-1. Albumen proteins and characteristics.

Protein	Amount (%)	pI	Function
Ovalbumin	54	4.5	Heat stabilization[2]
Ovotransferrin	12	6.06	Iron transport
Ovomucin	1.5	4.5-5.0	Structural, viscous
Ovomucoid	11	4.1	Proteinase inhibitor Inhibits trypsin[1]
Ovoinhibitor	1.50	5.1	Proteinase inhibitor
Cystatin	0.01	5.6 & 6.5	Thiol proteinase inhibitor
Ovostatin	0.5	4.9	Proteinase inhibitor
Lysozyme	3.4	10.7	Enzyme Lyses bacteria[1]
Ovoglobulin G2	1.0	4.9-5.3	Foaming agent[3]
Ovoglobulin G3	1.0	4.8	Foaming agent[3]
Riboflavin binding protein	1.0	4.0	Riboflavin transport
Avidin	0.5	10.0	Binds Biotin
Thiamin binding protein	-	-	Thiamin transport
Flavoprotein[1]	0.8	3.9-4.1	Binds riboflavin

Source: Stevens (1991), except where noted
[1]Taken from Gilbert (1971)
[2]Taken from MacDonnell *et al.,* (1955)
[3]Taken from Burley and Vadehra (1989)

The important functional constituents of albumen, are the globulins, ovomucin, and ovalbumen proteins (MacDonnell *et al.,* 1955). MacDonnell and co-workers (1955) explain that globulins act as foamers and are very important for good textured cakes because they contribute to volume, bubble size, and smooth texture. Ovomucin acts as a stabilizer and because of fast insolubilization at bubble surfaces, ovomucin allows for stabilization at short whipping times (MacDonnell *et al.,* 1955). The last protein ovalbumin is important for heat stabilization because it is a heat denaturable protein (MacDonnell *et al.,* 1955). To sum up the importance of these proteins, globulins will make a good foam, ovomucin will stabilize the foam, and ovalbumin will give structure to the baked foam.

CHAPTER II

BIBLIOGRAPHIC REVIEW

Interior Egg Quality

Interior egg quality can be measured by Haugh unit, albumen height, yolk index, albumen pH, and yolk pH (Haugh, 1937; Silversides and Villeneuve, 1994; Sauter *et al.,* 1951; Sharp and Powell, 1930). Other methods of determining interior egg quality used by the egg industry are candling and determining yolk shadowing (USDA, 1990).

Haugh Unit. The poultry industry has used the Haugh unit as the accepted method for determining albumen quality for decades (Eisen *et al.*, 1962; Kidwell *et al*., 1964). The Haugh unit was created by Raymond Haugh in 1937, and relates the weight of the egg to the albumen height (Haugh, 1937). Eggs starting with a high Haugh unit (> 80) have more of a decline in interior egg quality than eggs starting with a low Haugh unit (< 74) (Skala, 1968). Because of this, the Haugh unit has been questioned as an appropriate technique to measure interior egg quality. Some have suggested using alternate methods such as albumen height (Eisen *et al.,* 1962; Kidwell *et al.*, 1964; Silversides *et al.,* 1993; Silversides and Villeneuve, 1994).

One primary question has been how the Haugh unit formula was derived and if the regression had been tested (Eisen *et al.,* 1962). The Haugh unit does not adjust albumen height for egg weight correctly, and is biased for smaller eggs (Eisen *et al.,* 1962). For example, eggs that weigh between 52 and 56.5 grams have overestimated albumen heights, while the albumen height is underestimated for eggs weighing more than 56.5 grams (Eisen *et al.,* 1962).

Kidwell and co-workers (1964) investigated the regression of albumen height on egg weight by looking at fresh and stored eggs from two different breeds of laying hens. They found that using Haugh units may be useful for determining the quality of fresh eggs but not for stored eggs. They also found that an important feature of Haugh unit was its ability to transform albumen height in log form (Kidwell *et al.,* 1964). Silversides and co-workers (1993) compared the Haugh unit scores of fresh eggs from two different flocks and discovered that it was inconsistent between flocks and that albumen height alone was indicative of egg condition or the length of storage. They concluded that the Haugh unit was inadequate and that albumen height was a good replacement. Egg and albumen weights are not important factors when determining albumen height. Measuring the height of the albumen is sufficient to determine albumen quality, and that it is not necessary to weigh the eggs or apply the formula for albumen height (Silversides and Villeneuve, 1994).

Albumen Height. Scott and Silversides (2000) reported albumen height as a measure of albumen quality, rather than the Haugh unit, and combined albumen height and pH to measure the decline in albumen quality. The study was performed to determine the quality of eggs from different strains of hens during storage. They concluded that albumen weight and height decrease during storage as albumen pH increases (Scott and Silversides, 2000). Although albumen height is becoming an accepted method to determine albumen quality, consideration should also be given to the genetic origin of the bird (Scott and Silversides, 2000). For example, by using albumen height alone brown shell eggs are given an unfair advantage, albumen pH should also be taken (Scott and Silversides, 2000). This conclusion came from looking at two breed lines, ISA-Brown and ISA-White. Scott and

Silversides (2000) determined that hens that lay brown shell eggs have more albumen, thinner shells, and a smaller yolk than white shell eggs.

pH of the Albumen and Yolk. Healy and Peter (1925) discovered that the avian egg contained bicarbonate and carbon dioxide thus proving the existence of a bicarbonate buffer system in the egg. From pH 6.6 to 7.8, the amount of the buffer for every gram of protein is 4.8×10^{-5} (Brooks and Pace, 1938). The pH of albumen increases during storage due to the loss of water and carbon dioxide. The pH of the albumen is dependent upon its ability to maintain a balance of dissolved carbon dioxide, bicarbonate ion, carbonate ion, and protein (Powrie and Nakai, 1986). Water and CO_2 loss from the egg is dependent on many factors including the porosity of the shell (Mueller, 1958). Albumen pH is initially 7.6 and rises to approximately 9.5 during storage (Burley and Vadehra, 1989). Albumen pH gives an unbiased measurement when comparing eggs from different genetic bird strains because there are no differences between the pH of brown and white shell eggs appear (Scott and Silversides, 2000). Eggs with brown shells have more shell and albumen with less yolk than those with a white shell (Scott and Silversides, 2000).

Yolk pH changes during storage with an initial pH of 6.0 when fresh and rising to 7.2 after being stored at room temperature for 56 days (Burley and Vadehra, 1989; Healy and Peter, 1925).

Yolk Index. Haugh unit and albumen height indicates albumen quality, but yolk quality is determined by color, shape, and membrane strength (Stadelman, 1986). A fresh egg will have a yolk with a curved shape when placed in a dish, but as the egg ages the yolk will flatten (Sharp and Powell, 1930).

A measurement of the spherical nature of yolk, or yolk index, was developed by Sharp and Powell (1930). It is determined by dividing the height of the yolk by its width. The original method of determining yolk index was to break an egg into a petri dish and remove most of the albumen by a pipette. The remaining adhering albumen was then removed by gently wiping the yolk with a soft, wet cloth. The chalazae was cut off with shears and the yolk was set on a flat glass plate and left to "rest" for 5 minutes. Height and width of the yolk were measured after the 5-minute rest.

This method was refined by Funk (1948) because it was time consuming and low quality eggs would rupture before measurements could be made. The newly developed method took the height and width of the yolk when left in a natural position after breaking, thus leaving the albumen intact. Time wasn't considered before taking the measurements. The yolk index by this new method was higher than the original method, but was comparable when the new method value was reduced by 10%. Sauter and co-workers (1951) reviewed the method of Sharp and Powell (1930) and Funk (1948) and proposed that the new method saved time, but that the 10% reduction in the yolk index value was not sufficient to cover all eggs either stored or fresh. They recommended that for high interior quality eggs, the value should be reduced by 20%, for lower interior egg quality the reduction should be 15%, and stale eggs should be reduced by 10%.

A strong relationship exists between the yolk index and the strength and weight of the VM (Fromm, 1964).

Egg candling and yolk shadowing. The air cell is formed in an egg due to thermal contraction that occurs as the egg decreases in temperature from 105ºF, at the time it is laid, to ambient temperature (USDA, 1990). As the egg ages or is exposed to different

temperatures and humidities, the air cell will increase in size as water and carbon dioxide are lost. The size of the air cell is one parameter used to determine grade (AA, A, and B) (USDA, 1990). The AA quality is given to eggs that have up to 1/8 inch air cell depth, A quality eggs have up to 3/16 inch depth, and B quality is given to eggs that extend beyond grade A depth to no limit (USDA, 1990). Egg candling is the only method in which the egg industry can look at the air cell depth and determine egg quality (Sauter *et al,* 1953). Another indicator of interior egg quality, which can also be determined by candling, is yolk shadowing. This is considered by some to be the best method to determine interior egg quality (USDA, 1990). The yolk shadow allows several factors to be taken into account such as the condition of the albumen, and condition of the yolk (USDA, 1990). The movement of the yolk, when observed through candling, can demonstrate the firmness of the albumen (Baker and Forsythe, 1951). When the outline of the yolk shadow is not distinct, the candler can tell that the albumen is thick. As the yolk shadow becomes more visible and clear, the albumen begins to lose quality and deteriorates (USDA, 1990). Off colors and blemishes can also be seen by different colored shadows (USDA, 1990).

Relationships between observed candling scores and interior factors were studied by Stewart and co-workers (1932d,e,f,g,h; 1933). As the air cell increases, the quality of the albumen decreases, the yolk index decreases, and the yolk color will darken. As the yolk color increases the percentage of thin white increases (Stewart *et al.,* 1932d). Interior quality factors that are related to candling are the percentage of thin white and yolk color (Stewart *et al.,* 1932e). Yolk color is a good indicator of the condition of the albumen, but in some cases when eggs were downgraded due to color, the opened eggs reflected a high interior quality (Stewart *et al.,* 1932f). Variability between candlers is a concern. High

quality and low quality eggs grade with little variability, but eggs with a medium quality have more variability (Stewart *et al.,* 1932a,b,c).

Sauter and co-workers (1953) evaluated the relationships between candling grades and interior egg qualities. Significant correlations were found to exist between the candled quality and albumen index, yolk index, yolk color, albumen score, and pH. Baker and Vadehra (1972) found high correlations between the candling grade to albumen height and Haugh unit. The higher quality the egg is at candling, the higher the quality of the egg product (Sauter *et al.,* 1953).

Storage, Molt, Age, and Season on Interior Quality of Eggs

Storage. When eggs are stored at 37ºC for 45 days, the albumen proteins remain stable, but the albumen becomes progressively thinner (Feeney et al., 1952). Other changes that occur under the same storage conditions include a 20-25% decrease in lysozyme activity, and a decrease in concentration of antihemagglutinin as the albumen thins (Feeney *et al*., 1952).

Evans and co-workers (1949) studied the effects of egg proteins under cold storage (0ºC) in fresh eggs and those stored for 9, 18, 22, and 26 months. Each storage time showed a progressive loss in egg weight due to water loss as compared to fresh eggs. Most of the weight loss came from the albumen, with a lesser amount from the yolk. Protein content in the albumen increased over the storage period along with an increase in sulfur content of the albumen proteins. The albumen pH of stored eggs reached a maximum of 8.9 after 23 months of storage. Evans and co-workers (1949) also saw another change in eggs that are stored. A decrease in grams of proteins per egg occurred, but this was later proven

inaccurate by Evans and Davidson (1953) who reported that there was no loss of protein in stored eggs.

Evans and co-workers (1958) evaluated fresh eggs and eggs stored at 0ºC for 4, 8, and 12 months to monitor changes in egg proteins. After 12 months, proteins did not migrate from the albumen into the yolk.

Sharp and Powell (1930), who created the measurement of yolk index, determined the yolk index on eggs stored at 2, 7, 16, 25, and 37ºC for various days. Yolk index declined at all temperatures, but eggs stored at 2 and 7ºC had the least difference in yolk index value from fresh to day 100. Woodward and co-workers (1987) observed that when eggs were stored at 10 or 23ºC for 4 weeks, yolk index and Haugh unit declined, and rupture strength of the vitelline membrane decreased.

Molting. Molting increases the quality of the albumen (Tona *et al.,* 2002) due to an energized production in birds after a molt. When a flock is molted at 65-70 weeks, they return to the same production level as a 40-50 week flock (Bell, 2003). This is one of the major reasons why a producer chooses to molt a flock. Egg producers also consider the price and supply of eggs when they decide to molt (McDaniel and Aske, 2000). When a producer molts, it allows them to decrease production when the prices of eggs are low or the supply of eggs is high (McDaniel and Aske, 2000). Farms that do not molt will use 8.4 new flocks within a ten-year period while farms that molt will use 5.7 new flocks in the same ten year period (Bell, 2003). When using induced molting programs, to regain satisfactory egg production and shell quality, termination of production and retraction of reproductive organs are crucial (Berry and Brake, 1987). Appropriate weight loss of the flock is another critical measure to insure total regression of the reproductive tract. A common weight loss target is

27 to 31% (Baker *et al.,* 1983). All induced molting programs should be adjusted for each flock (Bar *et al.,* 2001). The benefits of induced molting are an increase in egg production, egg mass, and shell quality, and a decrease in shell breaking, mortality and culling (Bar *et al.,* 2001). Haugh unit values for eggs after molting are significantly higher than the values before molting (Tona *et al.,* 2002). Tona and co-workers (2002) found that albumen pH is not affected by molting. However, the albumen pH was significantly higher in eggs before molting as compared to eggs after molting.

Age of the bird. The weight of albumen and yolk increase with hen age (Rossi and Pompei, 1995). Haugh units will decrease as the bird ages throughout the lay period (Cunningham *et al.,* 1960). Age influences egg weight, the volume of albumen, and Haugh unit (Cunningham *et al.,* 1960). The amount of albumen decreases with age along with a decline in Haugh unit (Cunningham *et al.,* 1960). As the bird ages, the albumen yield decreases and becomes more watery (Fetcher *et al.,* 1983). Albumen height decreases during storage at 16ºC, 78% RH and as the hen ages (Lapao *et al.,* 1999). Aging of the bird also increases the yolk-albumen ratio, with an increase in yolk weight (Hussein *et al.,* 1993). Although yolk weight and percentage of yolk increase, there is more deformation of the yolk (Fletcher *et al.,* 1981). Yolk viscosity and emulsion stability are also affected by aging (Varadarajulu and Cunningham, 1972). Age not only affects the interior egg quality, but also the exterior quality. Marion and co-workers (1964) discovered that hens in their second year of production produced eggs with 0.7% less shell. Age also affects the vitelline membrane rupture strength. Younger hens have higher rupture strengths than older hens (Ngoka *et al.,* 1983).

Season. The season the egg is laid affects egg weight, but does not affect interior egg quality as measured by Haugh unit (Cunningham *et al.,* 1960). Egg weight increases from August to December, plateaus from February to May, declines in June and then increases again from July to August (Cunningham *et al.,* 1960). The months to maximize egg weight are March and April while the months of minimum egg quality are July and August due to high temperatures (Cunningham *et al.,* 1960).

Vitelline Membrane

With 30% of shell eggs going to further processing, it is important to understand the VM properties, proteins, and rupture strength to prevent unwanted yolk contamination in the albumen (Egg Industry, 1997; St. John and Flor, 1931). Trziszka and Smolinska (1982) determined the chemical composition of the VM as shown in Table II-1. Two methods of preparation altered the outcome of the chemical composition. The first method used a saline solvent to prevent proteins from the chalazae from contaminating the VM proteins for nitrogen determination. The protein content was higher for an alcohol-ether extraction than the saline method. The second method used an alcohol-ether mixture to extract lipid fractions from the yolk that remain on the inner membrane. Thus the lipid content was higher for the saline method than the alcohol-ether mixture.

TABLE II-1. Chemical composition of the vitelline membrane from a fresh egg[1,2].

Isolated by Saline			Isolated by Alcohol-Ether		
Protein	Lipids	Carbohydrates	Protein	Lipids	Carbohydrates
85.31	13.26	8.35	91.50	5.20	9.12

[1]Value reflects mean from ten eggs
[2]Source: Trziszka and Smolinska (1982)

Vitelline membrane structure. The VM is formed from secretions emitted from the follicular epithelium of the follicle and oviduct (McNally, 1943). The VM from a freshly ovulated egg consists of an inner layer that is derived from the collagenous membrane which lies in the epithelium of the follicle. The outer layer of the VM is laid down later by the oviduct secretions of mucin (McNally, 1943). The mucin acts with the collagenous membrane to create the whole membrane. Using a scanning electron micrograph (SEM), Bellairs and coworkers (1963) observed that the membrane is made up of an inner layer, and outer layer, with a continuous membrane imbedded in between the two. The inner layer is fibrous and composed of a meshwork of solid cylindrical fibers (Bellairs *et al.,* 1963). Formed in the ovary, its thickness ranges from 1.0-3.5 μ with an average of 2.7 μ (Bellairs *et al.,* 1963). The outer layer is also fibrous and composed of many sub layers that lay on top of one another; the layers of the outer VM are made of fibrils that swell at contact points (Bellairs *et al.,* 1963). The outer layer thickness ranges from 3-8 μ (Bellairs *et al.,* 1963). The continuous membrane is a granular section of mainly fibrous membrane and its thickness ranges from 500-1000 Å (Bellairs *et al.,* 1963).

Separation Methods of the Vitelline Membrane. Separation of the VM is necessary for characterizing individual proteins, determining their function(s), and observing interactions of not only the proteins involved but for the layers themselves. When the membrane is separated wet, the continuous membrane remains attached to the inner layer, but when separated dry, the continuous membrane remains attached to the outer layer (Bellairs *et al.,* 1963).

There are different methods for separating the VM (Bellairs *et al.,* 1963; Back *et al.,* 1982; Kido and Doi, 1988). Bellairs and co-workers (1963) separated the VM by cutting off

pieces of the membrane from the yolk and washing them. The pieces were then placed a petri dish filled with saline, and the saline siphoned off. This causes, one side of the membrane to stick to the petri dish and the other side can be pulled off. If separation is not easy, a scalpel can be used to aid in separation (Bellairs *et al.,* 1963). Another method separates the membranes by first rolling the yolk on a wet paper towel to remove adhering albumen, puncturing the yolk and extracting its contents (Back *et al.,* 1982). The membrane is then placed into a petri dish filled with double distilled water, and Toluidine blue dye is added to stain the outer layer a faint blue while the inner layer remains colorless (Back *et al.,* 1982). Yolks can be incubated in 0.01 N HCl for an hour at 37˚C, then punctured and the yolk contents carefully squeezed out (Kido and Doi, 1988). At this point, the membrane will be partially separated and tweezers can be used to finish the separation. A third method for separation uses a compilation of techniques from Back and co-workers (1982) and Kido and Doi (1988). With this method the yolk is separated from the albumen and then rolled on a wet paper towel to remove the adhering albumen layer as described by Back and co-workers (1982). The yolk was then ruptured and the contents removed by rinsing with a stream of double distilled water. In a method similar to Kido and Doi (1988), a dissecting microscope can be used to magnify the membrane so that the partially separated layers can be seen and pulled apart with tweezers.

Vitelline Membrane Proteins. Nine proteins have been identified in the VM. Those in the outer layer are ovomucin, lysozyme, lectin, vitelline membrane outer (VMO) I and VMO-II. The inner layer proteins are glycoprotein (GP) I, GP-II, GP-III, and GP-IV (Back *et al.,* 1982; Kido *et al.,* 1975; Kido *et al.,* 1976; Cook *et al.,* 1985; Kido and Doi, 1988).

Outer Layer Proteins. The proteins in the outer layer closely resemble the amino acid makeup of lysozyme, conalbumen, and avidin (Bellairs *et al.,* 1963). Ovomucin is an outer layer protein and has a molecular weight that is too high to characterize (Back *et al.*, 1982). Lysozyme is also an outer layer protein with molecular weight of 14,000 kDa (Kido and Doi, 1988). Ovomucin is an insoluble fraction in the outer layer (Back *et al.,* 1982). The disulfide linkages in ovomucin are key to maintaining the structural integrity of the VM (Kido and Doi, 1988). While ovomucin forms the skeleton of the outer layer, the structural integrity is dependent on lysozyme (Back *et al.,* 1982). Back and co-workers (1982) theorize that electrostatic interactions between ovomucin and lysozyme work together to provide normal structure and that the outer layer is an important antimicrobial layer. Lysozyme was characterized as the major protein in the VM and the lysozyme found in the VM is the same as found in the albumen (type C) (De Boeck and Stockx, 1986a). The function of lysozyme is still debated, but may serve more of a structural role than a catalyst (De Boeck and Stockx, 1986a). Lysozyme can be extracted by salt solutions or solubolized by charged detergents. The positively charged lysozyme residue has an ionic interaction with the other proteins in the outer layer such as ovomucin (De Boeck and Stockx, 1986b). The lysozyme-VM complex is a function of pH and when exposed to a high or low pH, the membrane dissociates (De Boeck and Stockx, 1986b). As the pH in the egg nears the pK values of 2.5 and 11.5, the interaction of the complex will dissociate (De Boeck and Stockx, 1986b). A lysozyme dimmer forms after a period of six weeks at 20ºC (Back, 1984) because no dimer formed from a pure lysozyme solution, it was concluded that lysozyme dimer formation is exclusive to activities that occur inside the egg (Back 1984).

Lectin was found and characterized by Cook and co-workers (1985). With a molecular weight of 62,000 kDa, the function of this protein is still unknown, but it may serve as a transporter or is bactericidal (Cook *et al.,* 1985). More research is needed to characterize the actual function. Lectin is a protein that can be separated into two classes, soluble or integrated into the membrane. Lectin in the VM is a soluble protein, therefore it can be isolated without the help of a detergent. Two proteins, later named VMO-I and VMO-II, were found to have molecular weights of 21,000 and 12, 000 kDa, respectively (Back *et al.,* 1982). However, later work reported that VMO-I had a molecular weight of 17,000 kDa (Kido and Doi, 1988). VMO-I decreases during storage and its function is not known, but there is a possibility that it has an interaction with a high molecular weight material found in the egg (Back, 1984).

Inner Layer Proteins. The inner layer proteins most closely resemble connective tissue (Bellairs *et al.,* 1963). Collagen and the inner layer proteins might actually be the cement that binds the fibers together (Bellairs *et al.,* 1963). Inner layer proteins are identified as GP-I, GP-II, GP-III, and GP-IV (Kido *et al.,* 1975; Kido and Doi, 1988). The molecular weight of GP-I is 32,000 kDa, GP-II is 260, 000 kDa, and GP-III has a molecular weight that was too high to characterize (Kido *et al.*, 1975). Other studies report that GP-III had a molecular weight of 300,000 kDa (Kido *et al.*, 1975; Back *et al.,* 1982). Further reports characterized molecular weights of GP-II as 183,000 kDa and GP-III as greater than 1,000,000 kDa in addition to reporting an insoluble fraction of GP-IV with a molecular weight too high to characterize (Kido and Doi, 1988). The structure of the VM is held together by hydrophobic interactions that can be disrupted by SDS. When SDS is removed, it reaggregates (Kido *et al.,* 1975). Disulfide bonds are also important in the structure of the

VM (Kido *et al.,* 1975). GP-I is highly insoluble in water and due to its hydrophobicity that is attributed to exposed hydrophobic segments at the surface and not the fact that 39% of the protein is composed of hydrophobic amino acids (Kido *et al.,* 1976). Kido and co-workers (1976) also report that along with a high percentage of hydrophobic amino acids, there is a high cystine content; GP-I may function as a lipoprotein in the membrane. GP-II has a high amount of proline, is hydrophobic, and changes most during storage (Kido *et al.,* 1977). GP-II deteriorates during storage; which has been attributed to the combination of hydrogen sulfide and an increase in albumen pH (Kido *et al.,* 1977). The hydrophobicity of this protein has been explained by the high percentage (40%) of hydrophobic amino acids; it functions as a cement in the VM (Kido *et al.,* 1977).

Properties of the vitelline membrane. Several factors can affect the properties and strength of the VM. Sharp and Powell (1930) reported that the weakening of the VM is caused by an increase in osmotic pressure due to water entering the yolk. Feeney and co-workers (1956) report that the weakening of the VM is caused by a chemical or biochemical reaction because the energy of activation is within this range at 18,000 calories. The breakdown of the VM is theorize to be a result of a breakdown of mucin that surrounds the VM (Feeney *et al.,* 1956). When this substance deteriorates the VM is also affected by the same factors and therefore starts to deteriorate (Feeney *et al.,* 1956). Storing the egg will cause the membrane to weaken and lose weight (Fromm, 1964; Fromm and Matrone, 1962; Moran, 1936; Fromm, 1967). After 3 days of storage at 35ºC, the network of fibers of the VM on a freshly laid egg start to disappear, due to the same factors that affect the chalaziferous layer (Fromm, 1967). Throughout storage, the yolk will increase in size, but the VM does not increase in size (Heath, 1976). Heath (1976) showed that under

refrigerated conditions, the VM weight remained stable while the VM from eggs stored under room temperature conditions decreased. On days 3, 6, and 9, the VM weight of eggs stored under refrigeration (7ºC) were significantly higher, as compared to the VM that were stored in room temperature (22ºC).

Elasticity of the VM was first thought to be associated with the age of the egg (Moran, 1936). When eggs were stored at 39˚C, after 15 days the VM was easy to rupture. Higher storage temperatures caused the membrane to become more elastic (Fromm and Matrone, 1962), but only one portion of the VM was tested. Ngoka and co-workers (1983) found that at lower temperatures the VM becomes more elastic and its rupture strength is higher for young hens as compared to eggs from older hens.

Heath (1975) studied the movement of water into the VM and noted that water moved from the yolk to the albumen within the first 3 days when stored at 6˚C. Moisture lost from the yolk was 2.8% but no significant changes occurred after 7 days. Another part of the study prevented gas exchange to reduce the pH to determine if that would prevent weakening of the VM. At both 6˚C and 28˚C, there was no change in the yolk index and no weakening of the VM. The study went further to look at yolk moisture when separated from the albumen. A different osmotic relationship occurred with more moisture movement across the VM.

Rupture Strength of the VM. A strong VM is important to prevent the detrimental effect that yolk contamination has on albumen proteins (St. John and Flor, 1931).

Fromm and Matrone (1962) evaluated VM strength using a 2 mm capillary tube and 23 mm Hg vacuum on days 0 through 5, 20 mm Hg on days 6 and 7, and 18 mm Hg on days 8 through 10. The strength required to rupture the membrane was determined by the

relationship between the time to rupture and the level of vacuum. Yolk rupture strength decreased with age, but elasticity of the membrane did not change (Fromm and Matrone, 1962). Temperature of the yolk was also found to have an important effect on the strength of the membrane as determined by the capillary vacuum method. As temperature increases, the time for rupture of the VM increases (Fromm and Matrone, 1962).

Fromm (1964) evaluated VM strength measuring different points along the membrane using the same method as Fromm and Matrone (1962). Fromm (1964) determined that there was variability in strength at different points along the membrane and that the strength of the membrane seemed to be related to the amount of the chalaziferous layer that surrounds it.

Kirunda and McKee (2000) studied the rupture strength of the VM by using a texture analyzer that allowed a larger contact surface with the yolk whereas previous methods had direct contact with only one position. Correlations between interior egg qualities and rupture strength indicated that as membrane strength decreased, yolk index and Haugh units decreased while yolk and albumen pH increased (Kirunda and McKee, 2000). Images from a scanning electron microscope indicated that the fibrous material from the outer layer of the VM starts to disappear after storage (Kirunda and McKee, 2000). Kirunda and McKee (2000) concluded that VM strength decreases because of the same factors that affect Haugh unit, yolk index, and the pH of albumen and yolk.

Jones and co-workers (2002) sought to look at the effects of cooling shell eggs with gaseous nitrogen, liquid nitrogen, and gaseous carbon dioxide. They evaluated its effects on rupture strength by using a texture analyzer. When eggs are cooled with a cryogenic cooling

medium, rupture strength increases and they have better Haugh units, but there is more of an incidence of cracked eggs (Jones *et al.,* 2002).

Woodward and co-workers (1987) studied the effects of feeding wheat instead of corn on the properties of yolk rupture strength. They found that yolk rupture strength was higher for wheat fed hens than for the hens fed a corn diet. They also observed that yolk index and rupture strength were closely related to each other.

CHAPTER III

THE EFFECTS OF STORAGE TIME ON VITELLINE MEMBRANE PROTEIN BANDING PATTERNS AND INTERIOR EGG QUALITY OF EGGS FROM PRE-MOLTED HENS

Synopsis

Vitelline membrane strength plays an important role in preventing contamination of albumen by yolk during separation. This is important to food safety. This study was conducted to determine if a relationship exists between vitelline membrane protein banding patterns on SDS-PAGE gels, interior egg quality, and vitelline membrane rupture strength. In Experiment 1, eggs were collected from a 72 wk commercial flock before molting. In Experiment 2, eggs were collected from a 26 wk commercial flock before molt. Twenty-one eggs were gathered and stored 0, 7, 14, 21, 28, 35, and 42 days (4°C, 69% RH) to evaluate SDS-PAGE protein banding patterns in each experiment. Three eggs were evaluated each storage day and the yolk from individual eggs were isolated and rolled on a wet paper towel to remove adhering albumen. The yolk was emptied and washed. The whole membrane was placed into double distilled water and divided into two sections. The first section was the whole membrane sample and the other section was separated by forceps into inner and outer membranes. The three components were then dissolved independently in 1 to 2 mL of 1% sodium dodecyl sulfate (SDS)/70 mM Tris/HCl, pH 6.8. Protein concentration was determined using the modified Lowry method and individual proteins separated on 4-20% gel gradient by SDS-PAGE. Protein banding patterns were analyzed using a densitometer. The proteins VMO-I and GP-II decreased during storage for both experiments, and banding patterns appeared between the 110 to 116 kDa range.

An additional 140 eggs were gathered at the same time from the same flocks and stored (4°C, 69% RH). In each experiment, twenty eggs were evaluated for quality on days 0, 7, 14, 21, 28, 35, and 42. Yolk index, albumen height, albumen pH, and yolk pH were determined. Vitelline membrane strength was measured using a compression anvil. Two different yolk preparation procedures were used to evaluate vitelline membrane rupture strength: 10 egg yolks with inner thin albumen layer, and 10 egg yolks rolled on a wet paper towel to remove inner thin albumen layer. Interior egg quality declined during refrigerated storage for both experiments. In Experiment 1, vitelline membrane strength declined significantly for the VM with the adhering albumen layer. In Experiment 2, the vitelline membrane strength declined significantly for the VM with and without the adhering albumen layer.

Introduction

The VM is the structure inside the egg that surrounds the yolk and keeps it compartmentalized (Trziszka and Smolinska, 1982). It is important because it prevents the yolk from contaminating the albumen in egg breaking plants and keeps microorganisms, such as *Salmonella* Enteritidis, from entering the nutrient rich yolk. In 2002, 203 million cases of eggs were produced in the United States, and 60.3 egg cases went on to further processing (NASS, 2003).

Age of the hen at the time that the egg is laid is an important factor and affects the quality of the eggs. The weight of albumen and yolk increase with hen age (Rossi and Pompei, 1995). Age of the hen influences egg weight, the volume of albumen, and Haugh unit (Cunningham *et al.,* 1960). As the bird ages, the albumen yield decreases, and becomes more watery (Fetcher *et al.,* 1983). Although yolk weight and percentage increase with the

age of the hen, there is more deformation of the yolk (Fletcher *et al.,* 1981). It has also been noted that eggs from younger hens have higher rupture strengths than eggs from older hens (Ngoka *et al.,* 1983).

Upon storage, albumen shows thinning from thick albumen to thin albumen (Feeney *et al*., 1952), and results in a loss of egg weight, mostly from the albumen, as well as an increase in yolk (Evans *et al.,* 1949). Yolk index declines over storage (Sharp and Powell, 1930).

The VM is composed of three layers, two fibrous layers, the outer and the inner membranes and imbedded between is the granular layer called the continuous membrane (Bellairs *et al*., 1963). The main proteins of the VM are ovomucin (Back *et al.,* 1982), lysozyme (Back *et al.,* 1982), lectin (Cook *et al*., 1985), VMO-I and II (Back *et al.,* 1982), GP-I, II, III and IV (Kido *et al*., 1975 & 1976; Kido and Doi, 1988).

It is hypothesized that over refrigerated storage proteins will breakdown and this will result in decreasing VM strength. Therefore, the objectives of this study were to evaluate VM strength and protein changes during 6 wk refrigerated storage. This was accomplished by separating proteins from the VM by SDS-PAGE, and measuring albumen height, albumen pH, yolk pH, yolk index, and yolk rupture strength. SDS-PAGE was performed on the whole VM layer, the inner VM, and the outer VM layer. Interior egg quality measurements were performed on yolks with an adhering albumen layer and on yolks with the adhering albumen layer removed.

Materials and Methods

For Experiment 1, eggs were gathered from the TAMU Poultry Research Farm in the fall, from W-36 Hy-Line hens at 72 wk of age. For Experiment 2, eggs were

gathered from a Texas commercial egg producer in the summer, from W-36 Hy-Line hens, at 26 wk of age, with a 90% production rate. A total of 161 eggs were gathered and stored in refrigerated conditions at 4˚C, 69% RH. One hundred forty eggs were used for interior egg quality, and 21 eggs were used for SDS-PAGE on days 0, 7, 14, 21, 28, 35, and 42.

Interior Egg Quality. Interior egg quality was evaluated by measuring albumen height, yolk index, albumen pH and yolk pH. A total of 20 eggs were used at each storage time. The eggs were weighed and returned to the refrigerator until needed for analysis. Eggs were opened onto a plate and albumen height determined using a tripod micrometer. The albumen was measured in the middle of the thick white surrounding the yolk as described by Silversides and Villeneuve (1994). Yolk index was taken using the tripod micrometer to measure the height of the yolk, and calipers were used to measure the diameter of the yolk at two different points (Funk, 1948). The yolk was separated from the albumen by pouring the egg in a cup and pouring off the albumen into another cup. A pH meter[1] equipped with a combination electrode was used to determined albumen pH. After rupturing the yolk, the pH of the yolk was taken.

Rupture Strength of the VM. After separating the yolk from the albumen, ten yolks were evaluated for rupture strength that had the adhering albumen layer removed by rolling the yolk on a wet paper towel. The remaining ten yolks had rupture strength determined with the adhering albumen layer left intact. To weigh the yolk, a glass cylinder dish and filter paper lining the bottom were put on a tared scale, the yolk placed into the dish and weighed to the nearest 0.01 g. Rupture strength was taken using a compression anvil and

Instron[2] with compression cell at 50 mm/min cross head speed, 500 g load range, and a 5 kg load cell. The rupture strength in kg was divided by the weight in grams of the yolk to give a final force calculation.

Separation of the VM, Protein Concentration and SDS-PAGE. Samples of the whole membrane, inner membrane layer, and outer membrane layer from three eggs were used to analyze the VM proteins. Yolk was separated from the albumen and the adhering albumen layer removed as described by Back and co-workers (1982). The yolk was ruptured by hand, yolk contents drained, and further washed with a stream of double distilled water. The VM was put into a petri dish filled with double distilled water and the membrane divided into two approximately equal portions under a dissecting microscope. One portion of the whole membrane was placed into 2 mL of 70 mM Tris-HCl, pH 6.8, 1% SDS. This became the sample used to determine whole membrane proteins. The other whole membrane portion was further separated with forceps into the inner membrane layer and outer membrane layer under the dissecting microscope. The inner and outer layer portions were placed separately in 2 mL of 70 mM Tris-HCl, pH 6.8, 1% SDS; these became the samples to determine inner layer and outer layer proteins. Samples were stirred with a magnetic stir rod for 15-17 hours, at 22ºC to dissolve the membrane.

Protein concentration was determined using the Modified Lowry Protein Assay. The dissolved membrane solution was decanted into centrifuge tubes and centrifuged at 20K, for 5 min at 4˚C using the Sorvall Untracentrifuge[3]. The supernatant was poured into glass tubes. Two hundred µL of each solution were pipetted into duplicate glass tubes. A blank

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[1]Corning pH meter, model 240, Corning Glassworks, Medfield, MA 02052.
[2]model 1011, Instron Corp., Canton, MA 02021.
[3]model OTD65B, DuPont Co., Wilmington, DE 19898.

tube was prepared for calibration using a solution of 70 mM Tris-HCl, pH 6.8, 1% SDS. One mL of Modified Lowry Reagent was put into the tubes, vortexed, then incubated for 10 minutes. After incubation, 0.1 mL of the Phenol reagent was added, vortexed, covered, and incubated for 30 min at room temperature. After incubation, the protein concentration was read at 750 nm using a Beckman Spectrophotometer[4], and protein concentration calculated. After the protein concentration was determined, samples were denatured and frozen until electrophoresised. Laemmli sample buffer was made with 62.5 mM Tris-HCl, (pH 6.8), 2% SDS, 25% glycerol to mix with the sample solutions. Four hundred μL of the sample were mixed with 400 μL of sample buffer, then 40 μL of bromophenol blue was added. The mixture was put into tubes and heated for 5-7 minutes at 100ºC in a water bath. The tubes were stored at 0ºC until ready for electrophoresis.

VM protein banding patterns were electrophoresed using the Protean II Ready Gel System[5] on 4 to 20% gradient gels at 106 to 107 V. For Experiment 1, approximately 2.8-8.4 μg of protein were loaded in each lane. For Experiment 2, approximately 2.97-3.03 μg of protein were loaded in each lane. When the dye front in the samples reached the bottom of the gel, the gels were held in double distilled water and stained with GelCode Blue Stain Reagent[6] for two hours. The gels were then washed in several changes of double distilled water and held (in double distilled water) overnight to enhance the stain. After staining, the gels were dried using Gel-Dry Solution[7] and DryEase mini-cellophane[8]. Gels were scanned to determine optical

[4]model DU-64, Beckman Instruments, Inc., Fullerton, CA 92634.
[5]Bio-Rad Laboratories, Inc., Hercules, CA 94547.
[6]Pierce Biotechnology, Inc., Rockford, IL 61105.
[7]Invitrogen Corp., Carlsbad, CA 92008.
[8]Novex, San Diego, CA 92121.

density with a Bio-Rad Multi-Analyst Densitometer[9]. Protein bands were evaluated subjectively by the volume (optical density x mm x mm) of the band divided by the protein μg loaded.

Statistical Analysis. Data were analyzed using the General Linear Model (GLM) to generate an ANOVA and significant means separated using the Duncan's Multiple Range Test at $P < 0.05$.

Results: Experiment 1

SDS-PAGE. The evaluations of the SDS-PAGE gels were subjective and optical density (OD) values from the densitometer readings were divided by the protein μg amount loaded onto each well to give the OD per μg protein (OD/μg) (Figure III-1 – Figure III-8). The OD/μg amount for the protein GP-II decreased throughout the storage period (Figure III-1). The protein GP-II isolated from the whole and inner membrane layer shows a decrease in OD/μg from days 0 through 28. The proteins GP-I, VMO-1, and lysozyme did not decrease in OD/μg (Figure III-1). Banding patterns appeared between 100-116 kDa range (Figure III-1). A breakdown of the proteins and banding patterns appear at day 21.

[9] model GS-690, BioRad Laboratories, Inc., Hercules, CA 94547.

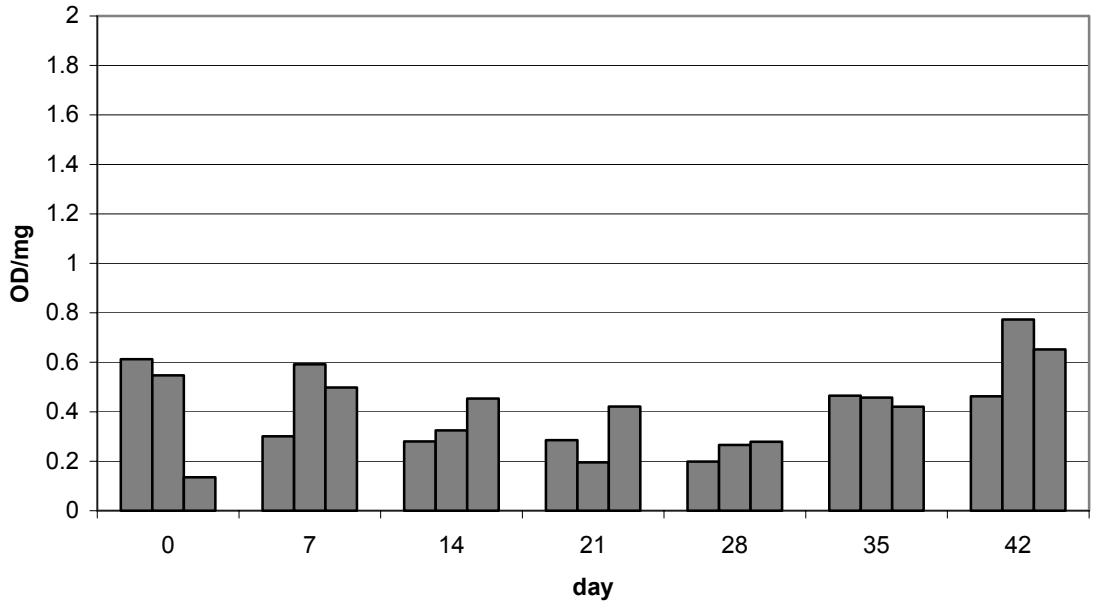

Figure III-1: Measurement of OD/μg values from SDS-PAGE gels for GP-II protein in the whole layer for 72 wk pre-molted hens; Experiment 1.

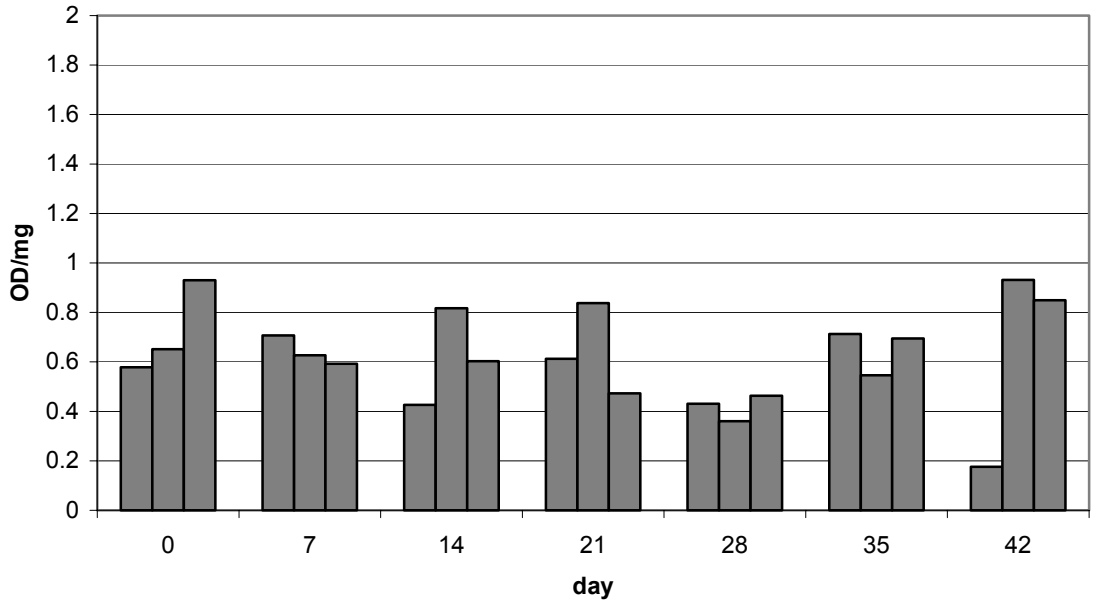

Figure III-2: Measurement of OD/μg values from SDS-PAGE gels for GP-II protein in the inner layer for 72 wk pre-molted hens; Experiment 1.

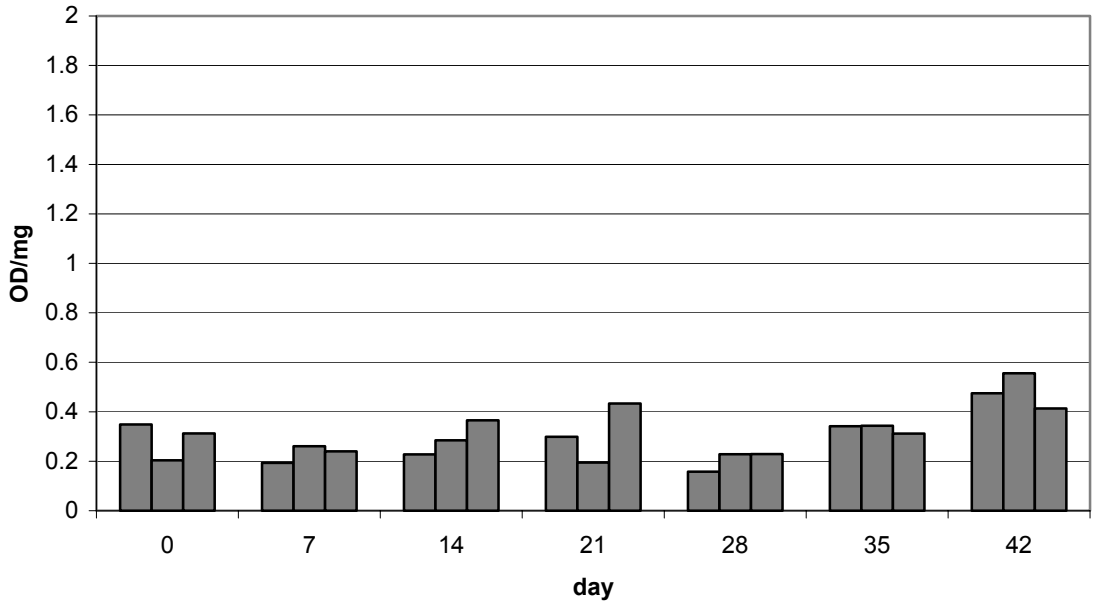

Figure III-3: Measurement of OD/μg values from SDS-PAGE gels for GP-I protein in the whole layer for 72 wk pre-molted hens; Experiment 1.

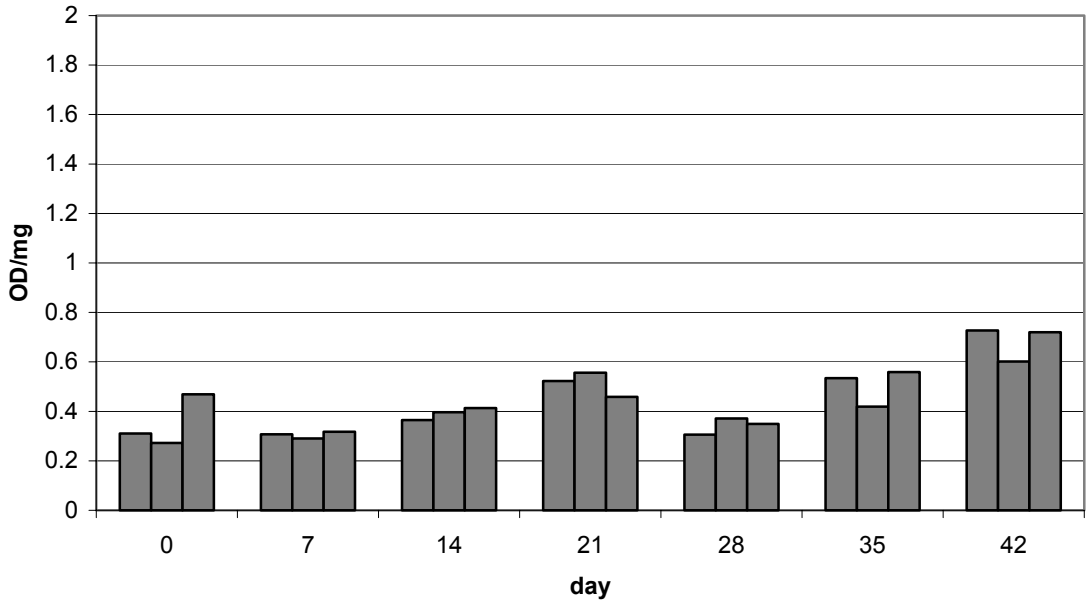

Figure III-4: Measurement of OD/μg values from SDS-PAGE gels for GP-I protein in the inner layer for 72 wk pre-molted hens; Experiment 1.

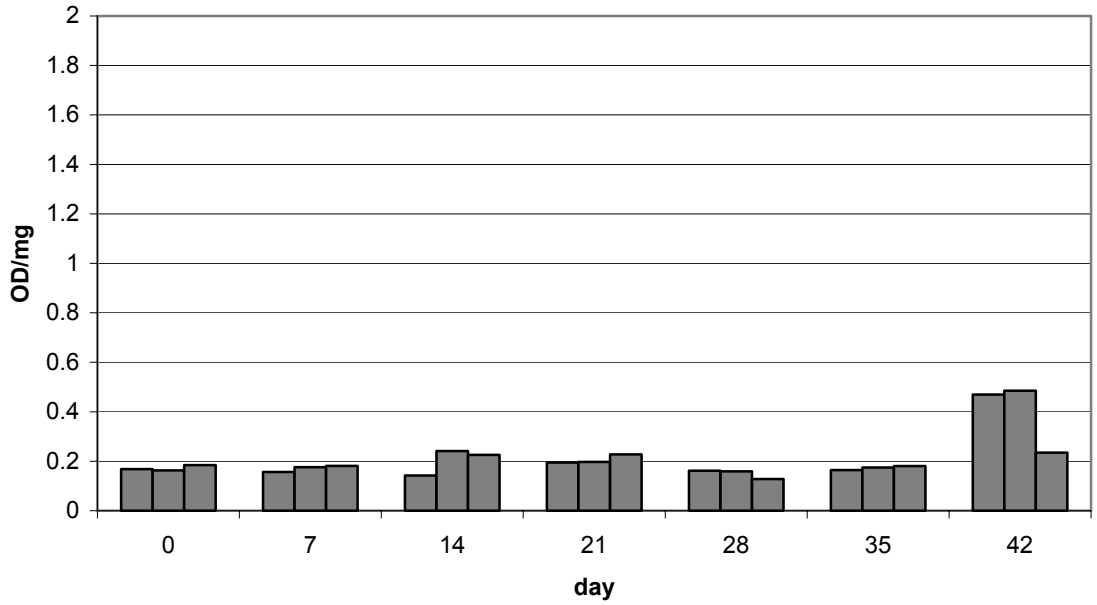

Figure III-5: Measurement of OD/μg values from SDS-PAGE gels for VMO-I protein in the whole layer for 72 wk pre-molted hens; Experiment 1.

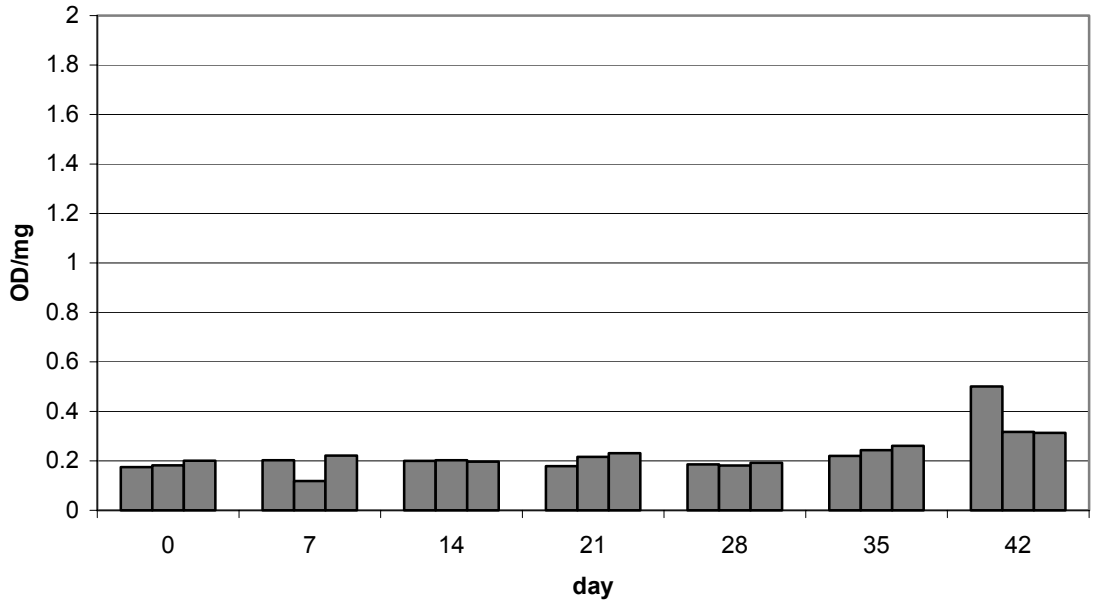

Figure III-6: Measurement of OD/μg values from SDS-PAGE gels for VMO-I protein in the outer layer for 72 wk pre-molted hens; Experiment 1.

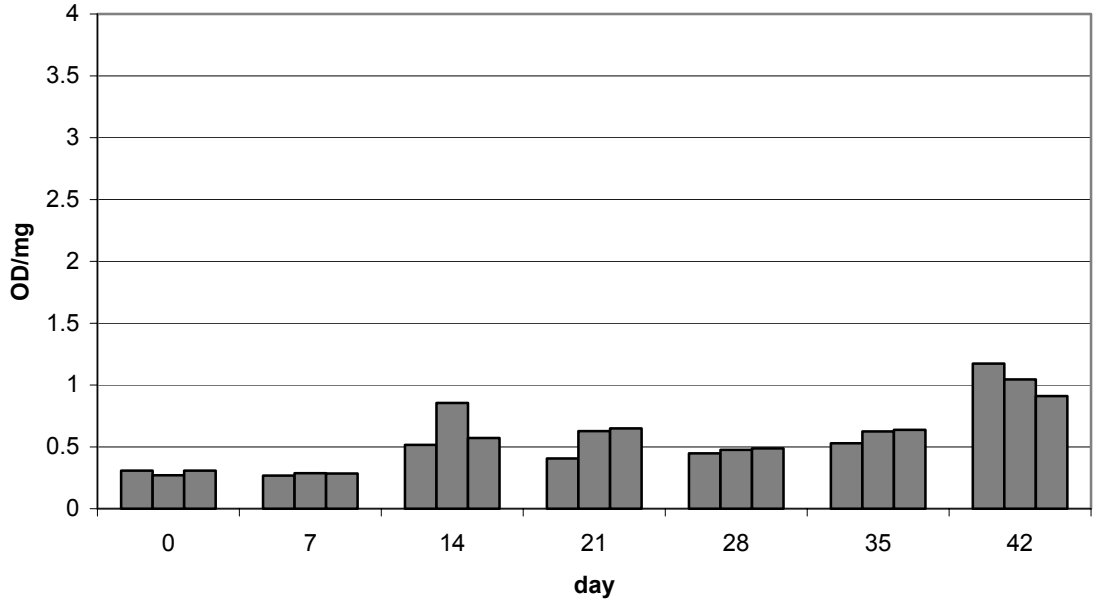

Figure III-7: Measurement of OD/μg values from SDS-PAGE gels for lysozyme protein in the whole layer for 72 wk pre-molted hens; Experiment 1.

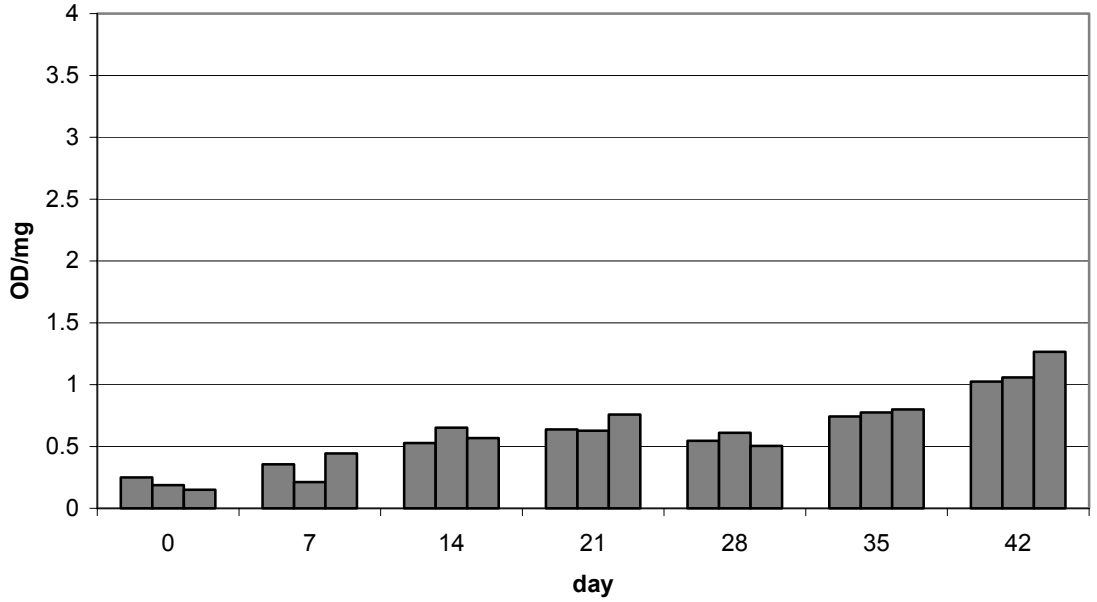

Figure III-8: Measurement of OD/μg values from SDS-PAGE gels for lysozyme protein in the outer layer for 72 wk pre-molted hens; Experiment 1.

Interior Egg Quality. Albumen height declined significantly from day 1 to day 7 and then plateaued until day 35, where there was another significant decline (Table III-1). Albumen pH increased significantly and incrementally on day 7, day 14, and day 35. Albumen pH plateaued from day 14 through day 28 and from day 35 through day 42 (Table III-1). Yolk index showed no significant changes except between day 21 and day 35 (Table III-1). Yolk pH remained stable from day 0 to day 14, but then increased from day 21 through day 42 (Table III-1).

TABLE III-1. Interior egg quality of refrigerated eggs from 72 wk old hens; Experiment 1[1].

Day	Albumen height	Albumen pH	Yolk Index	Yolk pH
0	6.87 ± 0.30[a]	7.32 ± 0.04[d]	0.51 ± 0.01[ab]	6.16 ± 0.02[b]
7	5.89 ± 0.34[b]	8.36 ± 0.05[c]	0.51 ± 0.01[ab]	6.27 ± 0.03[b]
14	5.97 ± 0.23[b]	8.68 ± 0.04[b]	0.51 ± 0.01[ab]	6.28 ± 0.02[b]
21	5.62 ± 0.24[b]	8.60 ± 0.05[b]	0.52 ± 0.01[a]	6.60 ± 0.06[a]
28	5.69 ± 0.20[b]	8.62 ± 0.02[b]	0.52 ± 0.00[ab]	6.57 ± 0.04[a]
35	4.77 ± 0.24[c]	8.98 ± 0.03[a]	0.50 ± 0.01[b]	6.54 ± 0.04[a]
42	5.23 ± 0.21[bc]	8.89 ± 0.04[a]	0.51 ± 0.01[ab]	6.51 ± 0.05[a]

[a-d] Means within same column with no common superscripts differ significantly ($p<0.05$).
[1]Means in same row for 20 eggs ± SE.

Rupture Strength. Rupture strength for the VM with the adhering albumen layer removed does not show any significant changes throughout the storage period (Table III-2). The VM with the adhering albumen layer does show significant changes throughout storage period (Table III-2). Rupture strength for the VM with the adhering albumen layer intact decreases significantly from day 0 to day 7 (Table III-2). Rupture strength is not significantly different on day 0 and day 14 and 21, but rupture strength significantly decreases on day 0 when compared to day 28, 35, and 42. The initial strength at day 0 for the VM with the albumen layer was 11.1 g/g on day 0 and ends with a rupture strength of 6.20 g/g. The data show a gradual decline over refrigerated storage.

TABLE III-2. Yolk rupture strength of refrigerated eggs from 72 wk old hens; Experiment 2[1].

Day	Yolk without albumen layer Rupture strength (g/g)	Yolk with albumen layer Rupture strength (g/g)
0	7.29 $\pm$ 0.83 ^{c,x}	11.10 $\pm$ 1.17^{a,y}
7	7.02 $\pm$ 0.77^{c}	7.19 $\pm$ 1.03^{c}
14	7.08 $\pm$ 0.62^{c}	8.81 $\pm$ 0.61^{abc}
21	6.82 $\pm$ 0.50^{c,x}	9.83 $\pm$ 0.84^{ab,y}
28	8.00 $\pm$ 0.76^{bc}	7.97 $\pm$ 0.94^{bc}
35	6.50 $\pm$ 0.60^{c}	7.02 $\pm$ 0.92^{c}
42	6.92 $\pm$ 0.75^{c}	6.20 $\pm$ 0.47^{c}

$^{a-c}$ Means within same column with no common superscripts differ significantly ($p<0.05$).
^{x,y} Means within same row with no common superscripts differ significantly ($p<0.05$).
[1]Means within rows and columns for 10 yolks $\pm$ SE.

Results: Experiment 2

SDS-PAGE. These gels were also evaluated by OD/μg (Figure III-9 – Figure III-16). The proteins GP-II and VMO-I show a trend of decreasing OD/μg values throughout the storage period (Figure III-8). The protein GP-I isolated from the whole and inner membrane also shows an OD/μg value decrease from day 0 as compared to day 42 (Figure III-8). Lysozyme does not decrease (Figure III-8). Banding patterns appear between 100-116 kDa range (Figure III-8). The breakdown of the proteins and banding patterns appear on the gels at day 21 (Figure III-11).

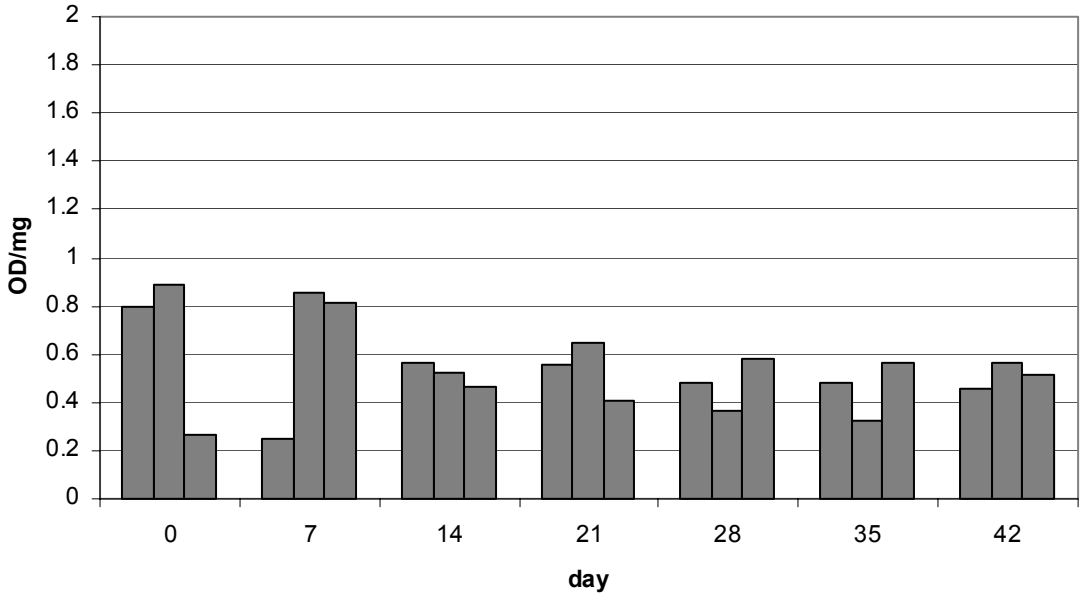

Figure III-9: Measurement of OD/μg values from SDS-PAGE gels for GP-II protein in the whole layer for 26 wk pre-molted hens; Experiment 2.

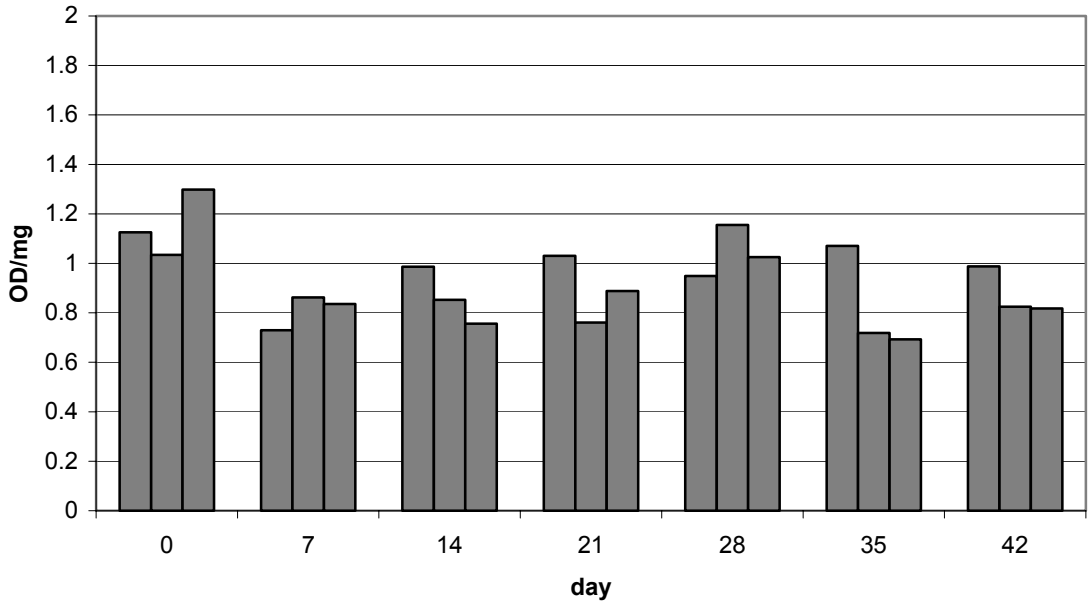

Figure III-10: Measurement of OD/μg values from SDS-PAGE gels for GP-II protein in the inner layer for 26 wk pre-molted hens; Experiment 2.

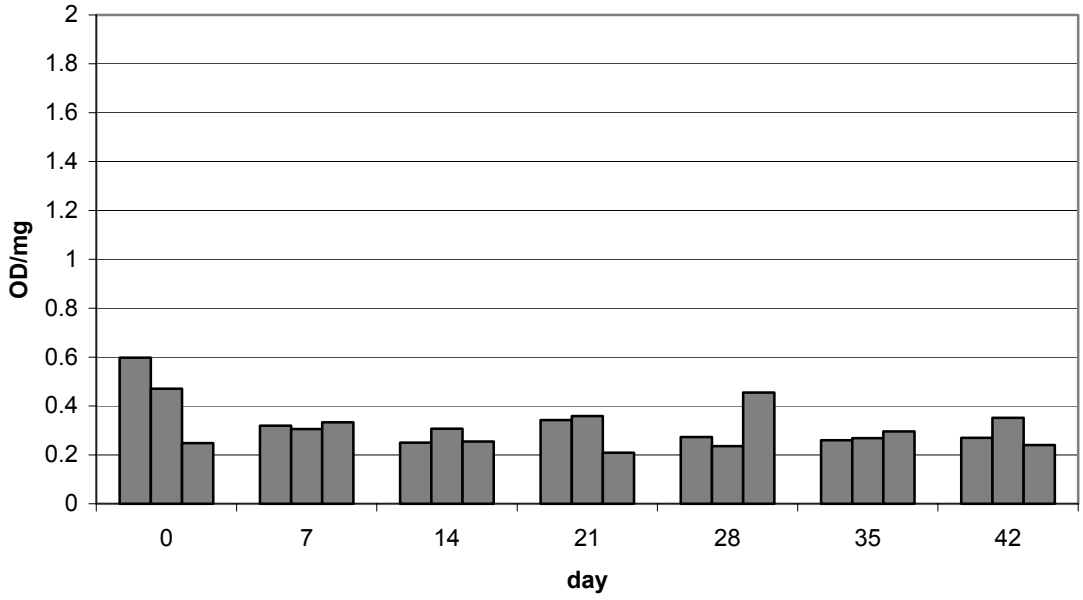

Figure III-11: Measurement of OD/μg values from SDS-PAGE gels for GP-I protein in the whole layer for 26 wk pre-molted hens; Experiment 2.

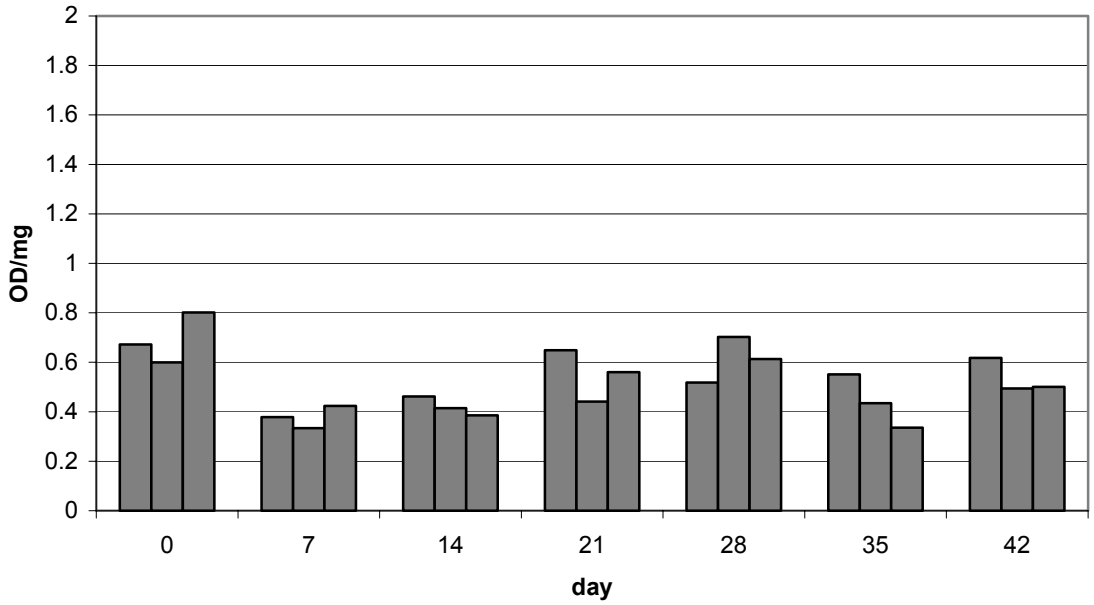

Figure III-12: Measurement of OD/µg values from SDS-PAGE gels for GP-I protein in the inner layer for 26 wk pre-molted hens; Experiment 2.

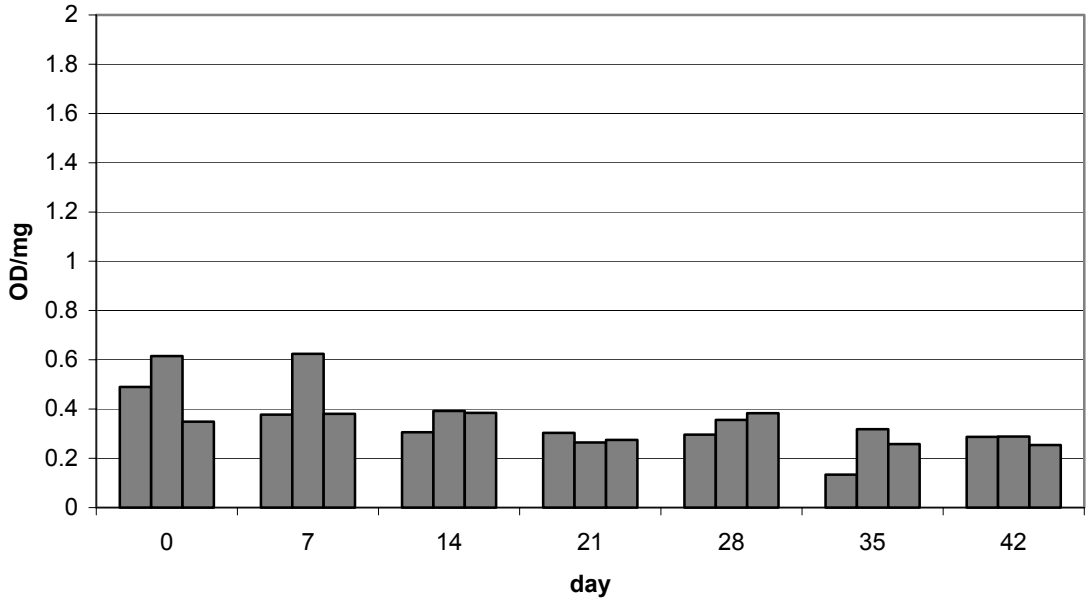

Figure III-13: Measurement of OD/µg values from SDS-PAGE gels for VMO-I protein in the whole layer for 26 wk pre-molted hens; Experiment 2.

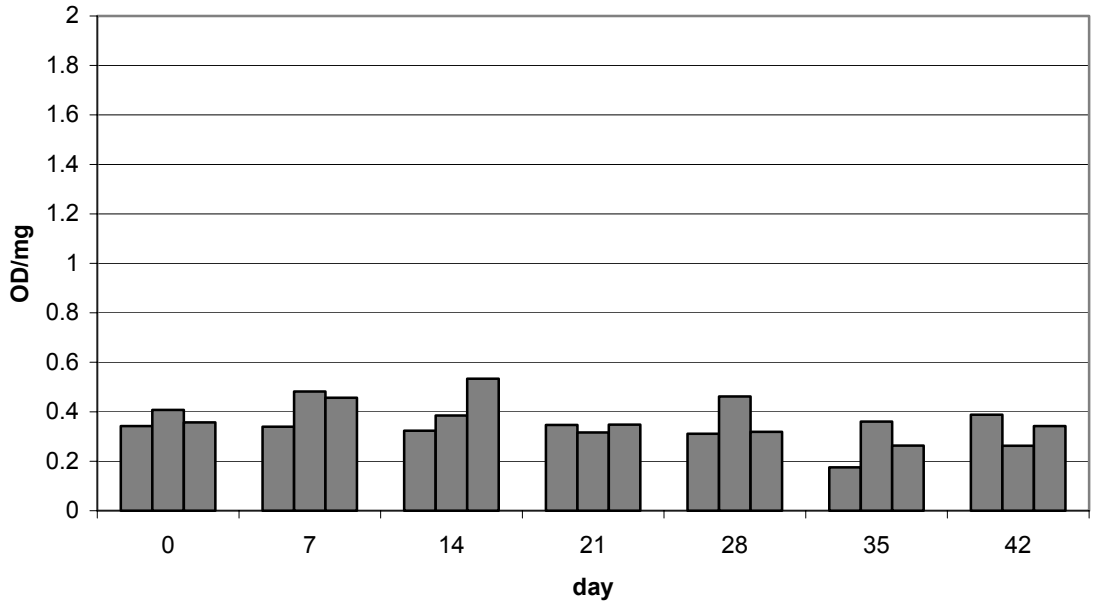

Figure III-14: Measurement of OD/µg values from SDS-PAGE gels for VMO-I protein in the outer layer for 26 wk pre-molted hens; Experiment 2.

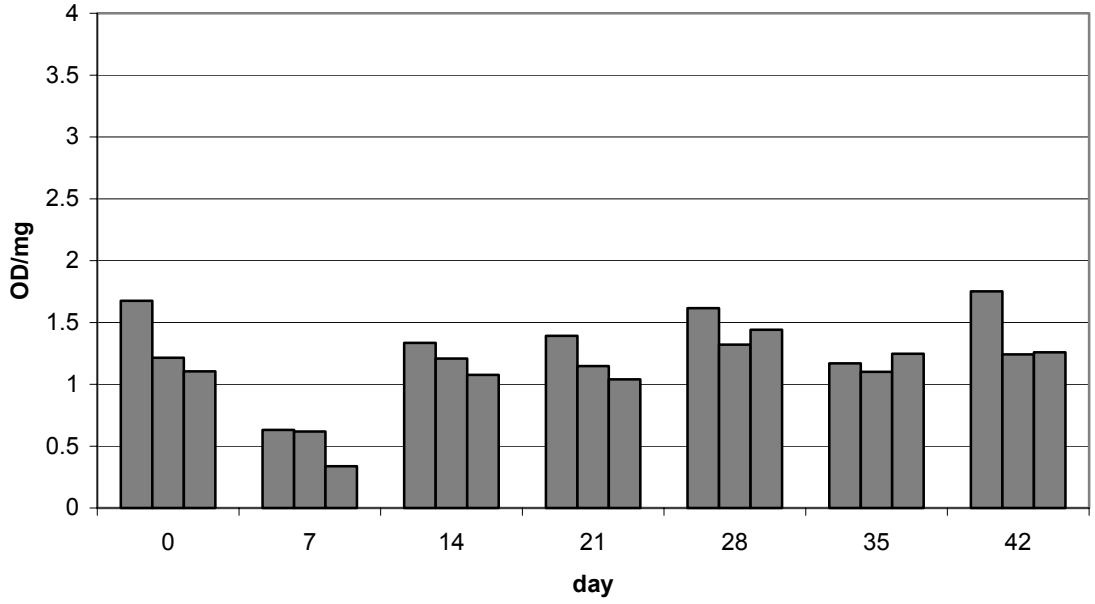

Figure III-15: Measurement of OD/µg values from SDS-PAGE gels for lysozyme protein in the whole layer for 26 wk pre-molted hens; Experiment 2.

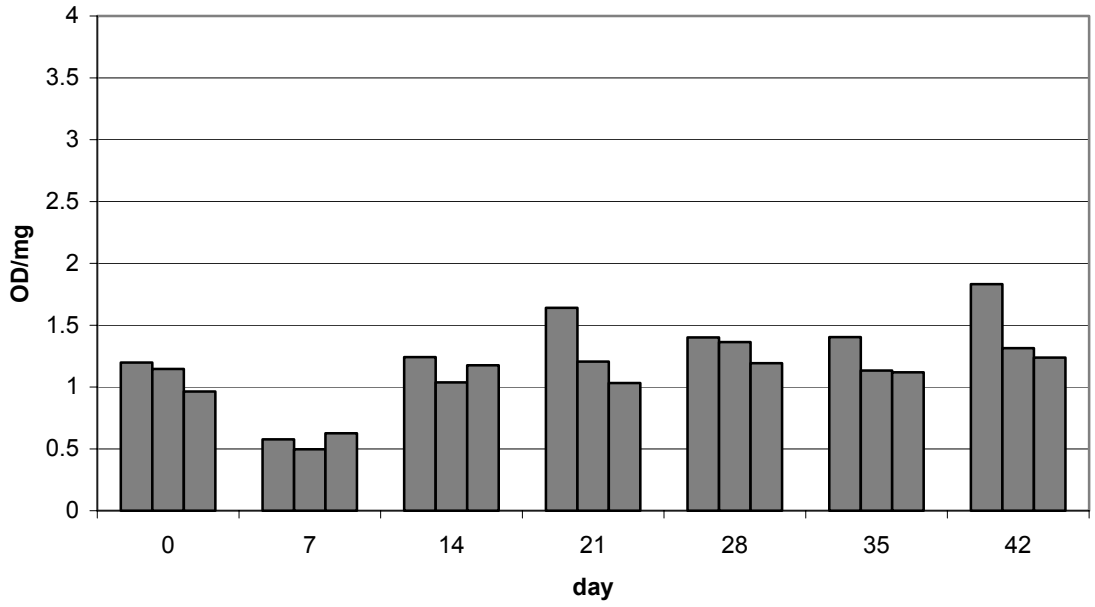

Figure III-16: Measurement of OD/μg values from SDS-PAGE gels for lysozyme protein in the outer layer for 26 wk pre-molted hens; Experiment 2.

Interior Egg Quality. Albumen height significantly increases for day 0 to day 7; however there is a progressive decrease in albumen height for days 14 through 42 (Table III-3). Albumen pH likewise increases incrementally from day 0 to day 42 (Table III-3). Albumen pH increases significantly from day 7 to day 14 and then again on day 28 through day 42. Yolk index has a significant increase from day 0 at 0.50 to day 7 at 0.59, but there is no significance from day 7 to day 42 (Table III-3). Yolk pH shows a significant increase from day 0 to day 7 then plateaus from day 7 to day 21 (Table III-3). There is a significant decrease form day 21 to day 28, but day 35 and 42 are significantly higher than the earlier storage times.

TABLE III-3. Interior egg quality of refrigerated eggs from 26 wk old hens; Experiment 2[1].

Day	Albumen height	Albumen pH	Yolk Index	Yolk pH
0	7.50 $\pm$ 0.24^{bc}	8.08 $\pm$ 0.05^{d}	0.50 $\pm$ 0.01^{b}	6.23 $\pm$ 0.02^{d}
7	8.19 $\pm$ 0.23^{a}	8.18 $\pm$ 0.03^{d}	0.59 $\pm$ 0.01^{a}	6.51 $\pm$ 0.05^{c}
14	7.81 $\pm$ 0.24^{ab}	8.66 $\pm$ 0.02^{c}	0.59 $\pm$ 0.01^{a}	6.59 $\pm$ 0.07^{bc}
21	7.14 $\pm$ 0.18^{cd}	8.91 $\pm$ 0.03^{ab}	0.57 $\pm$ 0.01^{a}	6.60 $\pm$ 0.05^{bc}
28	7.20 $\pm$ 0.23^{bcd}	8.87 $\pm$ 0.05^{b}	0.58 $\pm$ 0.01^{a}	6.32 $\pm$ 0.08^{d}
35	7.19 $\pm$ 0.15^{bcd}	8.99 $\pm$ 0.03^{a}	0.58 $\pm$ 0.01^{a}	6.86 $\pm$ 0.07^{a}
42	6.60 $\pm$ 0.13^{d}	8.98 $\pm$ 0.02^{a}	0.58 $\pm$ 0.01^{a}	6.75 $\pm$ 0.08^{ab}

[a-d] Means within same column with no common superscripts differ significantly ($p<0.05$).
[1]Means in same row for 20 eggs $\pm$ SE.

Rupture Strength. Rupture strength for the VM without the adhering albumen layer was not different throughout storage except day 35 was less than day 0 (Table III-4). Rupture strength for VM with the adhering albumen layer declined after day 0 but was not different for the remainder of the storage period (Table III-4). The VM with the adhering albumen layer and the VM without the adhering albumen layer are significantly different from one another on day 0 and again on day 35 where the VM without the adhering layer is lower.

TABLE III-4. Yolk rupture strength of refrigerated eggs from 26 wk old hens; Experiment 2[1].

Day	Yolk without albumen layer Rupture strength (g/g)		Yolk with albumen layer Rupture strength (g/g)	
0	11.98	$\pm$ 0.79^{b,x}	16.67	$\pm$ 1.16^{a,y}
7	10.81	$\pm$ 1.21^{bc}	12.57	$\pm$ 1.19^{b}
14	9.67	$\pm$ 0.89^{bc}	11.99	$\pm$ 1.16^{b}
21	12.21	$\pm$ 0.93^{b}	13.15	$\pm$ 1.03^{b}
28	10.75	$\pm$ 1.01^{bc}	13.0	$\pm$ 1.11^{b}
35	8.34	$\pm$ 0.95^{c,x}	12.57	$\pm$ 1.15^{b,y}
42	10.35	$\pm$ 1.29^{bc}	11.68	$\pm$ 1.55^{bc}

[a-c] Means within same column with no common superscripts differ significantly ($p<0.05$).
[x,y] Means within same row with no common superscripts differ significantly ($p<0.05$).
[1]Means within rows and columns for 10 yolks $\pm$ SE.

Discussion

Experiment 1. The evaluations of the SDS-PAGE gels were subjective because of the variability of the amount of protein added to each well and because of the technique. The SDS-PAGE gels in this experiment were performed to verify qualitative protein changes in the VM and to monitor these changes over storage. The OD/μg for GP-II (Kido *et al.,* 1977) decreased throughout the storage period as expected. Kido and co-workers (1977) observed breakdown of GP-II after 25 days of storage. The OD/μg value for lysozyme increased over time, which may be attributed to the breakdown of other proteins leaving residues of the same molecular weight as the proteins being monitored. VMO-I and GP-I did not change appreciably throughout the storage period, and the banding patterns that appear between 100-116 kDa range are possibly due to the breakdown of the GP-II protein. The protein breakdown could not be quantified by using this technique because of the variability in staining times, and because all gels are not completely identical and can contribute to interference when using OD. To be able to quantify the proteins, other techniques will need to be applied such as Western blotting or gel filtration.

The yolk index (Sharp and Powell, 1930), and albumen height (Scott and Silversides, 2000) decreased over the storage period as albumen pH (Burley and Vadehra, 1989), and yolk pH (Healy and Peter, 1925) increased as expected. Albumen pH increases during storage due to the loss of water and carbon dioxide because the pH of the albumen is dependent on the ability to maintain balance of dissolved carbon dioxide, bicarbonate ion, carbonate ion, and protein inside the egg (Powrie and Nakai, 1986). Changes in yolk index and yolk pH may be attributed to water migrating into the yolk. The yolk index value increased, while the yolk pH remained stable. However, the values of yolk index and yolk

pH increase which shows that the VM has reached its maximum point of swelling and has begun to flatten, reflecting a decreasing yolk index value while the yolk pH remains high. Similar results were seen in yolk index after 24 hours (Sharp and Powell, 1930).

The rupture strength values show that the albumen layer may be playing a more structural role in the VM because the VM with the albumen layer began with a much higher rupture strength value than the VM without the albumen layer. Aging of the vitelline membrane will cause it to weaken (Fromm, 1964; Fromm and Matrone, 1962; Moran, 1936; Fromm, 1967), which was shown in this study. Weakening of the VM is caused by an increase in osmotic pressure due to water entering the yolk (Sharp and Powell, 1930), which is evident in this study by the yolk index values. The theory of the breakdown of the VM is that the strength of the VM comes from the mucin substance that surrounds the VM and when this substance deteriorates the VM is also affected by the same factors and therefore starts to deteriorate (Feeney *et al.,* 1956). This may explain why the VM with the albumen layer intact began with higher rupture strength value than the VM that had the albumen layer removed. Removal of the albumen layer may cause gaps in the VM and therefore reduce its structural integrity. This reduction is exhibited as lower VM strength.

Another reason the rupture strength for the VM without the adhering albumen layer might be lower is due to the actual removal process. The rolling on the wet paper towel may be disrupting the VM structure and might also be physically tearing the membrane. This could also be a reason for seeing a difference.

As membrane strength decreases, yolk index and albumen quality decrease while yolk and albumen pH increased (Kirunda and McKee, 2000). This was shown in this study

where a decrease in the rupture strength was followed with decreases in interior egg qualities.

Experiment 2. The gel data for this experiment was also analyzed subjectively due to the same reasons as stated in Experiment 1. The OD value of the protein GP-II (Kido *et al.,* 1977) decreased throughout storage as expected. The breakdown of this protein is thought to be related to the effect of both pH and hydrogen sulfide (Kido *et al.,* 1977). VMO-I (Back, 1984) showed a trend for decreasing OD/μg values throughout the storage period as expected (Figure III-8). Back (1984) saw a decrease in VMO-I after storage in conditions that would weaken the VM. The banding patterns that appear between 100-116 kDa is possibly due to the breakdown of the GP-II protein. Changes observed were decreases in values throughout storage from GP-II, VMO-I, and GP-I proteins.

The yolk index (Sharp and Powell, 1930), and albumen height (Scott and Silversides, 2000) decreased through storage as albumen pH (Burley and Vadehra, 1989), and yolk pH (Healy and Peter, 1925) increased over the storage period. Previous studies have shown that albumen pH starts at approximately 7.6 and rises to approximately 9.5 during storage (Burley and Vadehra, 1989). This study shows a higher albumen pH, beginning at 8.08 and rising to 8.98 at the end of the experiment. This may be due to the age of the birds in this experiment. Younger birds have a higher quality of albumen that is less watery (Fetcher *et al.,* 1983), and maintains a higher albumen height (Lapao *et al.,* 1999). The thickness of the shell is also greater (Marion *et al.*, 1964) and can be another reason for maintaining high quality albumen.

With the rupture strength of the 26 wk birds, the VM without the adhering layer is always lower than the VM with the layer, again showing the importance of the albumen VM

interaction. Aging of the vitelline membrane will cause it to weaken (Fromm, 1964; Fromm and Matrone, 1962; Moran, 1936; Fromm, 1967), as has been shown in this study. As membrane strength decreases, yolk index and albumen quality decrease while yolk and albumen pH increased (Kirunda and McKee, 2000), and this occurred in this study.

With the decreases in interior egg qualities there is a decrease in rupture strength and decrease in protein values. The vitelline membrane is influenced by factors that cause thinning of the albumen. The ovomucin-lysozyme is involved in the structure of the albumen and may also play that same role in the outer layer of the VM. As pH increases in the egg, the protein-protein complex of ovomucin and lysozyme will lose its association, thus losing its functionality. Results in the studies show that the adhering albumen layer may play a role in giving strength to the VM. When preparing samples for SDS-PAGE, several techniques were applied to achieve separation, but separation was only possible when the yolk was rolled on a wet paper towel to remove the albumen layer. This is an important fact because this shows that the albumen layer may have some type of interaction in holding the VM together. The decrease in VM strength might also be caused by the physical removal of the adhering albumen layer which is disrupting the VM by the physical rolling and also possibly tearing the membrane.

Experiments Combined. Noticeable differences can be seen between the 72 wk flock and the 26 wk flock. When evaluating the OD/μ values, it is evident that the 26 wk hens have values higher than the 72 wk hens in every protein. The 26 wk hens also have higher albumen height values, and yolk index values than the older flock. The younger flock also have rupture strength values that start off higher than the older flock. Age has been a factor that effects the quality of eggs and rupture strength (Rossi and Pompei, 1995;

Cunningham *et al.,* 1960; Fetcher *et al.,* 1983; Fletcher *et al.,* 1981; Ngoka *et al.,* 1983), which has been shown in this study.

CHAPTER IV

THE EFFECTS OF STORAGE TIME ON VITELLINE MEMBRANE PROTEIN BANDING PATTERNS AND INTERIOR EGG QUALITY OF EGGS FROM MOLTED HENS

Synopsis

A strong VM is important to prevent contamination of albumen by yolk during separation. It also helps to keep pathogenic bacteria from coming into contact with the nutrient rich yolk. This experiment was conducted to determine if a relationship exists between vitelline membrane protein banding patterns on SDS-PAGE, interior egg quality, and vitelline membrane rupture strength. In Experiment 1, eggs were collected from a 74 wk commercial flock after molting. In Experiment 2, eggs were collected from an 82 wk commercial flock after molt. Twenty-one eggs were gathered and stored (4°C, 69% RH). Three eggs were evaluated on days 1, 7, 14, 21, 28, 35, and 42 for changes in SDS-PAGE protein banding patterns. The yolk from each egg was isolated and rolled on a wet paper towel to remove adhering albumen. The yolk was emptied and washed. The whole membrane was placed into double distilled water and divided into two sections. The first component was the whole membrane sample and the other was separated by forceps into inner and outer membranes. The three components were dissolved in 1% sodium dodecyl sulfate (SDS)/70 mM Tris/HCl, pH 6.8. Protein concentration was determined using the Lowry method and individual proteins separated on 4-20% gel gradient by SDS-PAGE. Protein banding patterns were analyzed using a densitometer. Reductions of VMO-I and GP-II proteins occurred during both experiments. Banding patterns appeared between the 100-116 kDa range for both experiments.

An additional 140 eggs were gathered at the same time from the same flocks and stored (4°C, 69% RH). Twenty eggs were evaluated on days 1, 7, 14, 21, 28, 35, and 42 for quality. Yolk index, albumen height, albumen pH, and yolk pH were determined. Vitelline membrane strength was measured using a compression anvil. Two different yolk separation procedures were used to evaluate rupture strength: 10 egg yolks with inner thin albumen layer, and 10 egg yolks rolled on a wet paper towel to remove inner thin albumen layer. Interior egg quality declined during refrigerated storage for both experiments. For Experiment 1, no significant changes occurred for VM without the adhering albumen layer and no changes occurred for the VM with the adhering albumen layers. For Experiment 2, The VM without the adhering albumen layer showed a significant decrease from day 1 to the rest of the storage time. The VM with the adhering albumen layer showed a significant decrease between day 1 and day 42.

Introduction

The role of the VM in the egg is to keep the yolk compartmentalized (Trziszka and Smolinska, 1982), which prevents yolk contamination of the albumen in egg breaking plants and it keeps microorganisms, such as *Salmonella* Enteritidis, from entering the nutrient rich yolk. This separation is important because of the amount of liquid egg that is produced each year. In 2002, 203 million cases of eggs were produced in the United States, and 60.3 egg cases went on to further processing (NASS, 2003).

Quality of the egg is affected by storage conditions and the age of the hen. Storage conditions influence albumen thinning, loss of egg weight due to albumen thinning, and decline in yolk index (Feeney et al., 1952; Evans *et al.,* 1949; Sharp and Powell, 1930). Age affects egg weight, the volume of albumen, and the albumen becomes more watery

(Cunningham *et al.,* 1960; Fetcher *et al.,* 1983). Rupture strength is also affected by age. Younger hens have higher rupture strength values than older hens (Ngoka *et al.,* 1983).

Molting the hens also affect interior egg quality. Molting birds increase the quality of the albumen (Tona *et al.,* 2002). After a molt birds go back into an energized production, and when a flock is molted at 65-70 weeks, they go back into producing like a 40-50 week flock (Bell, 2003). The effects of induced molting are an increase in egg production, egg mass, and shell quality, and decreases in shell breaking, mortality and culling (Bar *et al.,* 2001). Haugh unit values for birds after molting are significantly higher than the values before molting (Tona *et al.,* 2002).

The VM has of three layers: a fibrous outer layer followed by a granular continuous membrane, and then the inner layer which is also fibrous (Bellairs *et al*., 1963). The proteins of the vitelline membrane that have been discovered so far in the outer layer are ovomucin (Back *et al.,* 1982), lysozyme (Back *et al.,* 1982), lectin (Cook *et al*., 1985), VMO-I and II (Back *et al.,* 1982); and in the inner layer GP-I, II, III and IV (Kido *et al*., 1975 & 1976; Kido and Doi, 1988).

It is hypothesized that over refrigerated storage proteins will breakdown and this will result in a decrease in VM strength. Therefore, the objectives of this study werre to evaluate VM strength and protein changes during 6 wk refrigerated storage. This was accomplished by separating proteins from the VM by SDS-PAGE, and albumen height, albumen pH, yolk pH, yolk index, and yolk rupture strength for interior egg quality. SDS-PAGE was performed on the whole VM layer, inner VM and outer VM layer. Interior egg quality measurements were performed on yolks with an adhering albumen layer and on yolks with the adhering albumen layer removed.

Materials and Methods

For Experiment 1, eggs were gathered from a Texas commercial egg producer in January, from Hy-Line cross hens at 74 wk of age. The method of molt was feed deprivation; any further description is proprietary. For Experiment 2, eggs were gathered from a Texas commercial egg producer in June from W-36 Hy-Line hens at 82 wk of age, with a 80% production rate. The method of molt was feed deprivation; any further description is proprietary. A total of 161 eggs were gathered and stored in a refrigerator at 4˚C, 69% RH, and used for interior egg quality (140 eggs), and SDS-PAGE (21 eggs) on days 1, 7, 14, 21, 28, 35, and 42.

Interior Egg Quality. Interior egg quality was evaluated by measuring albumen height, yolk index, albumen pH and yolk pH. A total of 20 eggs were used at each storage time. The eggs were weighed and returned to the refrigerator until needed for analysis. Eggs were opened onto a plate and albumen height determined using a tripod micrometer. The albumen was measured in the middle of the thick white surrounding the yolk. Yolk index was taken using the tripod micrometer to measure the height of the yolk, and calipers were used to measure the diameter of the yolk at two different points. The yolk was separated from the albumen by pouring the egg in a cup and pouring off the albumen into another cup. A pH meter[1] equipped with a combination electrode was used to determined albumen pH. After rupturing the yolk, the pH of the yolk was taken.

Rupture Strength of the VM. After separating the yolk from the albumen, ten yolks were evaluated for rupture strength that had the adhering albumen layer removed by rolling the yolk on a wet paper towel. The remaining ten yolks had rupture strength determined

[1]Corning pH meter, model 240, Corning Glassworks, Medfield, MA 02052.

with the adhering albumen layer left intact. To weigh the yolk, a glass cylinder dish and filter paper lining the bottom were put on a tared scale and then the yolk was put into the dish and weighed. Rupture strength was taken using a compression anvil and Instron[2] with compression system at 50 mm/min cross head speed, 500 g load range, and a 5 kg load cell. The rupture strength in kg was divided by the weight in grams of the yolk to give a final force calculation.

Separation of the VM, Protein Concentration and SDS-PAGE. Samples of the whole membrane, inner membrane layer, and outer membrane layer from three eggs were used to analyze the VM proteins. Yolk was separated from the albumen and the adhering albumen layer removed as described by Back and co-workers (1982). The yolk was ruptured by hand, yolk contents drained, and further washed with a stream of dd water. The VM was put into a petri dish filled with dd water and the membrane divided into two approximately equal portions under a dissecting microscope. One portion of the whole membrane was put into 2 mL of 70 mM Tris-HCl, pH 6.8, 1% SDS. It became the sample used to determine whole membrane proteins. The other whole membrane portion was further separated with forceps into the inner membrane layer and outer membrane layer under the dissecting microscope. The inner and outer layer portions were placed separately in 2 mL of 70 mM Tris-HCl, pH 6.8, 1% SDS; these became the samples to determine inner layer and outer layer proteins. Samples were stirred with a magnetic stir rod for 15-17 hours, at 22ºC to dissolve the membrane.

Protein concentration was determined using the Modified Lowry Protein Assay. The dissolved membrane solution was decanted into centrifuge tubes and centrifuged at 20K, for

[2]model 1011, Instron Corp., Canton, MA 02021.

5 minutes at 4˚C using the Sorvall Untracentrifuge[3]. The supernatant was poured into glass tubes. Two hundred µL of each solution was pipetted into duplicate glass tubes. A blank tube was prepared for calibration using a solution of 70 mM Tris-HCl, pH 6.8, 1% SDS. One mL of Modified Lowry Reagent was put into the tubes, vortexed, then incubated for 10 minutes. After incubation, 0.1 mL of the Phenol reagent was added, vortexed, and then incubated for 30 minutes while covered. After incubation, the protein concentration was read at 750 nm using a Beckman Spectrophotometer[4], and protein concentration calculated to find the actual µg of protein that should be added to SDS-PAGE gel. After protein concentration is determined, samples were denatured and frozen until electrophoresised. Laemmli sample buffer was made with 62.5 mM Tris-HCl, (pH 6.8), 2% SDS, 25% glycerol to mix with the sample solutions. Four hundred µL of the sample was mixed with 400 µL of sample buffer, then 40 µL of bromophenol blue was added. The mixture was put into tubes and heated for 5-7 minutes at 100ºC in a water bath. The tubes were stored at 0ºC until ready for electrophoresis.

The Protean II Ready Gel System[5] for SDS-PAGE was used to electrophorese the samples for VM protein banding patterns on 4 to 20% gradient gels at 106-107 V. For Experiment 1, approximately 1.90-2.32 µg of proteins were loaded in each lane. For Experiment 2, approximately 2.97-3.03 µg of proteins were loaded in each lane. Standards were run on each side of the gel with the inner lanes showing the first egg with whole membrane, followed by the inner layer, followed by the outer layer; the second egg followed with the same pattern; followed by the third egg with the same pattern. When the dye front

[3]model OTD65B, DuPont Co., Wilmington, DE 19898.
[4]model DU-64, Beckman Instruments, Inc., Fullerton, CA 92634.
[5]Bio-Rad Laboratories, Inc., Hercules, CA 94547.

in the samples reached the bottom of the gel, the gels were held in dd water and stained with GelCode Blue Stain Reagent for two hours. The gels were then washed in several changes of dd water and held (in dd water overnight) to enhance the stain. After staining, the gels were dried using Gel-Dry Solution and Novex cellophane. The gels were scanned to determine optical density with the Bio-Rad Multi-Analyst Densitometer[6]. Protein bands were evaluated subjectively by the volume (optical density x mm x mm) of the band divided by the protein μg loaded.

Statistical Analysis. Data were analyzed using the General Linear Model (GLM) to generate and an ANOVA and significant means separated using the Duncan's Multiple Range Test at $P < 0.05$.

Results: Experiment 1

SDS-PAGE. Gels were analyzed subjectively and values of protein bands were found by dividing the optical density (OD) reading from the amount of protein loaded into each well (μg) (Figure IV-1 – IV-8). The OD/μg value for the proteins GP-II and VMO-I decreased throughout the storage period (Figure IV-1). The GP-II protein isolated from the whole layer showed a decreased OD value on day 1 compared to day 42. The OD value of the protein GP-I isolated from the whole membrane decreased from day 1 to day 42 (Figure IV-1). VMO-1 protein isolated from the whole layer also shows a decreased OD value over the storage period (Figure IV-1). Lysozyme also had decreased OD values from day 1 to day 42 (Figure IV-1). When viewing the gels, banding patterns appear between to 100-116

[6]model GS-690, Bio-Rad Laboratories, Inc., Hercules, CA 94547.

kDa range (Figure IV-1). The breakdown of the proteins and banding patterns appear on the gels at day 21 (Figure IV-4).

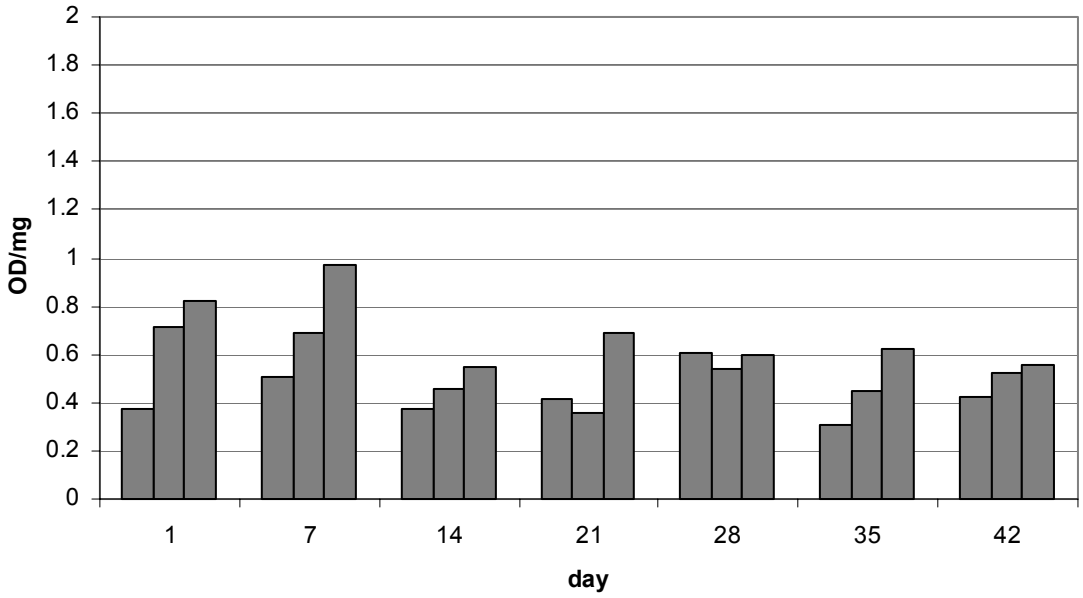

Figure IV-1: Measurement of OD/μg values from SDS-PAGE gels for GP-II protein in the whole layer for 74 wk molted hens; Experiment 1.

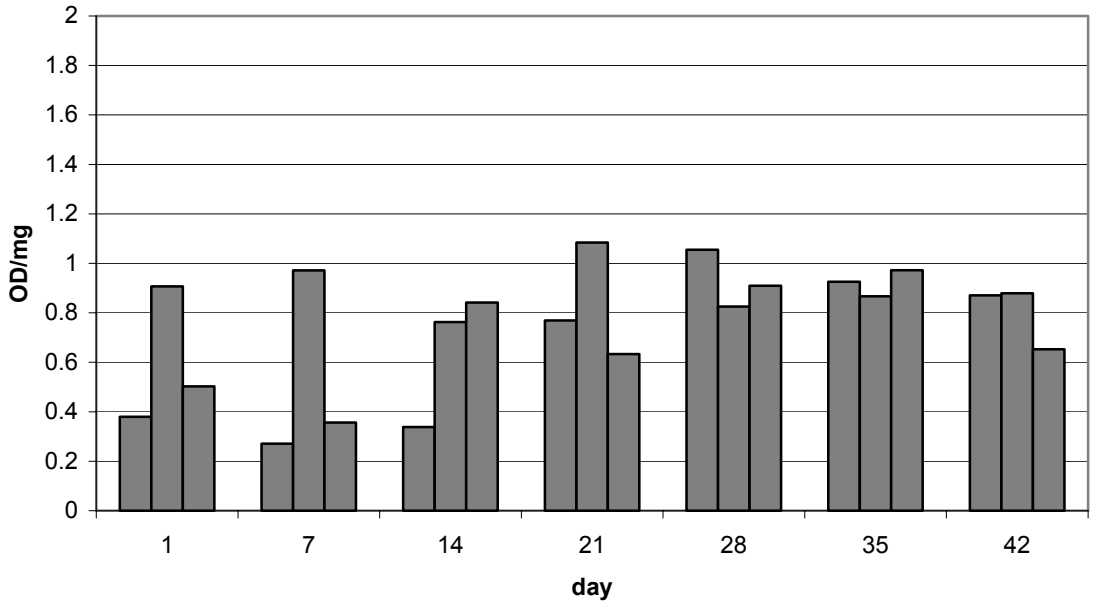

Figure IV-2: Measurement of OD/µg values from SDS-PAGE gels for GP-II protein in the inner layer for 74 wk molted hens; Experiment 1.

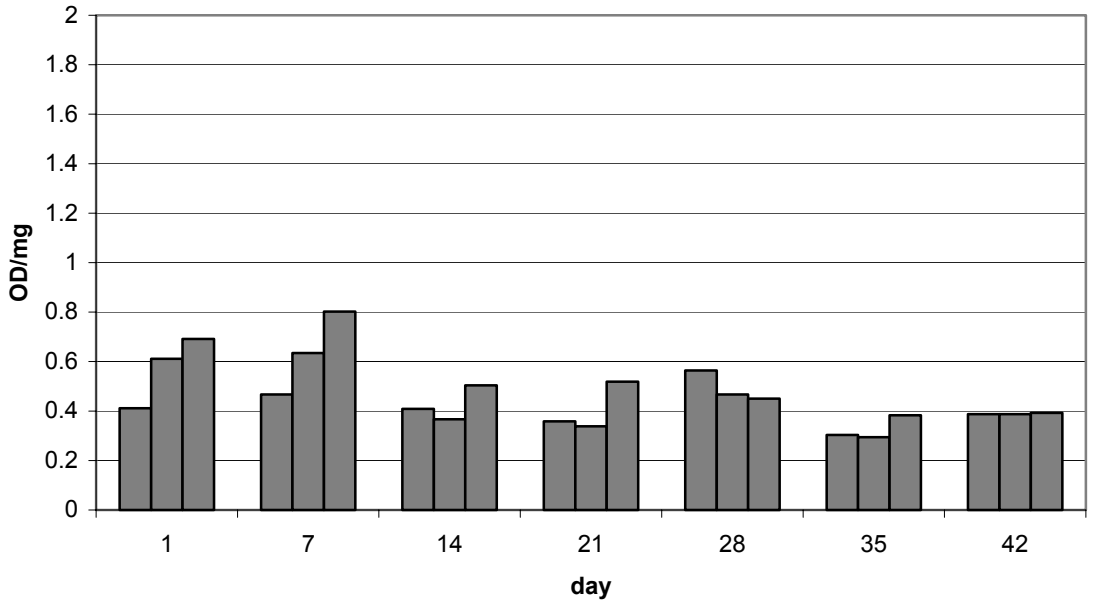

Figure IV-3: Measurement of OD/µg values from SDS-PAGE gels for GP-I protein in the whole layer for 74 wk molted hens; Experiment 1.

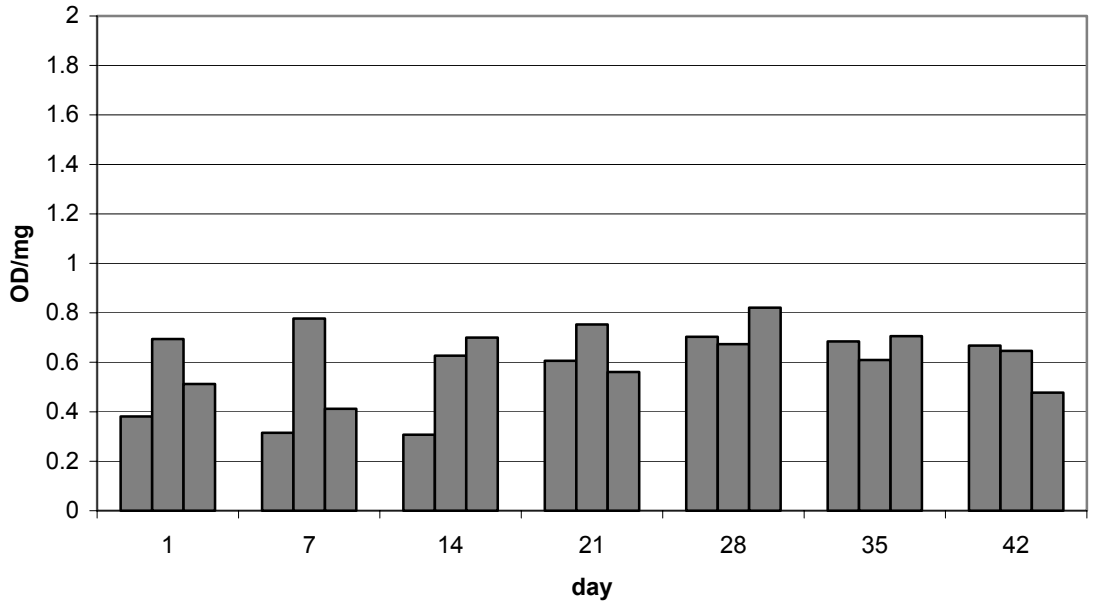

Figure IV-4: Measurement of OD/µg values from SDS-PAGE gels for GP-I protein in the inner layer for 74 wk molted hens; Experiment 1.

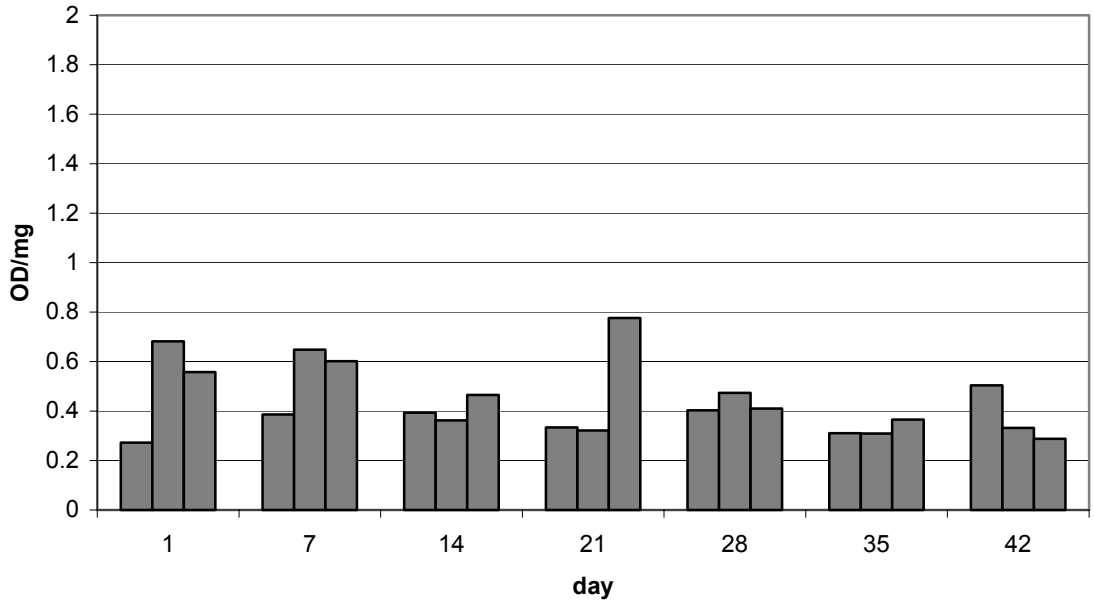

Figure IV-5: Measurement of OD/µg values from SDS-PAGE gels for VMO-I protein in the whole layer for 74 wk molted hens; Experiment 1.

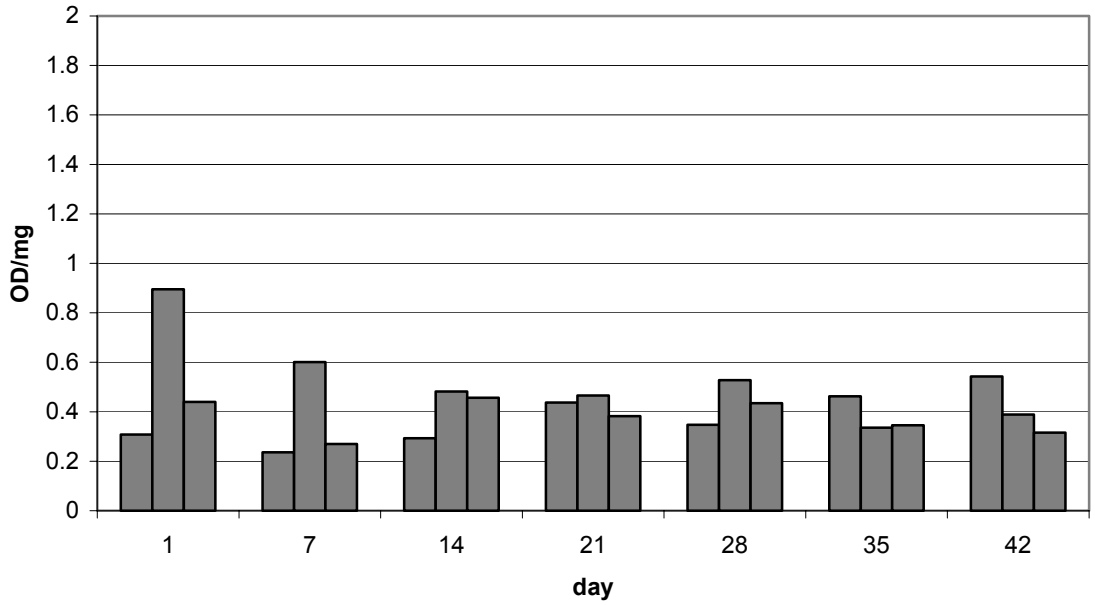

Figure IV-6: Measurement of OD/µg values from SDS-PAGE gels for VMO-I protein in the outer layer for 74 wk molted hens; Experiment 1.

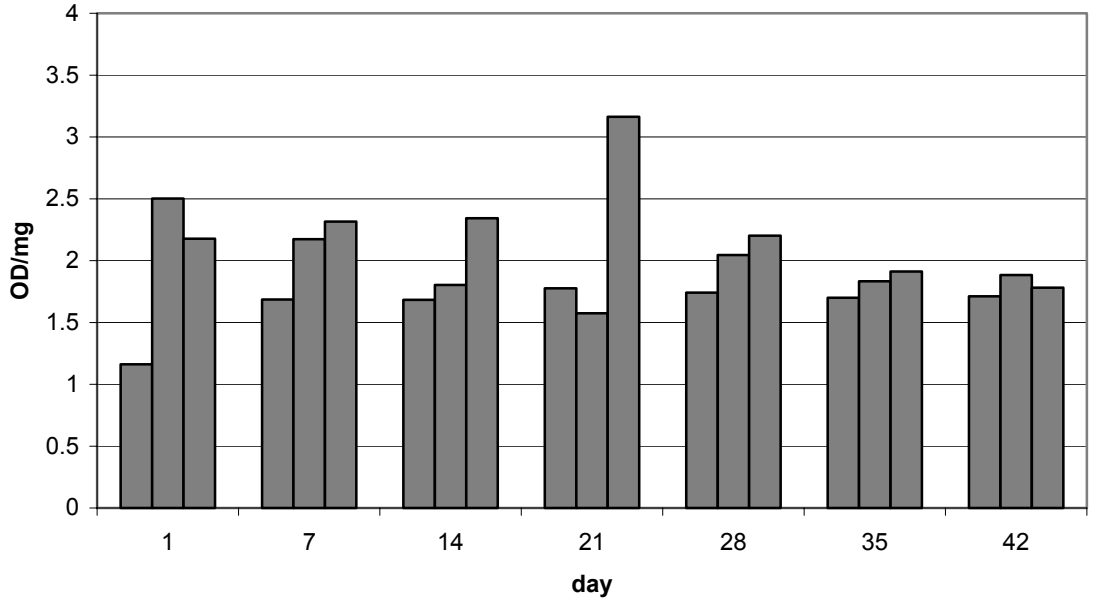

Figure IV-7: Measurement of OD/µg values from SDS-PAGE gels for lysozyme protein in the whole layer for 74 wk molted hens; Experiment 1.

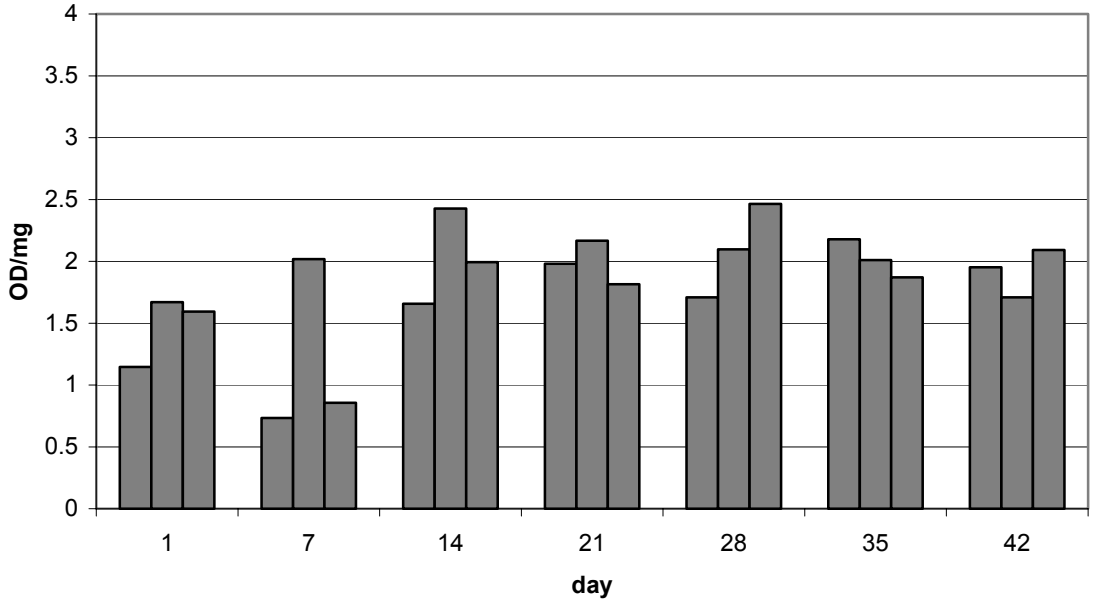

Figure IV-8: Measurement of OD/μg values from SDS-PAGE gels for lysozyme protein in the outer layer for 74 wk molted hens; Experiment 1.

Interior Egg Quality. Albumen height decreases significantly from day 1 to day 7 (Table IV-1). Albumen height then plateaus on day 7 to day 35, with another decrease on day 42. Albumen pH increases significantly within the first 2 weeks from day 1 to day 7 to day 14 (Table IV-1). Day 21, 28, and 35 are not significant from each other, but are still increasing in pH. Albumen pH for day 42 is significantly higher than day 1 through day 21. Yolk index increases significantly from day 1 up to day 14 (Table IV-1). Day 14 to day 21 shows a decrease, although not significant, but days 28 through 42 are significantly lower from day 14. Yolk pH increases from day 1 to day 14, where day 14 is significantly higher than day 1 (Table IV-1). Yolk pH increases significantly from day 14 to day 21; yolk pH for day 21 through day 42 remains stable.

TABLE IV-1. Interior egg quality of refrigerated eggs from 74 wk old hens; Experiment 1[1].

Day	Albumen height	Albumen pH	Yolk Index	Yolk pH
1	7.81 ± 0.29^{a}	7.72 ± 0.04^{e}	0.49 ± 0.00^{d}	6.15 ± 0.01^{c}
7	7.22 ± 0.14^{b}	7.99 ± 0.04^{d}	0.52 ± 0.01^{bc}	6.24 ± 0.02^{bc}
14	6.66 ± 0.19^{bc}	8.21 ± 0.04^{c}	0.54 ± 0.00^{a}	6.32 ± 0.03^{b}
21	6.62 ± 0.26^{bc}	8.30 ± 0.04^{bc}	0.53 ± 0.01^{ab}	6.57 ± 0.08^{a}
28	6.40 ± 0.15^{c}	8.34 ± 0.05^{ab}	0.52 ± 0.01^{bc}	6.60 ± 0.04^{a}
35	6.38 ± 0.15^{c}	8.39 ± 0.02^{ab}	0.52 ± 0.01^{b}	6.57 ± 0.06^{a}
42	5.47 ± 0.20^{d}	8.45 ± 0.04^{a}	0.50 ± 0.00^{c}	6.62 ± 0.06^{a}

[a-d] Means within same column with no common superscripts differ significantly ($p<0.05$).
[1]Means in same row for 20 eggs $\pm$ SE.

Rupture Strength. Rupture strength for the VM without the adhering albumen layer has no significant changes during storage (Table IV-2). The rupture strength starts out with a low rupture strength at 7.71 g/g. The VM with the adhering albumen layer intact also shows no significant changes over refrigerated storage (Table IV-2). The VM with the albumen layer does start out with a higher rupture strength at 8.18 g/g. The VM with the albumen layer and the VM without the albumen layer are significantly different between each other on days 21 and 28 where the VM without the albumen layer is lower. With the exception of day 7, the VM with the albumen layer has a higher rupture strength value than the VM without the albumen layer.

TABLE IV-2. Yolk rupture strength of refrigerated eggs from 74 wk old hens; Experiment 1[1].

Day	Yolk without albumen layer Rupture strength (g/g)	Yolk with albumen layer Rupture strength (g/g)
1	7.71 ± 0.77^{abc}	8.18 ± 0.85^{abc}
7	7.72 ± 0.62^{abc}	7.38 ± 0.65^{abc}
14	6.33 ± 0.55^{bc}	7.55 ± 0.99^{abc}
21	$6.37 \pm 0.41^{bc,x}$	$9.16 \pm 0.77^{a,y}$
28	$5.78 \pm 0.72^{c,x}$	$8.27 \pm 0.80^{ab,y}$
35	5.87 ± 0.50^{bc}	8.20 ± 0.88^{ab}
42	7.01 ± 0.62^{abc}	7.46 ± 0.77^{abc}

[a-c] Means within same column with no common superscripts differ significantly ($p<0.05$).
[x,y] Means within same row with no common superscripts differ significantly ($p<0.05$).
[1]Means within rows and columns for 10 yolks $\pm$ SE.

Results: Experiment 2

SDS-PAGE. These gels were also evaluated by OD/μg (Figure IV-9 – Figure IV-16). The protein GP-II had a decreasing value throughout storage (Figure IV-8). The value for GP-I also decreased during storage (Figure IV-8). The proteins VMO-I and lysozyme do not have decreasing OD values throughout storage (Figure IV-8). Banding patterns appeared between to 100-116 kDa range at day 21(Figure 1).

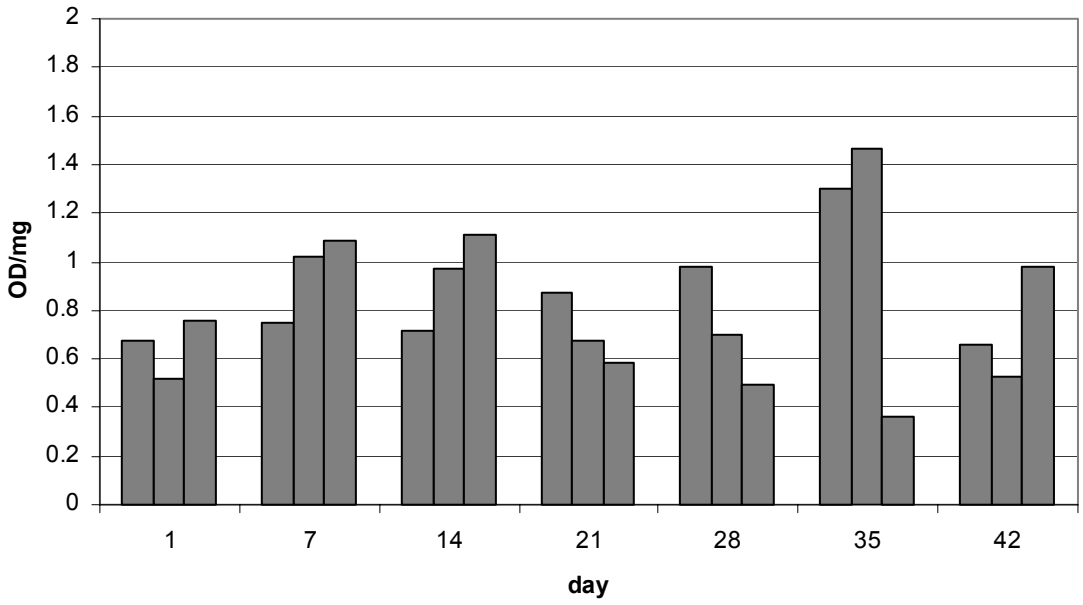

Figure IV-9: Measurement of OD/μg values from SDS-PAGE gels for GP-II protein in the whole layer for 82 wk molted hens; Experiment 2.

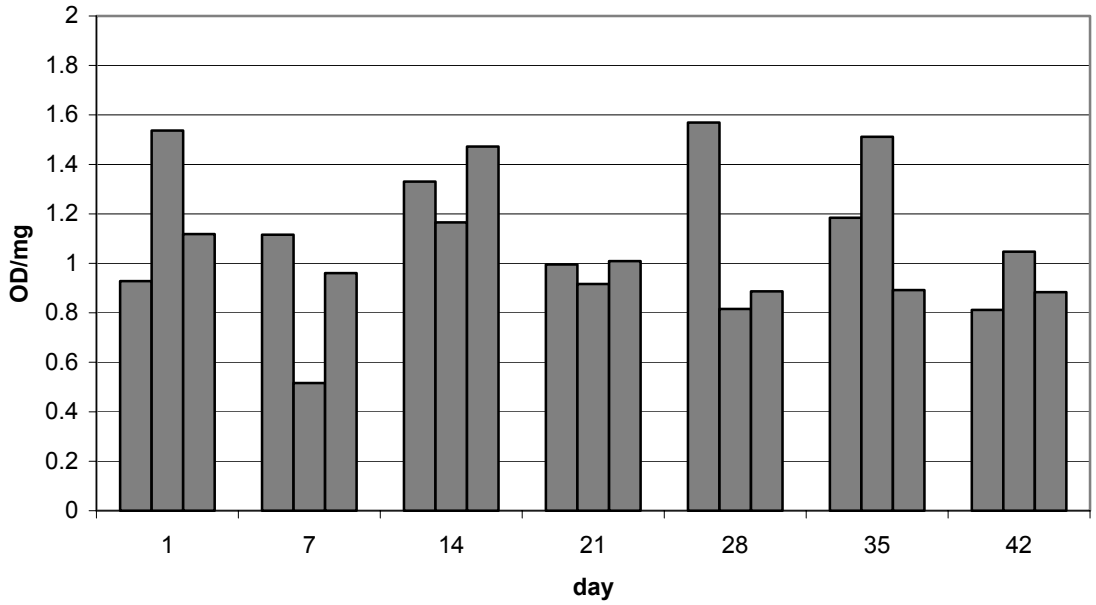

Figure IV-10: Measurement of OD/µg values from SDS-PAGE gels for GP-II protein in the inner layer for 82 wk molted hens; Experiment 2.

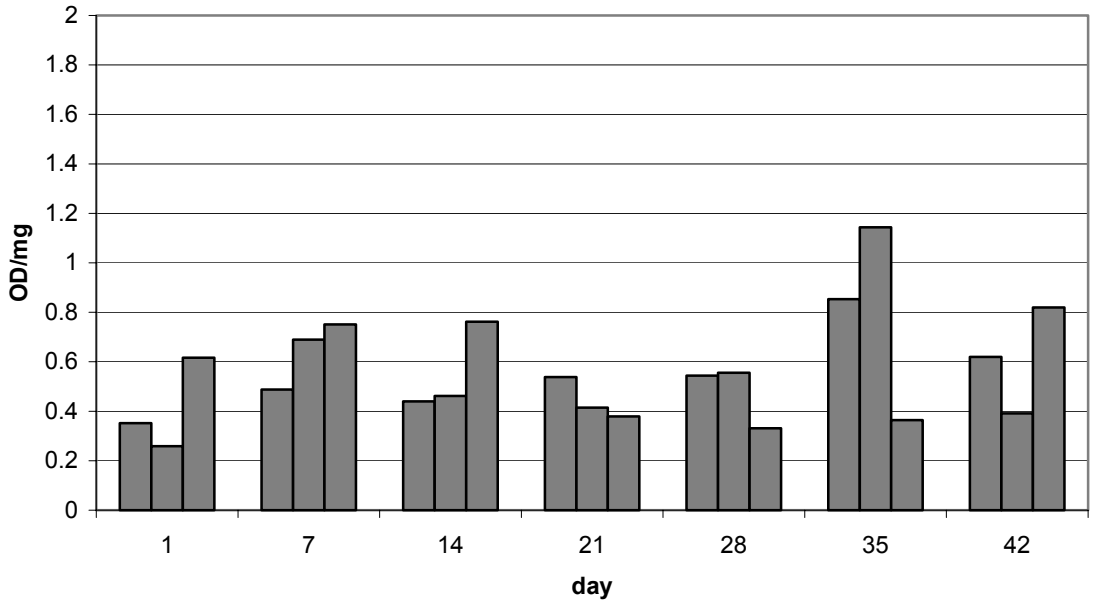

Figure IV-11: Measurement of OD/µg values from SDS-PAGE gels for GP-I protein in the whole layer for 82 wk molted hens; Experiment 2.

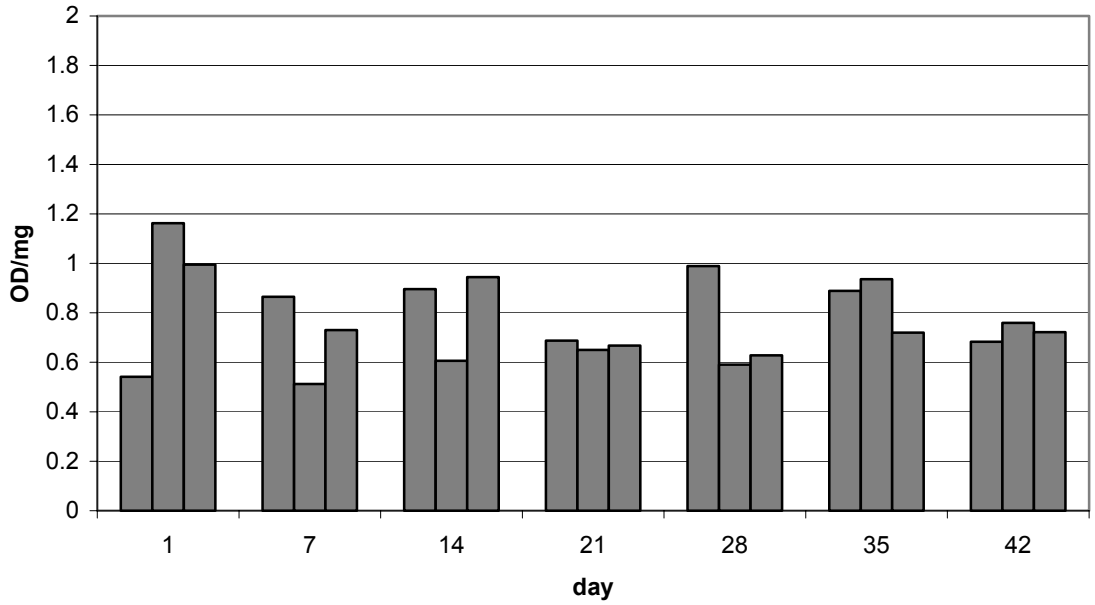

Figure IV-12: Measurement of OD/µg values from SDS-PAGE gels for GP-I protein in the inner layer for 82 wk molted hens; Experiment 2.

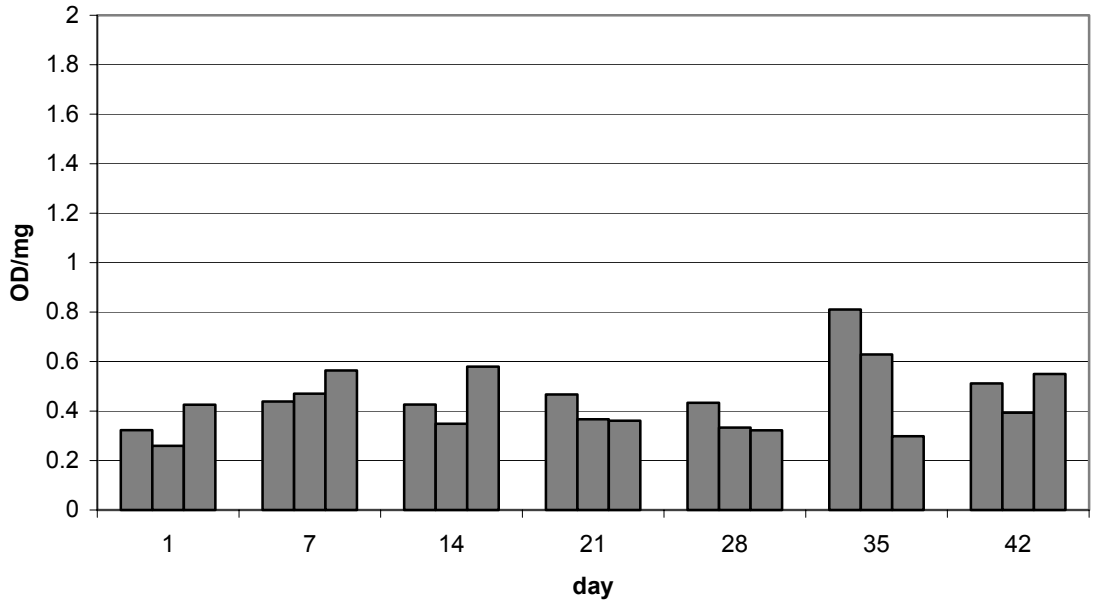

Figure IV-13: Measurement of OD/µg values from SDS-PAGE gels for VMO-I protein in the whole layer for 82 wk molted hens; Experiment 2.

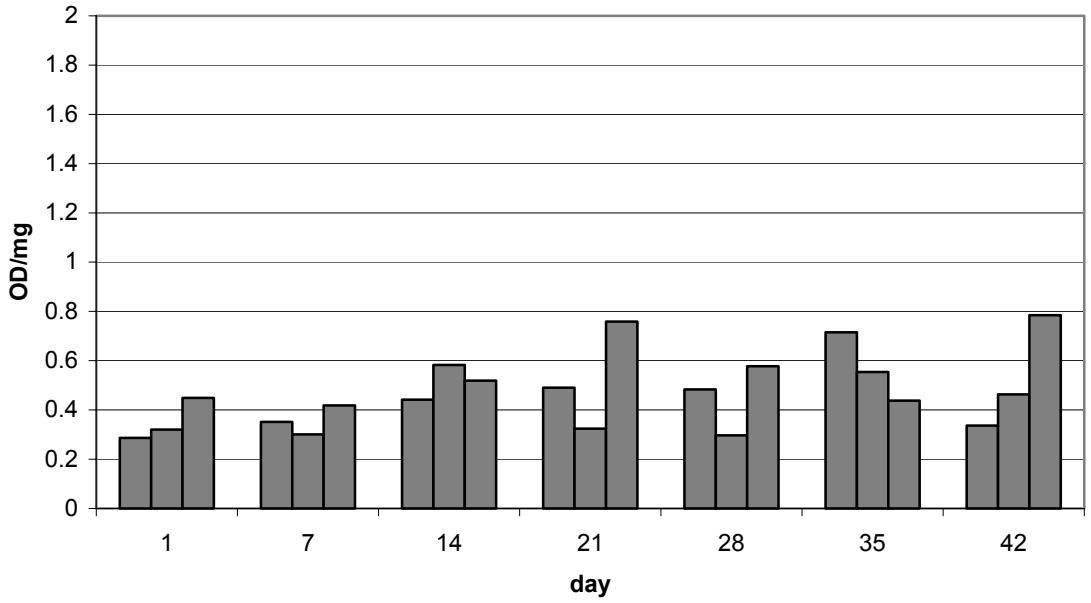

Figure IV-14: Measurement of OD/µg values from SDS-PAGE gels for VMO-I protein in the outer layer for 82 wk molted hens; Experiment 2.

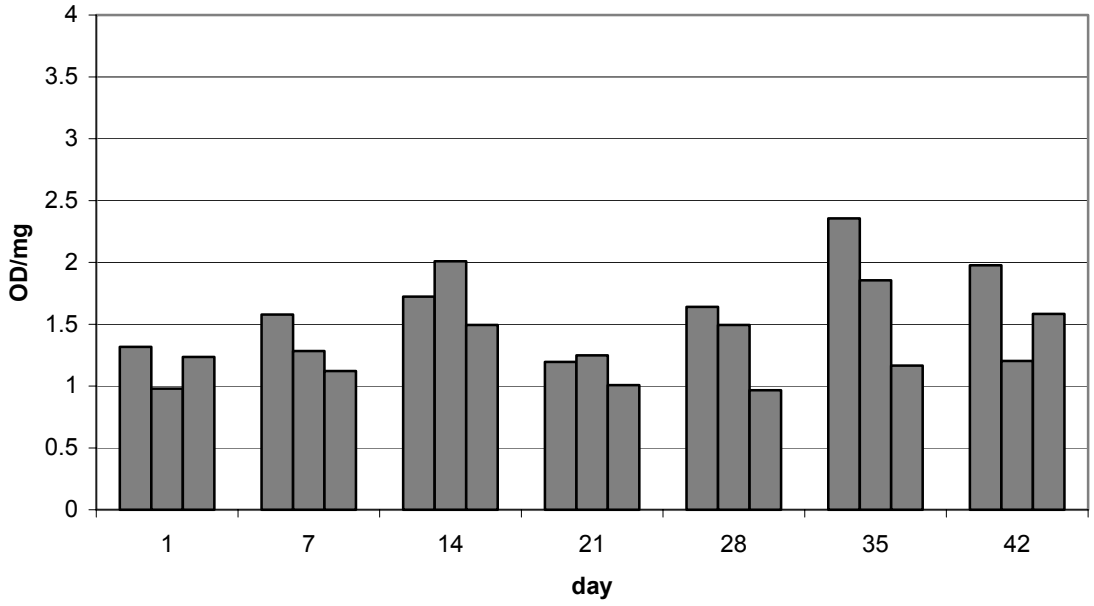

Figure IV-15: Measurement of OD/µg values from SDS-PAGE gels for lysozyme protein in the whole layer for 82 wk molted hens; Experiment 2.

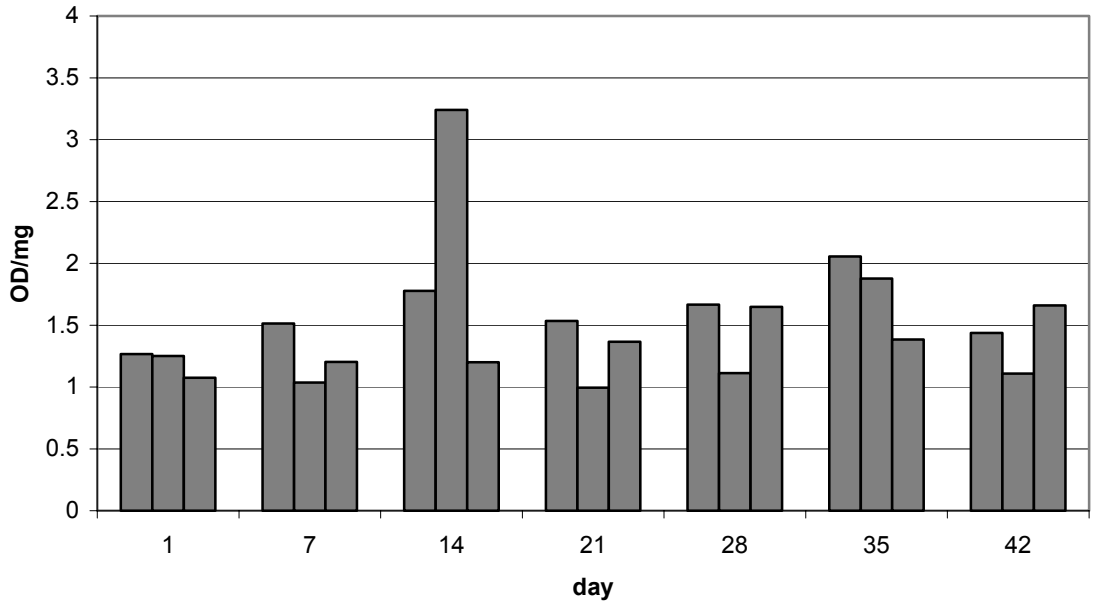

Figure IV-16: Measurement of OD/µg values from SDS-PAGE gels for lysozyme protein in the outer layer for 82 wk molted hens; Experiment 2.

Interior Egg Quality. Albumen height remains stable for day 1 and 7, but then significantly decreases on day 14 (Table IV-3). Day 14 through day 42 plateaus, but day 42 is significantly lower than days 14 and 28. Albumen pH increases significantly from day 1 throughout day 21 (Table IV-3). Day 21 through day 42 remains stable although days 28 and 35 are significantly different. Yolk index is only significantly different on days 7 and 42 (Table IV-3). The yolk index does show a trend of an increase from day 1 to day 7 and then a decrease from day 7 to 21. Day 28 through day 42 shows a trend of decreasing yolk index. Yolk pH increases from day 1 to day 14, where day 14 is significantly higher than day 1 (Table IV-3). Yolk pH for day 14 through day 28 decreases although not significantly, but there is a significant increase from day 28 through day 42.

TABLE IV-3. Interior egg quality of refrigerated eggs from 82 wk old hens; Experiment 2[1].

Day	Albumen height	Albumen pH	Yolk Index	Yolk pH
1	7.01 ± 0.18^{a}	8.01 ± 0.05^{e}	0.52 ± 0.01^{ab}	6.27 ± 0.05^{d}
7	6.94 ± 0.16^{a}	8.30 ± 0.05^{d}	0.53 ± 0.01^{a}	6.35 ± 0.04^{bcd}
14	6.41 ± 0.19^{b}	8.67 ± 0.03^{c}	0.52 ± 0.01^{ab}	6.48 ± 0.04^{bc}
21	5.85 ± 0.20^{bc}	8.93 ± 0.02^{ab}	0.51 ± 0.00^{ab}	6.45 ± 0.05^{bcd}
28	5.95 ± 0.19^{b}	8.90 ± 0.07^{b}	0.52 ± 0.00^{ab}	6.29 ± 0.09^{cd}
35	5.87 ± 0.19^{bc}	9.05 ± 0.03^{a}	0.51 ± 0.01^{ab}	6.50 ± 0.08^{b}
42	5.34 ± 0.18^{c}	9.00 ± 0.04^{ab}	0.51 ± 0.01^{b}	6.73 ± 0.08^{a}

[a-e] Means within same column with no common superscripts differ significantly ($p<0.05$).
[1]Means in same row for 20 eggs $\pm$ SE.

Rupture Strength. Rupture strength for the VM without the adhering albumen layer is significantly different from day 1 compared to the remaining storage times (Table IV-4). The rupture strength starts out high at 9.57 g/g. The VM with the adhering albumen layer is not significantly different throughout storage from day 1 through day 35, but is significantly different between day 1 and 42 (Table IV-4). The rupture strength for the VM with the albumen layer starts on day 1 at 7.90 g/g. The only significant difference between the VM without the adhering albumen layer and the VM with the albumen layer is on day 21 where the VM with the albumen layer is higher. Not expected in the rupture results was that the VM without the albumen layer had a higher rupture strength on day 1, day 7, and day 42 than the VM with the albumen layer, but days 14 through 35 the VM with the albumen layer was higher.

TABLE IV-4. Yolk rupture strength of refrigerated eggs from 82 wk old hens; Experiment 2[1].

Day	Yolk without albumen layer Rupture strength (g/g) [1]	Yolk with albumen layer Rupture strength (g/g) [2]
1	9.57 ± 0.74^{a}	7.90 ± 1.01^{ab}
7	7.02 ± 0.40^{bcd}	6.78 ± 0.63^{bcd}
14	5.63 ± 0.68^{cd}	7.35 ± 0.98^{bc}
21	$4.84 \pm 0.66^{d,x}$	$7.22 \pm 0.45^{bc,y}$
28	6.15 ± 0.48^{bcd}	7.30 ± 0.54^{bc}
35	5.25 ± 0.68^{cd}	6.54 ± 0.57^{bcd}
42	5.59 ± 0.65^{cd}	4.89 ± 0.62^{d}

[a-d] Means within same column with no common superscripts differ significantly ($p<0.05$).
[x,y] Means within same row with no common superscripts differ significantly ($p<0.05$).
[1]Means within rows and columns for 10 yolks $\pm$ SE.

Discussion

Experiment 1. The evaluations of the SDS-PAGE gels were subjective because of the variability of the amount of protein added to each well and because of the technique. The SDS-PAGE gels in this experiment were used characterize proteins that migrate in a 4 to 20% gradient. By using this gradient range, not all of the proteins in the VM could be characterized. The OD/μg value for the protein GP-II (Kido *et al.,* 1977) decreased throughout storage as expected. Kido and co-workers (1977) saw a decrease in this protein after 25 days in storage, and the breakdown is thought to be related to increasing pH and dissolution of hydrogen sulfide bonds. Another protein, VMO-I (Back, 1984) decreased throughout the storage period which was also expected. Back (1984) saw a degradation of the VMO-I protein after storage in 20°C when using no treatment to prevent loss of CO_2 and water. This leads to changes that weaken the VM. The protein breakdown could not be quantified by using this technique because of the variability in staining times, and because all gels are not completely identical and can contribute to interference when using OD. To be able to quantify the proteins in this study, another technique will such as Western blotting should be used.

In this experiment interior egg quality parameters decreased during the 6 wk storage period. Similar results were found for yolk index (Sharp and Powell, 1930), albumen pH (Burley and Vadehra, 1989), albumen height (Scott and Silversides, 2000), and yolk pH (Healy and Peter, 1925) during refrigerated storage. Albumen pH increased during the storage period due to the loss of water and carbon dioxide, which causes the pH of the albumen to rise (Powrie and Nakai, 1986). Yolk index was developed to measure the

spherical nature of the yolk (Sharp and Powell, 1930; Funk, 1948; Sauter *et al.,* 1951), and this is represented in the yolk index values. The yolk index value increases and yolk pH remains until the values of yolk index drop and yolk pH increase which shows that the VM has reached its maximum point of swelling and it has begun to flatten reflecting in a decreasing yolk index value when the yolk pH remains high.

Aging of the vitelline membrane will cause it to weaken (Fromm, 1964; Fromm and Matrone, 1962; Moran, 1936; Fromm, 1967), which has been shown in this study. Weakening of the VM is caused by an increase in osmotic pressure due to water entering the yolk (Sharp and Powell, 1930), which is evident in this study by the increasing yolk index values. The theory of the breakdown of the VM is that the strength of the VM comes from the mucin substance that surrounds the VM and when this substance deteriorates the VM is also affected by the same factors and therefore starts to deteriorate (Feeney *et al.,* 1956). This may explain why the VM with the albumen layer started out with higher rupture strength value than the VM that had the albumen layer removed and that the rupture strength values of the VM with the albumen layer were higher.

The loss of the adhering albumen layer might not be the reason though that there is a reduced rupture strength. The actual removal of the albumen layer not only physically manipulates the VM but could also produce tearing of the membrane. This would contribute to the reduced rupture strength.

The rupture strength values for the VM decline as does the interior egg qualities for the first few weeks. As the interior egg quality factors continue to decrease, the rupture strength values for the VM with the adhering albumen layers does not change significantly. The protein values also decreased throughout the storage period, but rupture strength for the

VM with the albumen layer did not steadily decrease which would also be expected. This may be due to the quality of the albumen. This experiment used older birds which have albumen that is less viscous (Fetcher *et al.,* 1983), and have lower albumen heights (Lapao *et al.,* 1999) than younger birds. The albumen quality may have been too low to have an impact on the structural integrity of the VM.

Experiment 2. This experiment was also analyzed subjectively due to the same reasons as Experiment 1. The OD/μg value for the protein GP-II (Kido *et al.,* 1977) decreased throughout the storage period as expected. Previous reports show degradation of GP-II after 25 days of storage due to PH increases and hydrogen sulfide (Kido *et al.,* 1977). The GP-I protein also showed a decrease in OD value, but VMO-I and lysozyme did not have any decrease, but this may be attributed to breakdown of other proteins, which have the same molecular weight as the proteins being observed.

The interior egg quality parameters yolk index (Sharp and Powell, 1930), albumen height (Scott and Silversides, 2000) decreased as albumen pH (Burley and Vadehra, 1989) and yolk pH (Healy and Peter, 1925) increased over the storage period. Yolk pH and yolk index did not readily reflect the migration of water into the yolk through the VM. There were no significant differences in the yolk index showing the maximum swelling point of the VM, and the yolk pH reflected this by having a yolk pH that fluctuated throughout storage.

The rupture strength value for the VM with the albumen layer was initially lower than the VM with the yolk alone which is unexpected. The values for the VM with the albumen layer also did not reflect the idea of the albumen layer playing an important structural role. Aging of the vitelline membrane will cause it to weaken (Fromm, 1964;

Fromm and Matrone, 1962; Moran, 1936; Fromm, 1967), which has been shown in this study. Weakening of the VM is caused by an increase in osmotic pressure due to water entering the yolk (Sharp and Powell, 1930), the yolk index values did reflect this although the yolk pH values fluctuated. The theory of the breakdown of the VM is that the strength of the VM comes from the mucin substance that surrounds the VM and when this substance deteriorates the VM is also affected by the same factors and therefore starts to deteriorate (Feeney *et al.,* 1956). By looking at the albumen height and pH it is evident to see that the albumen is deteriorating as the rupture strength values also decrease along with the decrease in the inner layer proteins GP-I and GP-II.

With differences in rupture strength value between the VM with the albumen layer and the VM without the VM layer, the physical removal of the albumen layer could be the cause of differences. Tearing can occur along with the act of rolling the yolk.

Experiments Combined. The age of the birds from Experiment 1 and Experiment 2 are too similar to see noticeable age differences between them. Age does play a role in the outcomes for both experiments because they are 72 wk and 82 wk hens. For both experiments, when preparing samples for SDS-PAGE, several techniques were applied to achieve separation, but separation was only possible when the yolk was rolled on a wet paper towel to remove the albumen layer. This is an important fact because this shows that the albumen layer does have some type of interaction in holding the VM together.

This may be explained by the influenced of factors that cause thinning of the albumen also weaken the VM. The ovomucin-lysozyme is involved in the structure of the albumen and may also play that same role in the outer layer of the VM. As pH increases in

the egg, the protein-protein complex of ovomucin and lysozyme will lose its association, thus losing its functionality.

The SDS-PAGE technique used in this study generally characterized the protein bands, but to quantify and better characterize the proteins other techniques will need to be employed. Using different stains will also help to see some band that did not appear with the stain used in these studies.

CHAPTER V

CONCLUSION

The vitelline membrane prevents the yolk from contaminating the albumen in egg breaking plants and it also aids in keeping microorganisms, such as *Salmonella* Enteritidis, from entering the nutrient rich yolk. Molting, age of the hen, and storage of the eggs play an important role in influencing the interior egg quality and rupture strength of the VM. These studies used eggs from pre-molt and molted hens to demonstrate these factors; the age differences in pre-molted birds reflected the differences.

Results in the studies show that the adhering albumen layer may play a role in giving strength to the VM. When preparing samples for SDS-PAGE, several techniques were applied to achieve separation, but separation was only possible when the yolk was rolled on a wet paper towel to remove the albumen layer. This is important because it shows that the albumen layer does have an important role in holding the VM together.

The SDS-PAGE technique used in these experiments was a useful tool to generally characterize the protein bands, but to quantify and better characterize the proteins other techniques will need to be employed. Using different stains will also help to see some band that did not appear with the stain used in these studies.

The vitelline membrane is influenced by factors that cause thinning of the albumen. The ovomucin-lysozyme is involved in the structure of the albumen and may also play that same role in the outer layer of the VM. As pH increases in the egg, the protein-protein complex of ovomucin and lysozyme will lose its association, thus losing its functionality.

Interior quality declines due to a combination of water loss and carbon dioxide, which allows the albumen pH to rise. When losing the bicarbonate buffer, pH become more

alkaline and affects both protein functionality and protein interactions. As pH of the albumen and yolk increase, the albumen becomes less rigid and the spherical nature of the yolk starts to deform and flatten.

The proteins in the vitelline membrane play an important role in the function of the membrane. Deterioration has been seen in the proteins characterized by this study. There are other proteins involved in the membrane that were not characterized in this study and so they may also play an influential role in the structure of the VM.

Rupture strength of the VM is reduced over time. Rupture strength is also reduced when the adhering albumen layer is present. The measurement of the strength of the VM is important because it demonstrates the extent of force a yolk can with stand as the egg ages. This is important when considering the age of the egg when brought to egg breaking facilities so that it may be known when the yolk is more vulnerable to rupture and contaminate albumen. Other storage condition factors that influence interior egg quality such as relative humidity, temperature, and carton type may also be important as they relate to water and CO_2 loss.

The loss of the adhering albumen layer might not be the reason though that there is a reduced rupture strength. The actual removal of the albumen layer not only physically manipulates the VM but could also produce tearing of the membrane. This would contribute to the reduced rupture strength.

Further studies that follow a flock from the first and second production periods would be useful. The age differences could better be compared and the effect of interior egg quality and VM strength might be better understood.

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APPENDIX

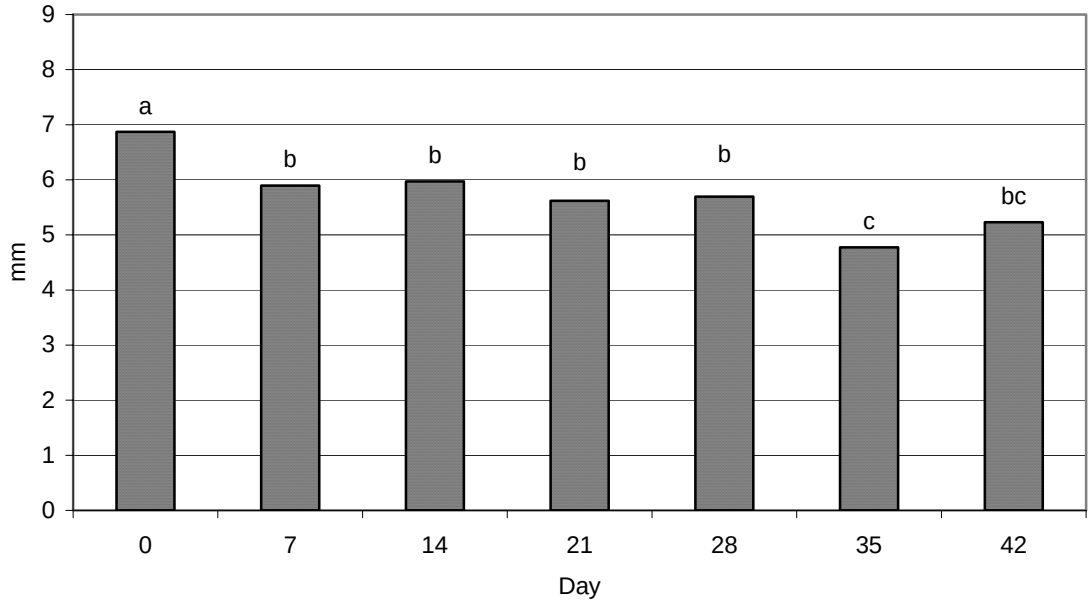

Figure A-1: Measure of albumen height over refrigerated storage from eggs from 72 wk pre-molted hens; Experiment 1. Bars represent 20 eggs sampled from day 0 through day 42. Significance value of $p < 0.05$.

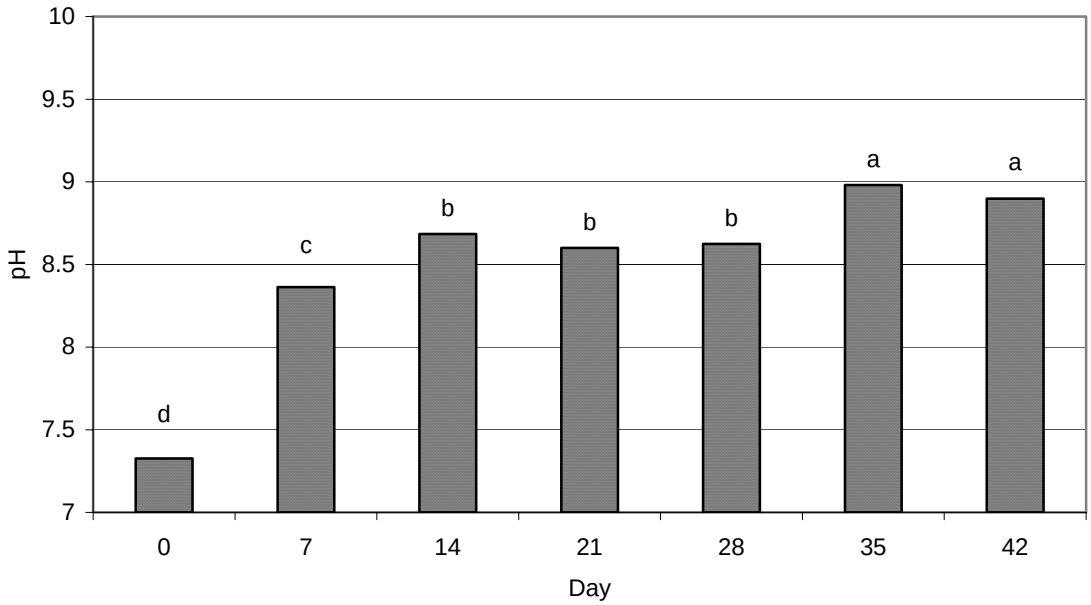

Figure A-2: Measure of albumen pH over refrigerated storage from eggs from 72 wk pre-molted hens; Experiment 1. Bars represent 20 eggs sampled from day 0 through day 42. Significance value of $p < 0.05$.

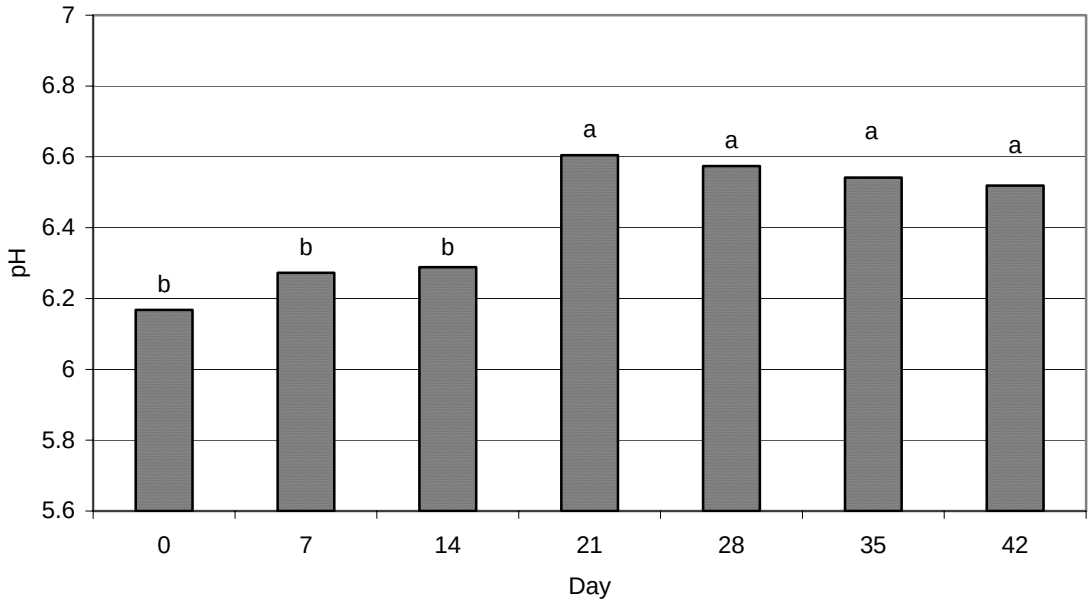

Figure A-3: Measure of yolk pH over refrigerated storage from eggs from 72 wk pre-molted hens; Experiment 1. Bars represent 20 eggs sampled from day 0 through day 42. Significance value of $p < 0.05$.

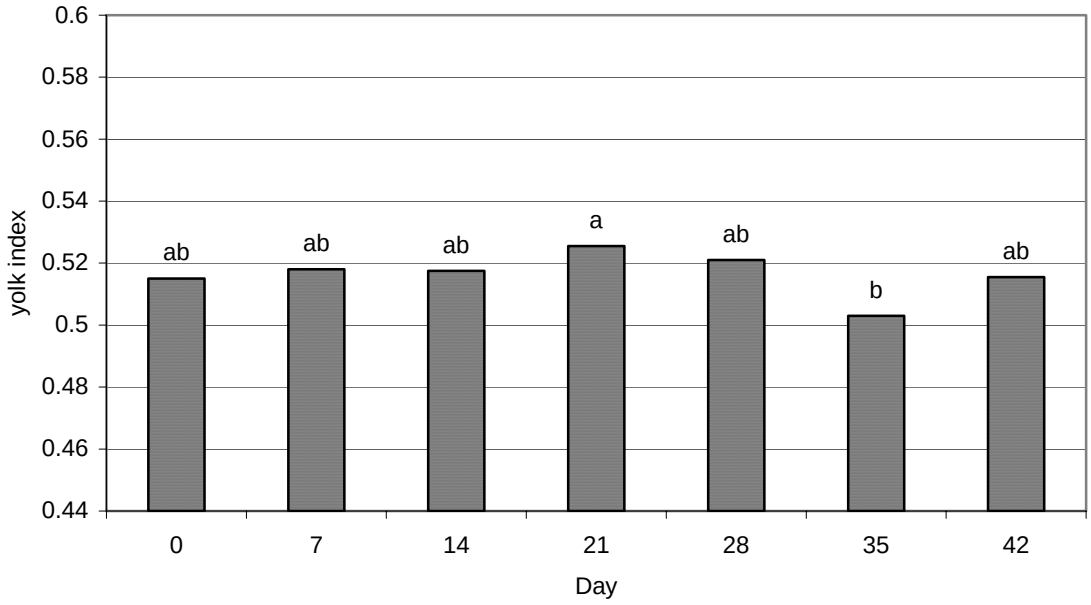

Figure A-4: Measure of yolk index over refrigerated storage from eggs from 72 wk pre-molted hens; Experiment 1. Bars represent 20 eggs sampled from day 0 through day 42. Significance value of $p < 0.05$.

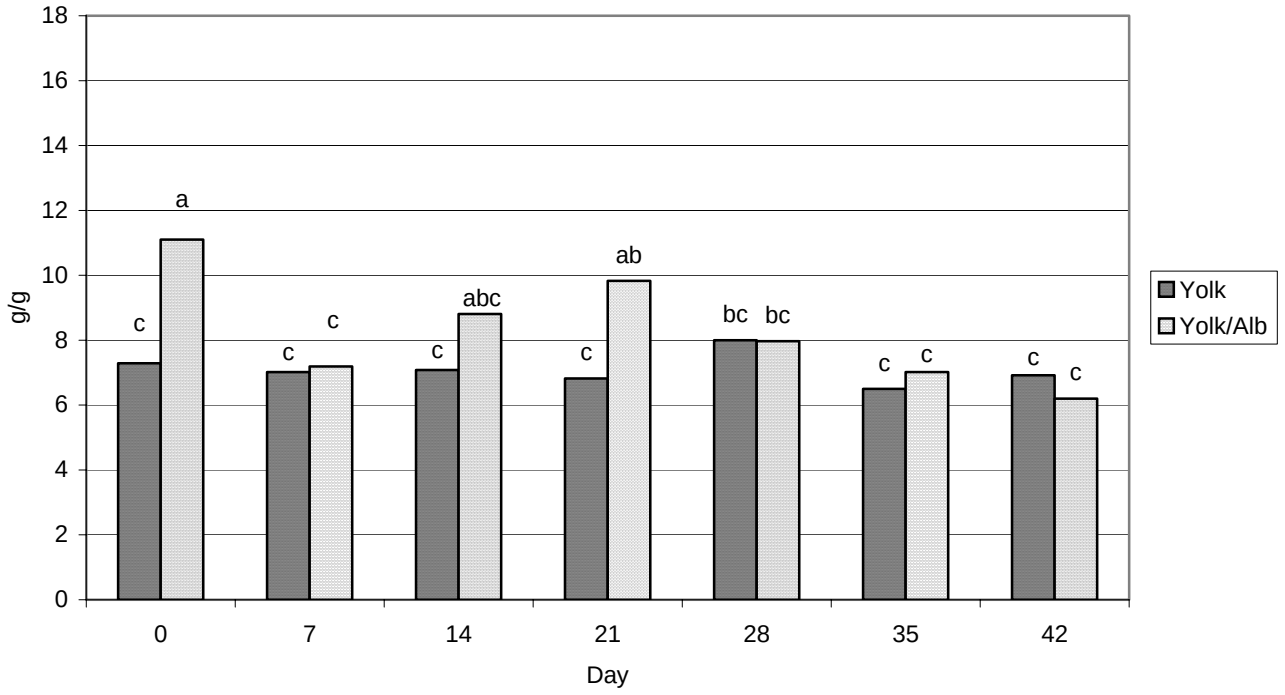

Figure A-5: Measure of vitelline membrane rupture strength over refrigerated storage from eggs from 72 wk pre-molted hens; Experiment 1. The dark bars represent ten yolks with the adhering albumen layer removed, and the light bars represent yolks with the adhering albumen layer intact. Yolks were sampled from day 0 through day 42. Significance value of $p < 0.05$.

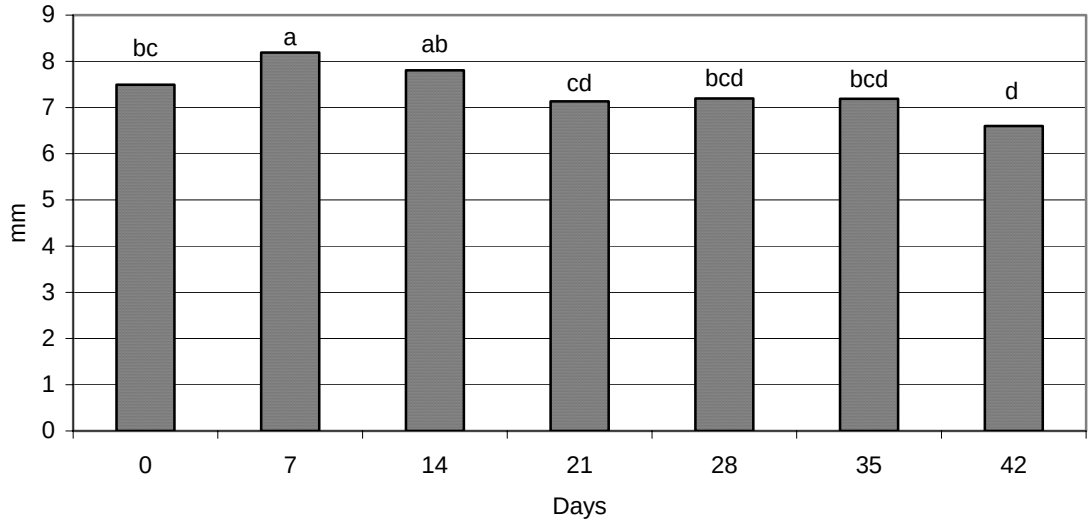

Figure A-6: Measure of albumen height over refrigerated storage from eggs from 26 wk pre-molted hens; Experiment 2. Bars represent 20 eggs sampled from day 0 through day 42. Significance value of $p < 0.05$.

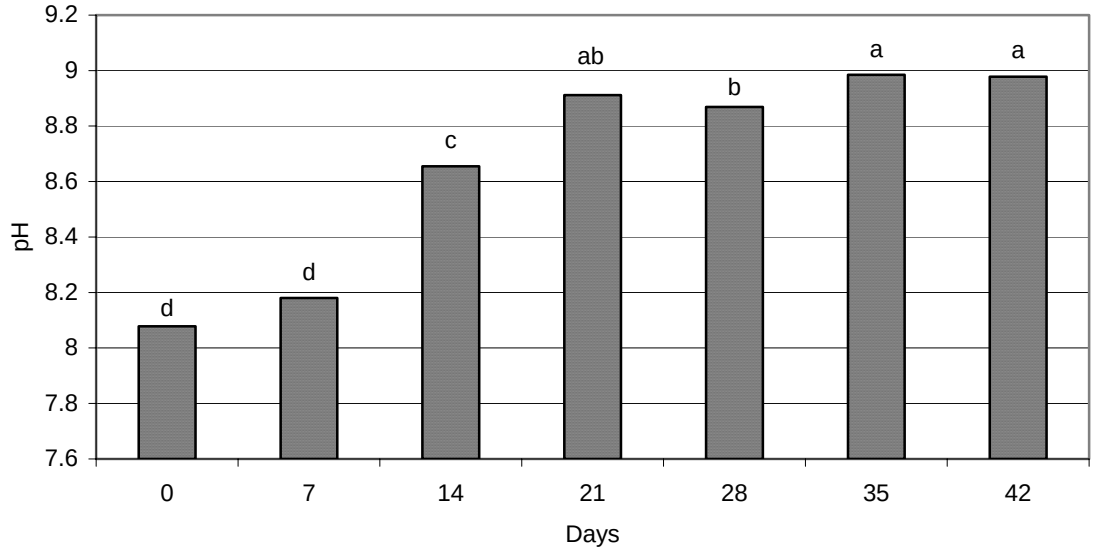

Figure A-7: Measure of albumen pH over refrigerated storage from eggs from 26 wk pre-molted hens; Experiment 2. Bars represent 20 eggs sampled from day 0 through day 42. Significance value of $p < 0.05$.

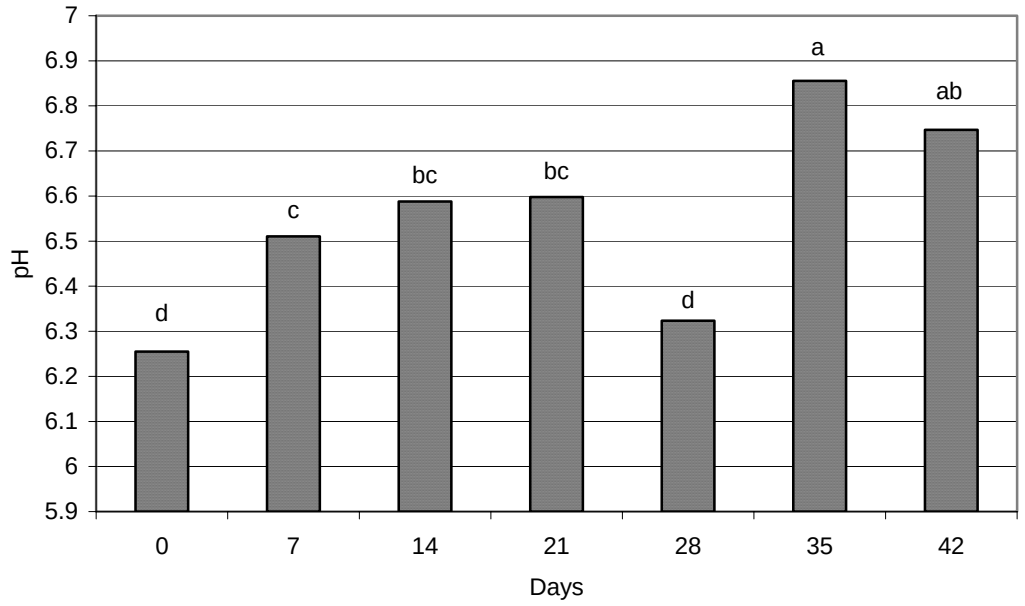

Figure A-8: Measure of yolk pH over refrigerated storage from eggs from 26 wk pre-molted hens; Experiment 2. Bars represent 20 eggs sampled from day 0 through day 42. Significance value of $p < 0.05$.

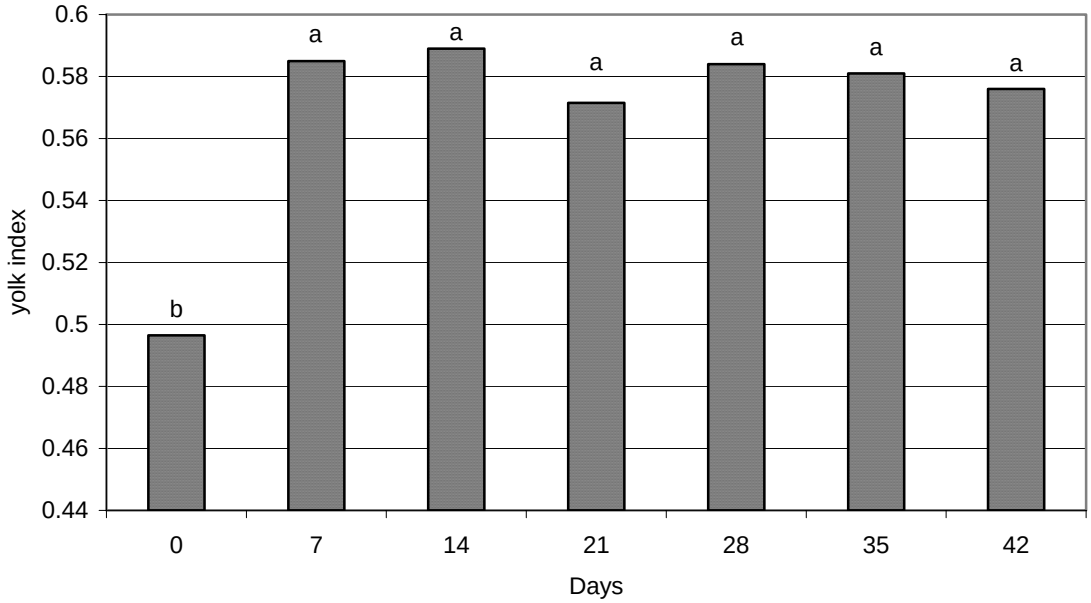

Figure A-9: Measure of yolk index over refrigerated storage from eggs from 26 wk pre-molted hens; Experiment 2. Bars represent 20 eggs sampled from day 0 through day 42. Significance value of $p < 0.05$.

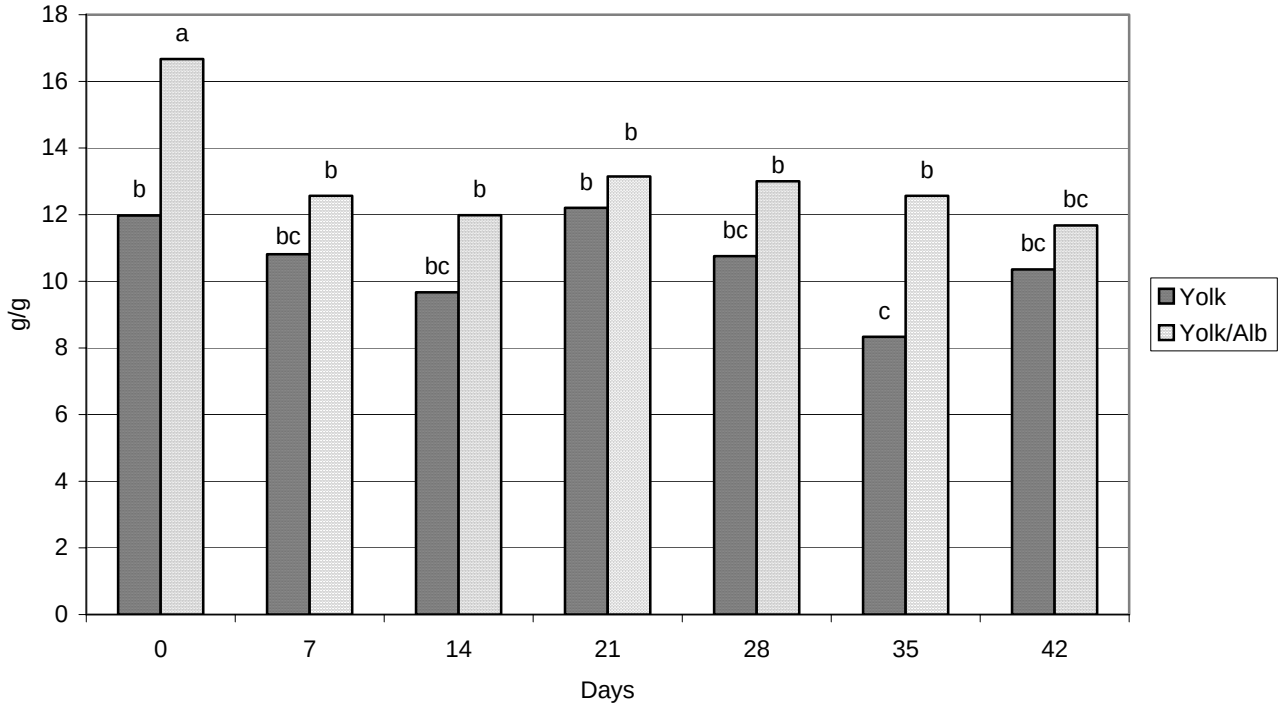

Figure A-10: Measure of vitelline membrane rupture strength over refrigerated storage from eggs from 26 wk pre-molted hens; Experiment 2. The dark bars represent ten yolks with the adhering albumen layer removed, and the light bars represent yolks with the adhering albumen layer intact. Yolks were sampled from day 0 through day 42. Significance value of $p < 0.05$.

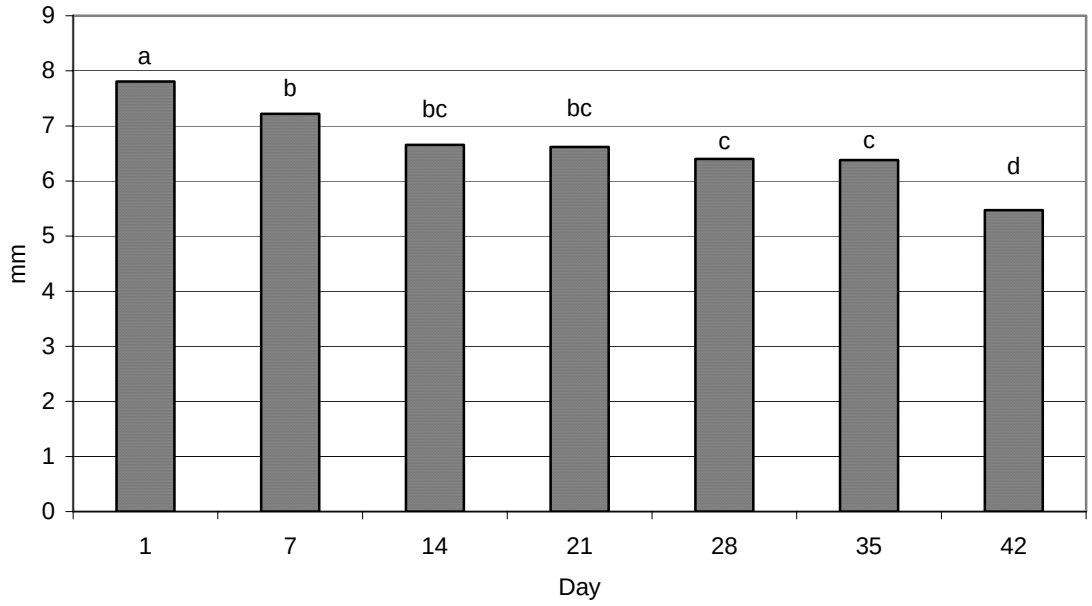

Figure A-11: Measure of albumen height over refrigerated storage from eggs from 74 wk molted hens; Experiment 1. Bars represent 20 eggs sampled from day 1 through day 42. Significance value of $p < 0.05$.

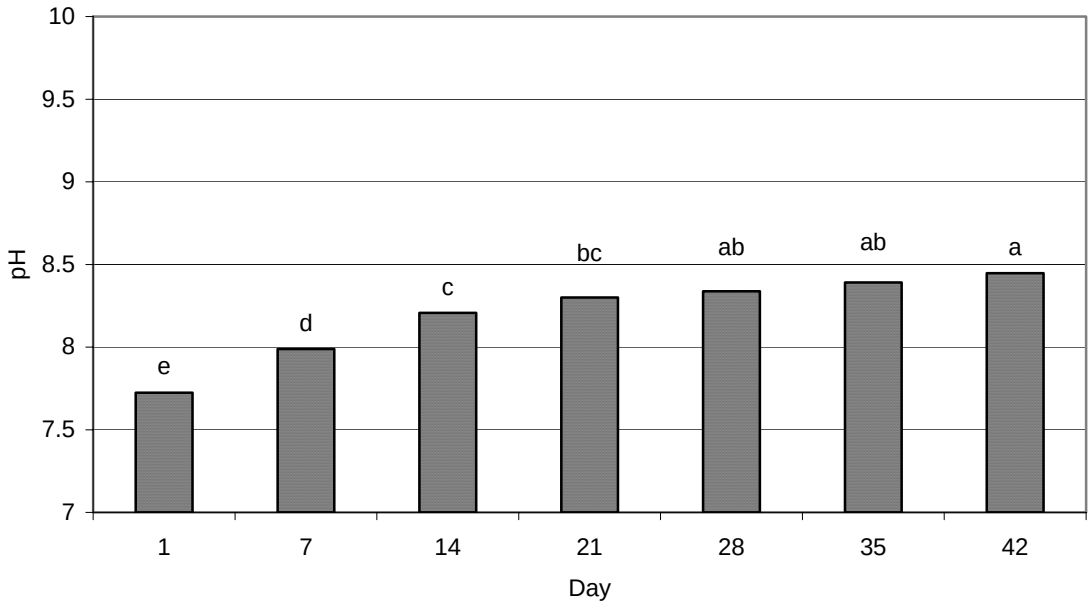

Figure A-12: Measure of albumen pH over refrigerated storage from eggs from 74 wk molted hens; Experiment 1. Bars represent 20 eggs sampled from day 1 through day 42. Significance value of $p < 0.05$.

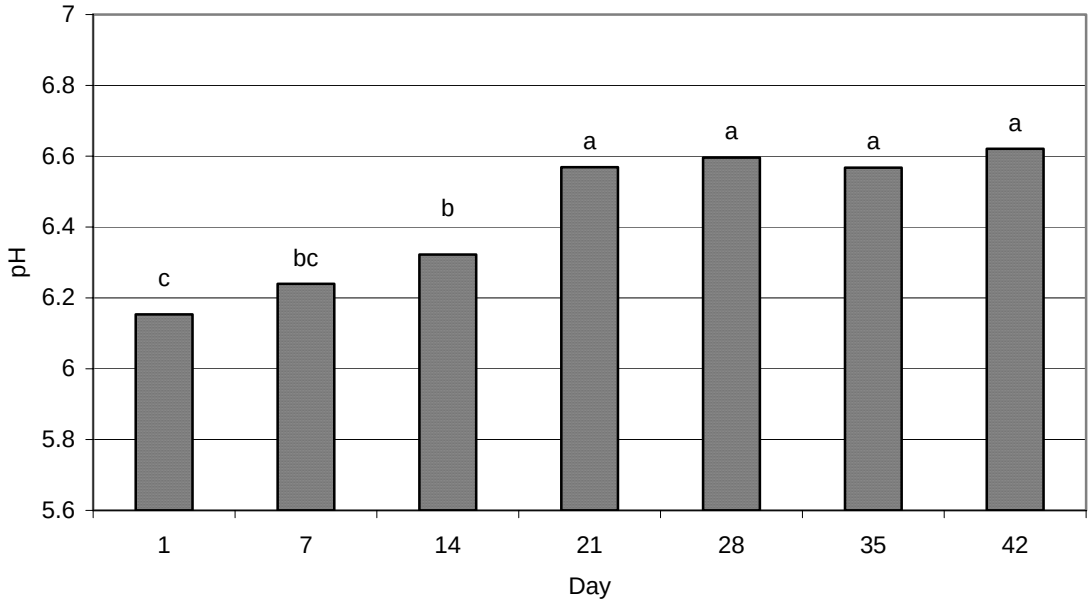

Figure A-13: Measure of yolk pH over refrigerated storage from eggs from 74 wk molted hens; Experiment 1. Bars represent 20 eggs sampled from day 1 through day 42. Significance value of $p < 0.05$.

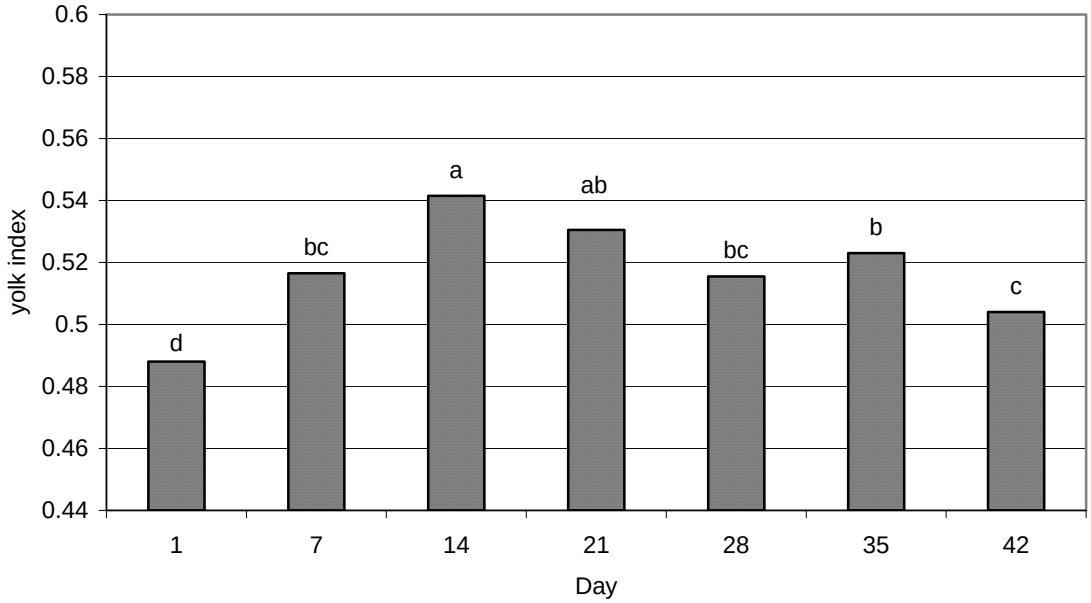

Figure A-14: Measure of yolk index over refrigerated storage from eggs from 74 wk molted hens; Experiment 1. Bars represent 20 eggs sampled from day 1 through day 42. Significance value of $p < 0.05$.

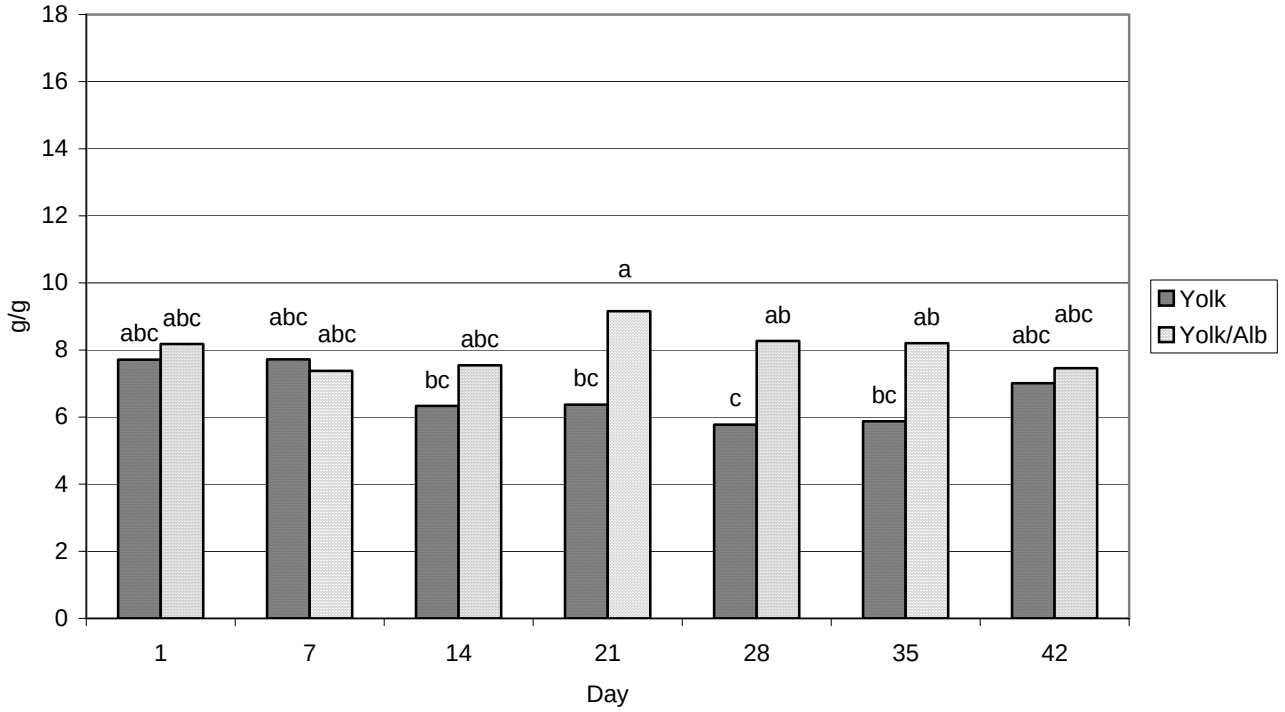

Figure A-15: Measure of vitelline membrane rupture strength over refrigerated storage from eggs from 74 wk molted hens; Experiment 1. The dark bars represent ten yolks with the adhering albumen layer removed, and the light bars represent yolks with the adhering albumen layer intact. Yolks were sampled from day 1 through day 42. Significance value of $p < 0.05$.

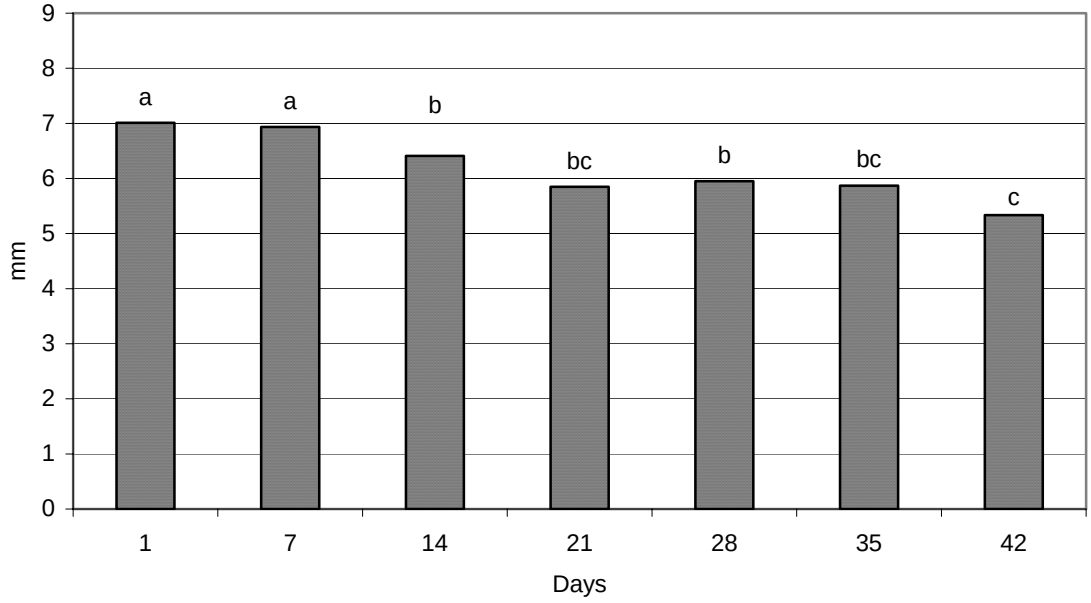

Figure A-16: Measure of albumen height over refrigerated storage from eggs from 82 wk molted hens; Experiment 2. Bars represent 20 eggs sampled from day 1 through day 42. Significance value of $p < 0.05$.

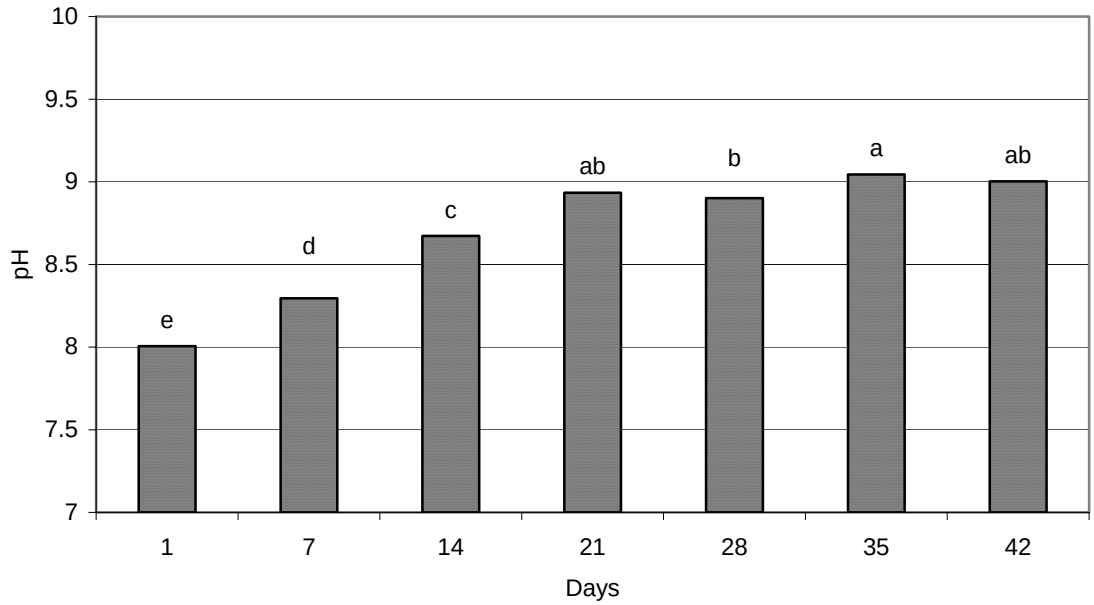

Figure A-17: Measure of albumen pH over refrigerated storage from eggs from 82 wk molted hens; Experiment 2. Bars represent 20 eggs sampled from day 1 through day 42. Significance value of $p < 0.05$.

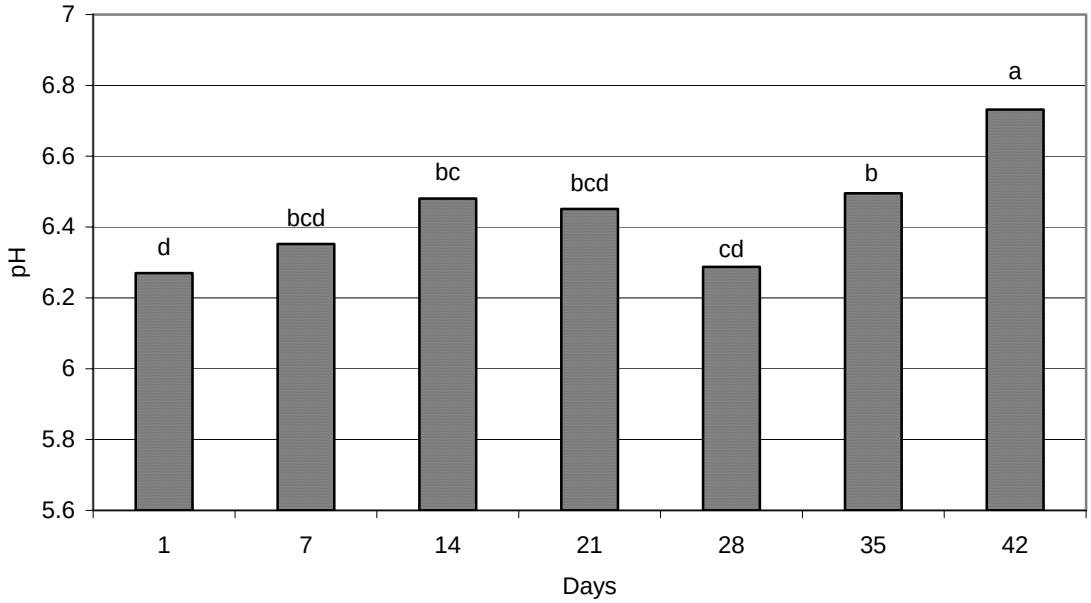

Figure A-18: Measure of yolk pH over refrigerated storage from eggs from 82 wk molted hens; Experiment 2. Bars represent 20 eggs sampled from day 1 through day 42. Significance value of $p < 0.05$.

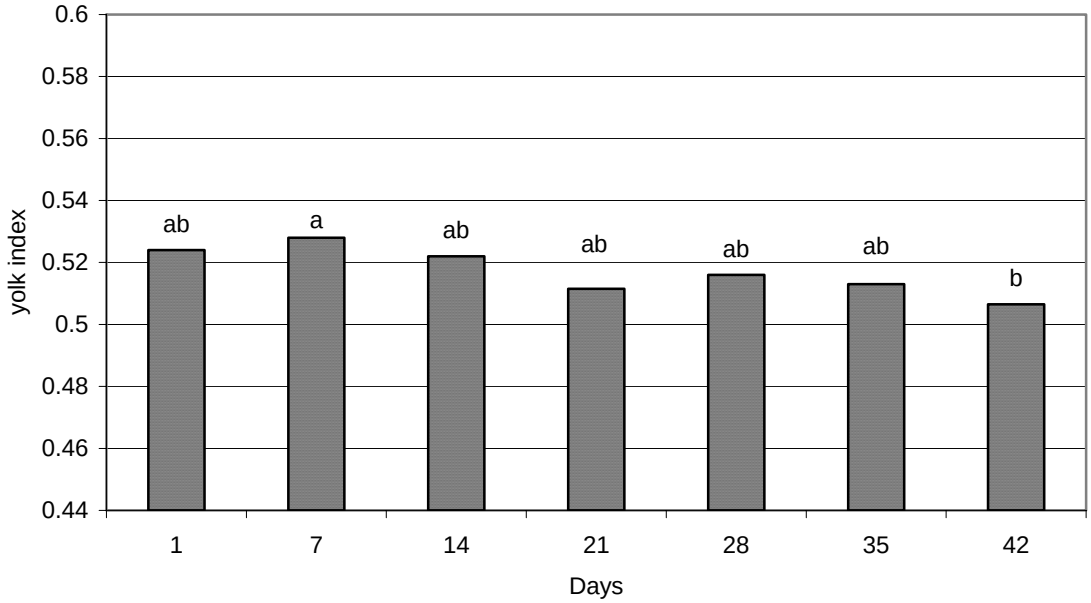

Figure A-19: Measure of yolk index over refrigerated storage from eggs from 82 wk molted hens; Experiment 2. Bars represent 20 eggs sampled from day 1 through day 42. Significance value of $p < 0.05$.

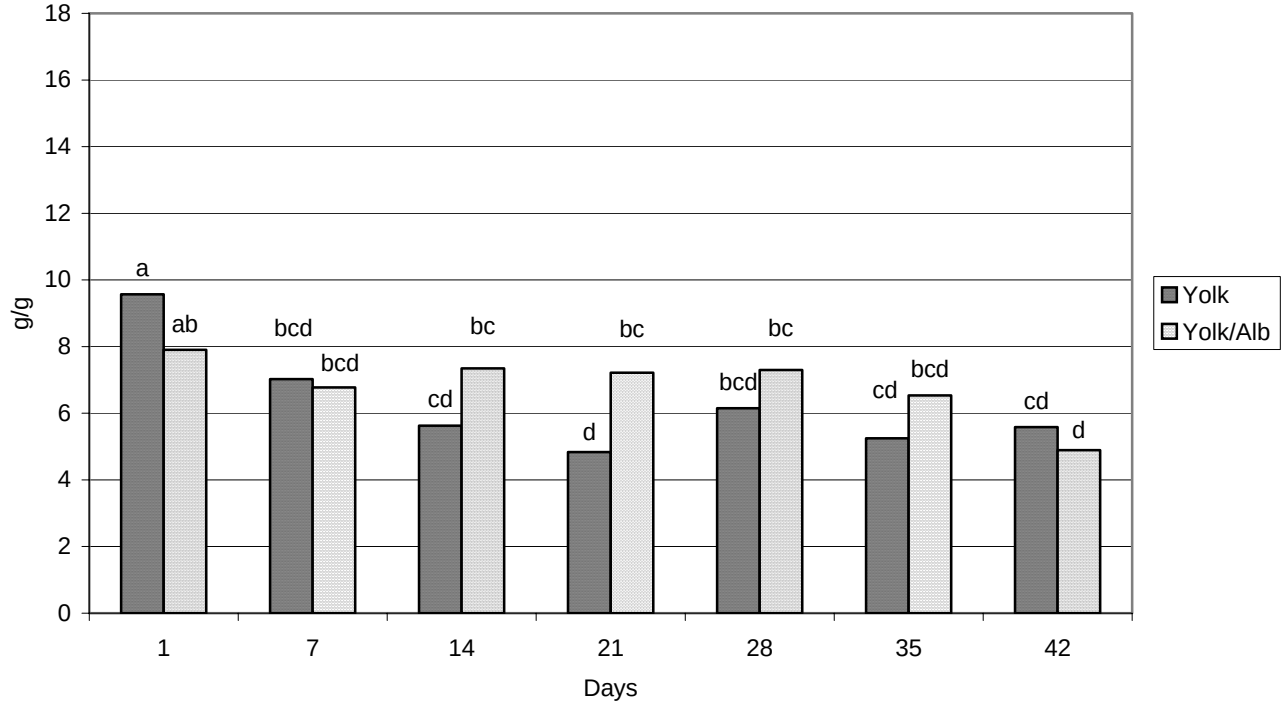

Figure A-20: Measure of vitelline membrane rupture strength over refrigerated storage from eggs from 82 wk molted hens; Experiment 2. The dark bars represent ten yolks with the adhering albumen layer removed, and the light bars represent yolks with the adhering albumen layer intact. Yolks were sampled from day 1 through day 42. Significance value of $p < 0.05$.

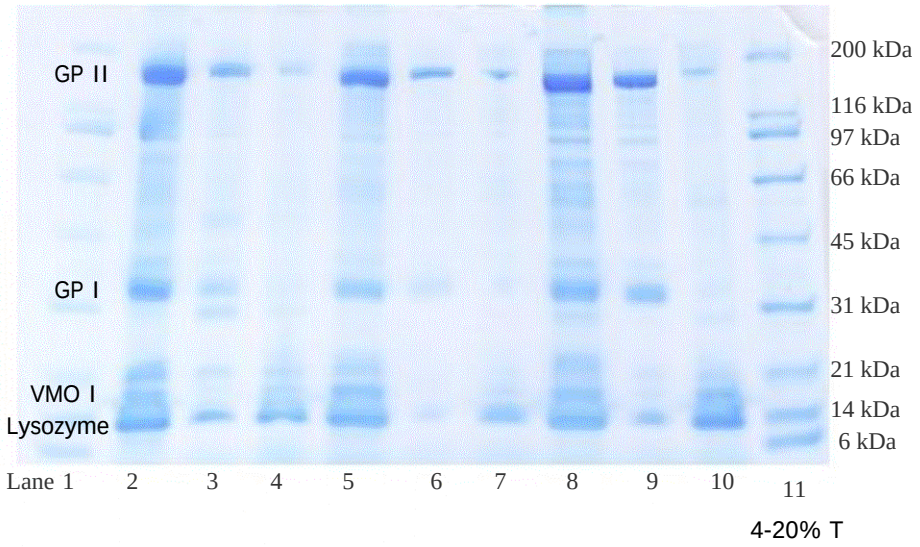

Figure A-21. Day 0 gel of vitelline membrane proteins from 72 wk pre-molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

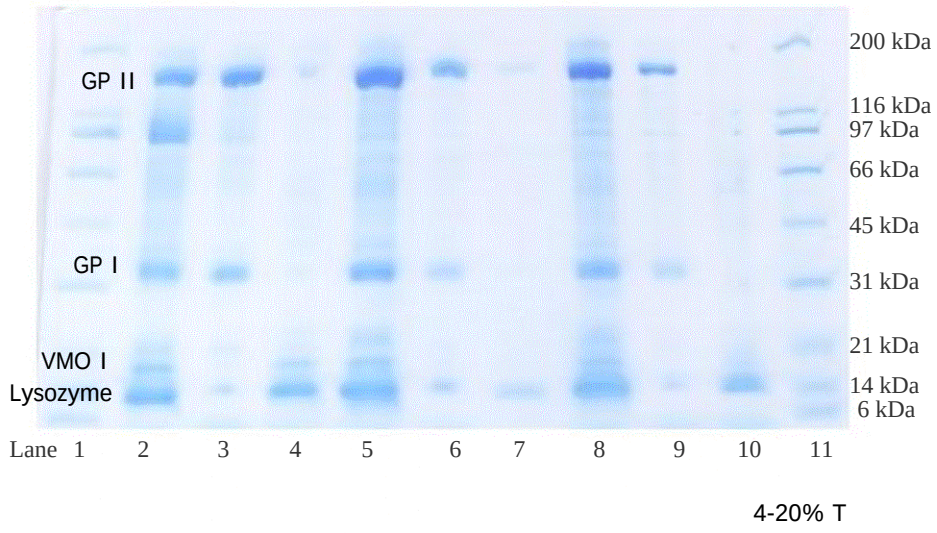

Figure A-22. Day 7 gel of vitelline membrane proteins from 72 wk pre-molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

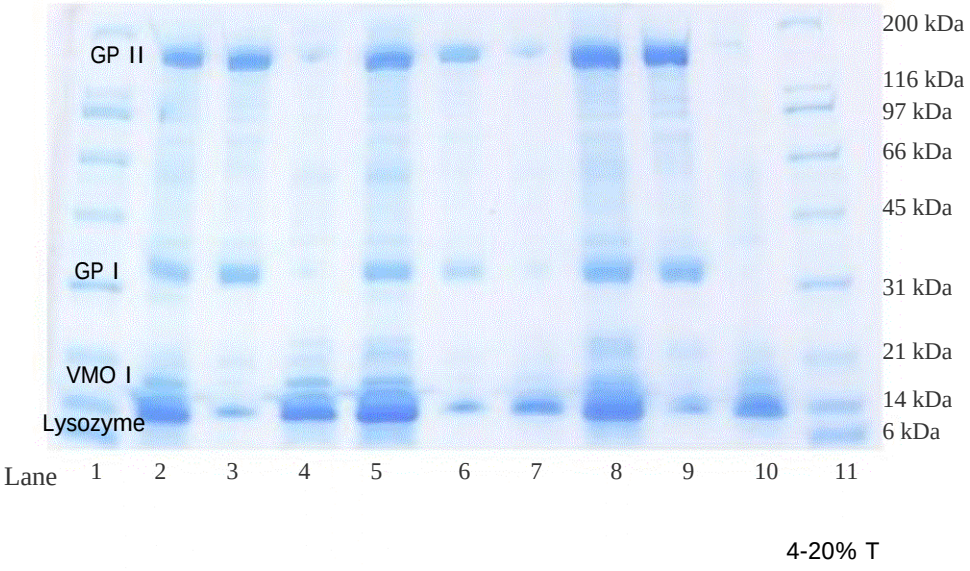

Figure A-23. Day 14 gel of vitelline membrane proteins from 72 wk pre-molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

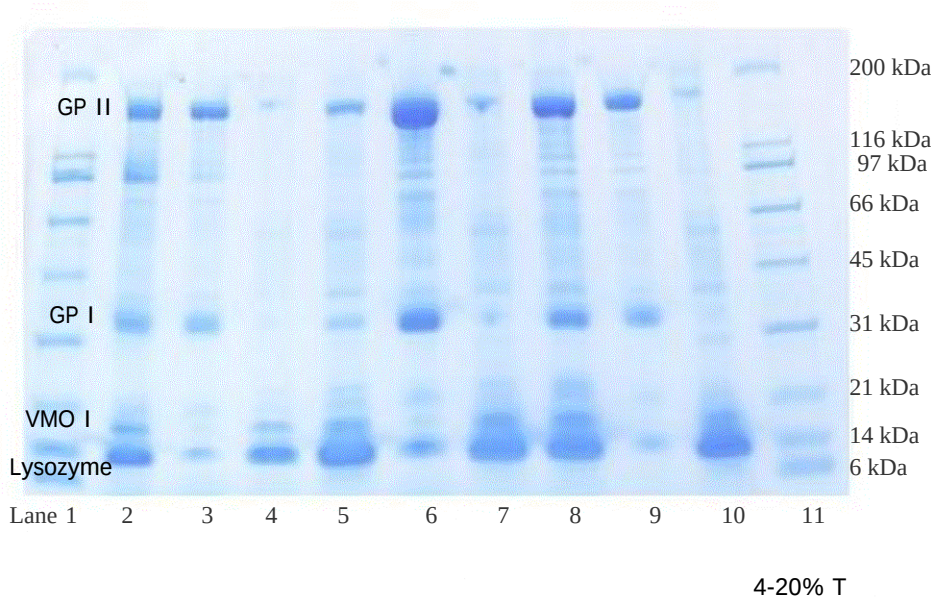

Figure A-24. Day 21 gel of vitelline membrane proteins from 72 wk pre-molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

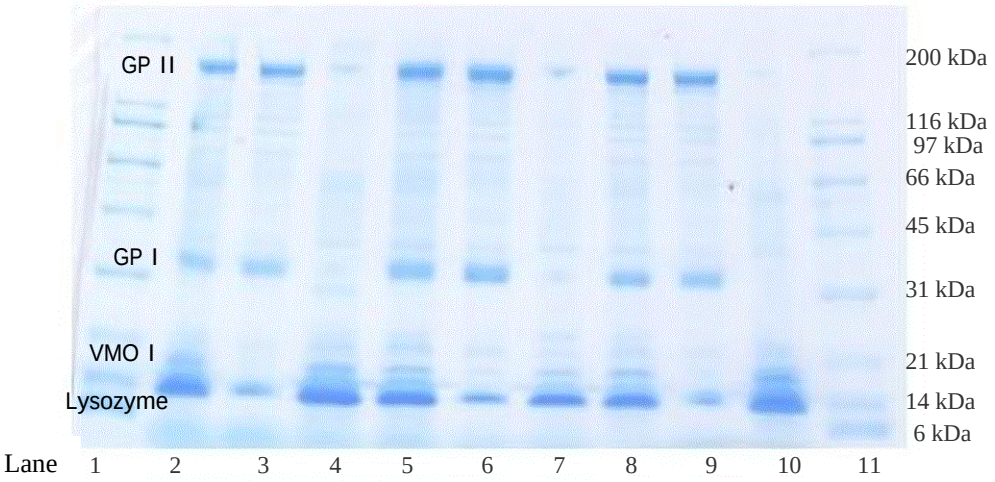

Figure A-25. Day 28 gel of vitelline membrane proteins from 72 wk pre-molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

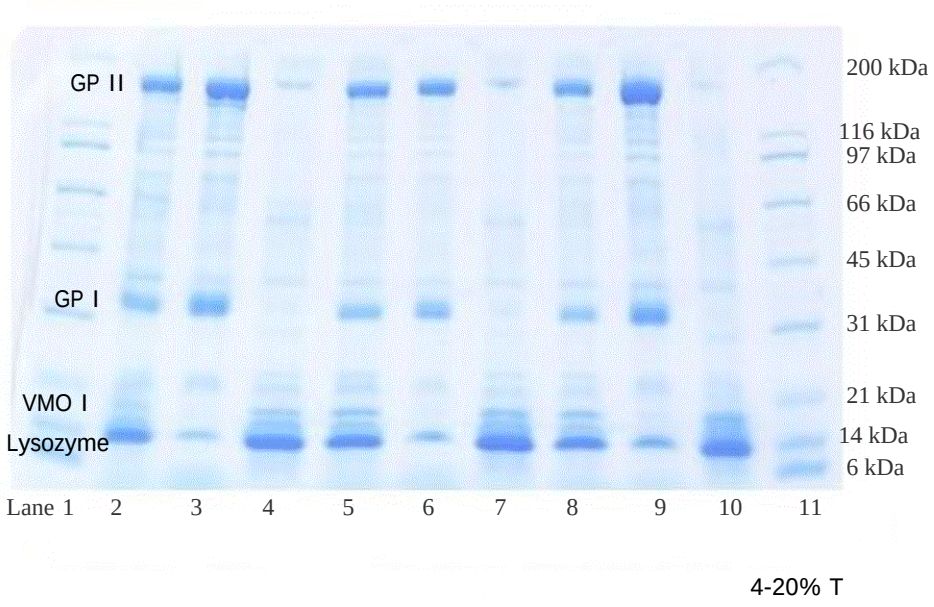

Figure A-26. Day 35 gel of vitelline membrane proteins from 72 wk pre-molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

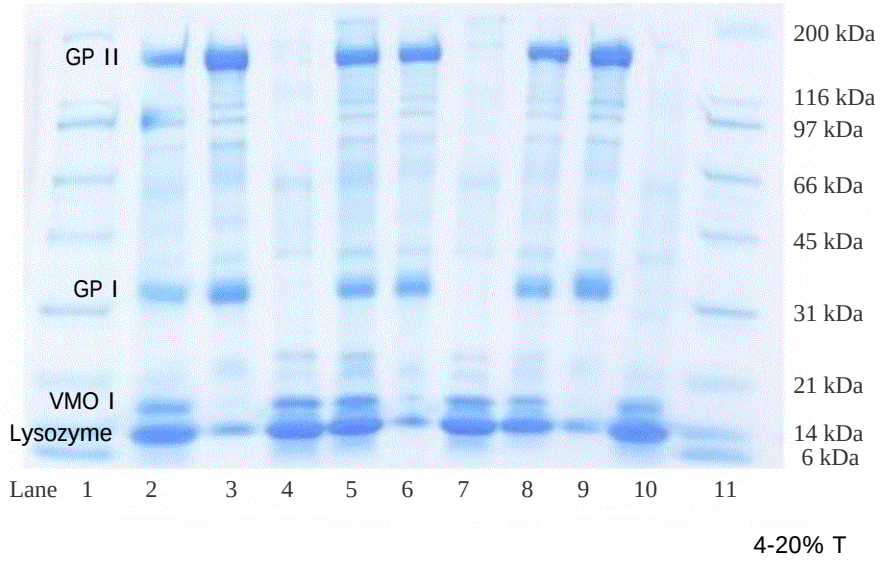

Figure A-27. Day 42 gel of vitelline membrane proteins from 72 wk pre-molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

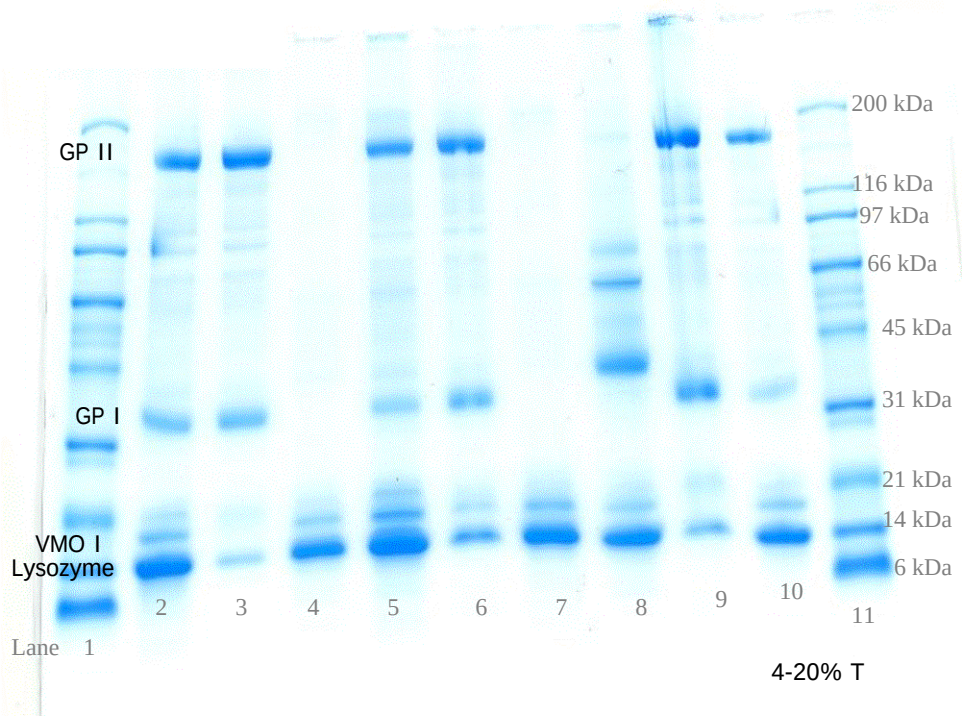

Figure A-28. Day 0 gel of vitelline membrane proteins from 26 wk pre-molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

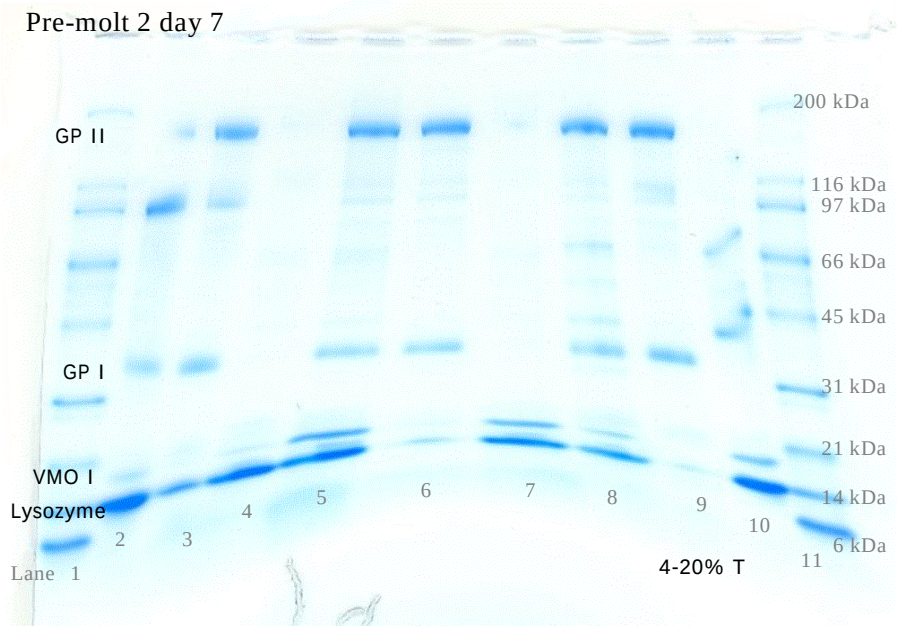

Figure A-29. Day 7 gel of vitelline membrane proteins from 26 wk pre-molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

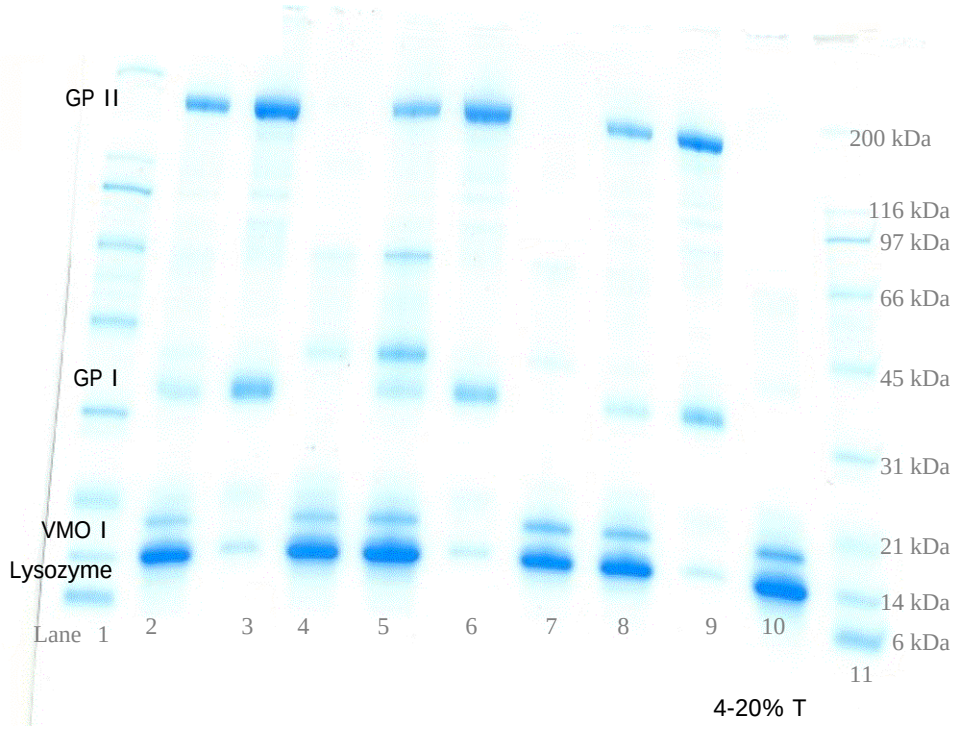

Figure A-30. Day 14 gel of vitelline membrane proteins from 26 wk pre-molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

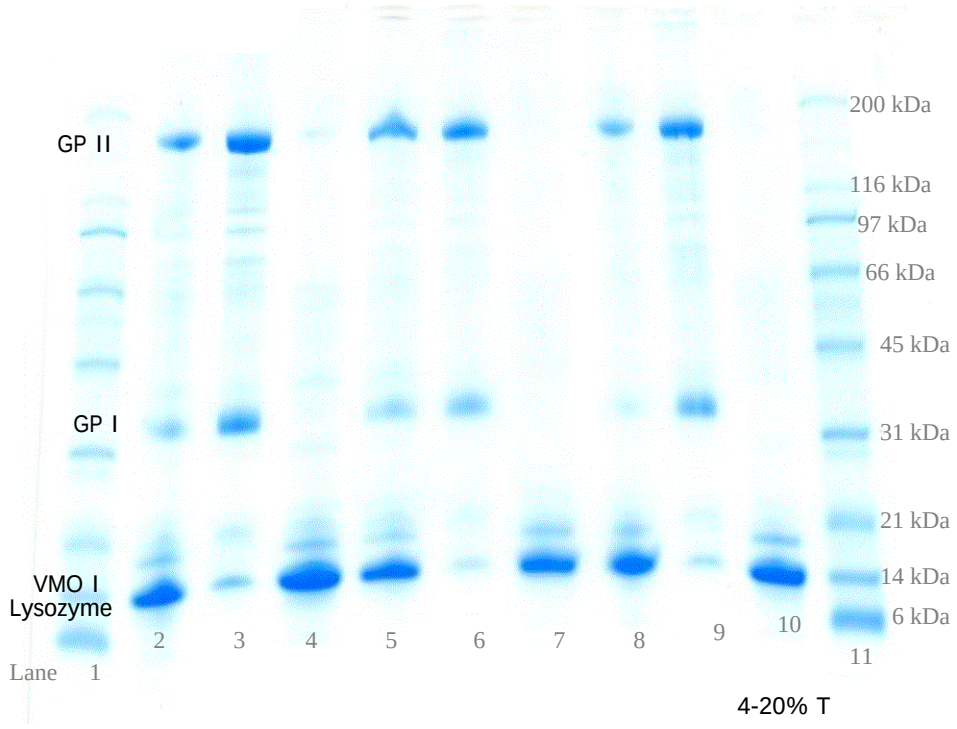

Figure A-31. Day 21 gel of vitelline membrane proteins from 26 wk pre-molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

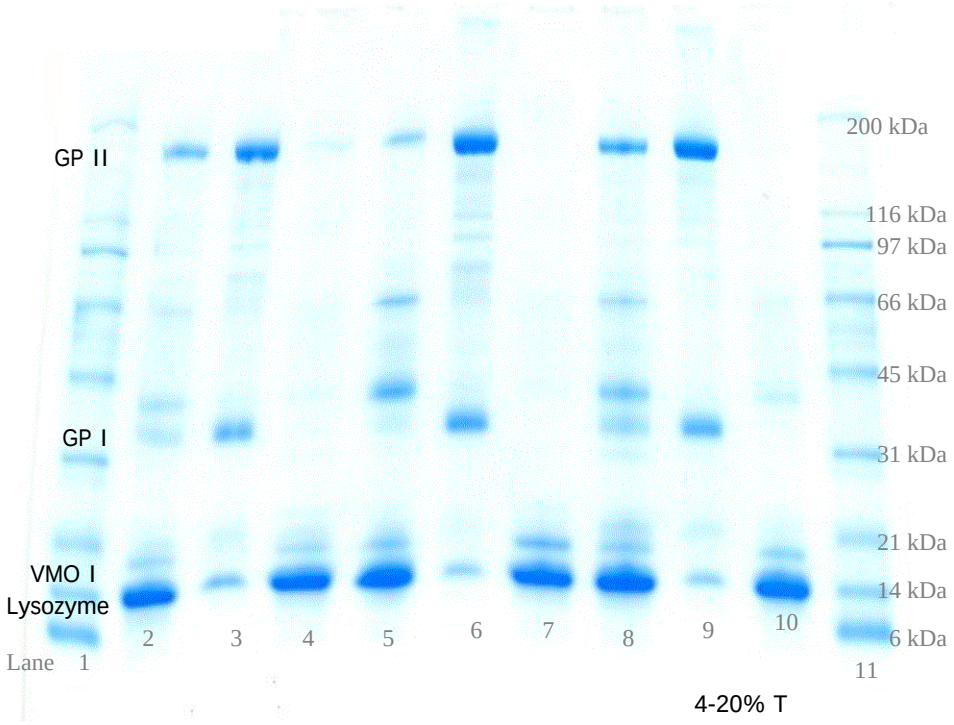

Figure A-32. Day 28 gel of vitelline membrane proteins from 26 wk pre-molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

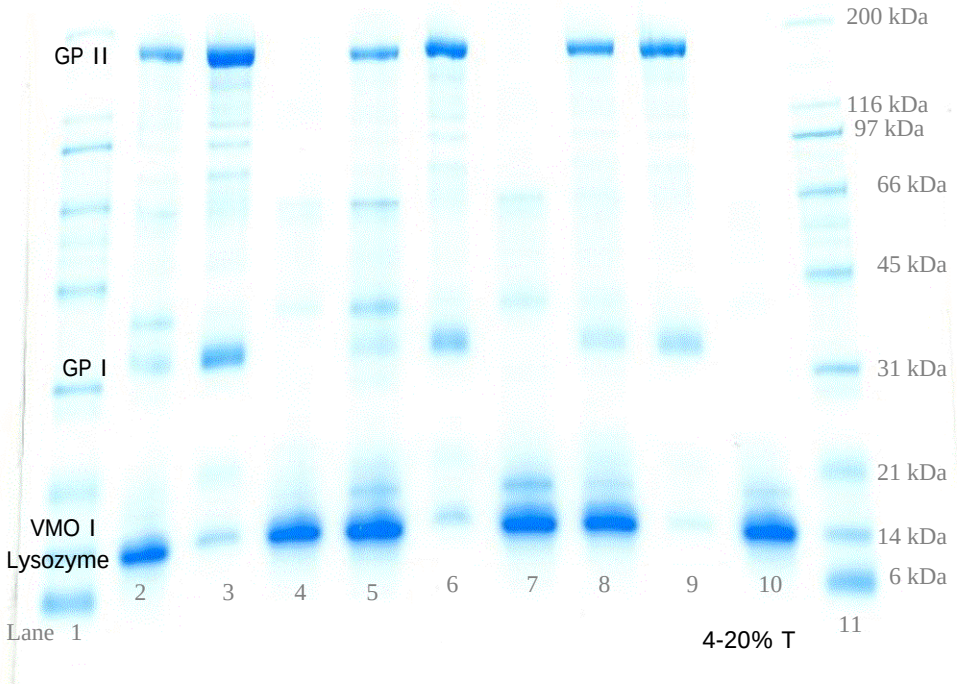

Figure A-33. Day 35 gel of vitelline membrane proteins from 26 wk pre-molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

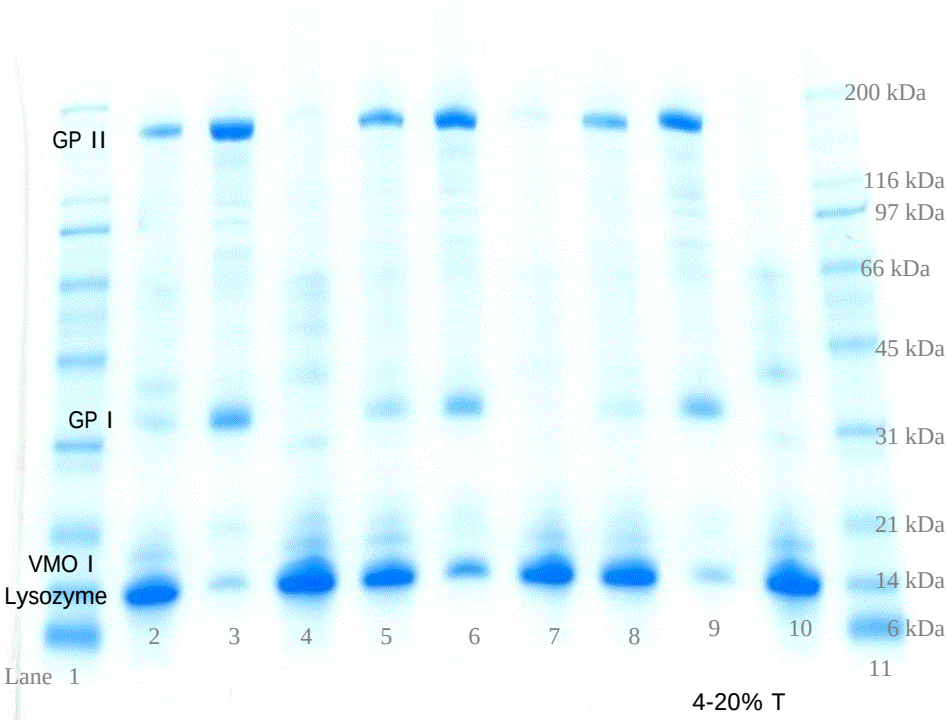

Figure A-34. Day 42 gel of vitelline membrane proteins from 26 wk pre-molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

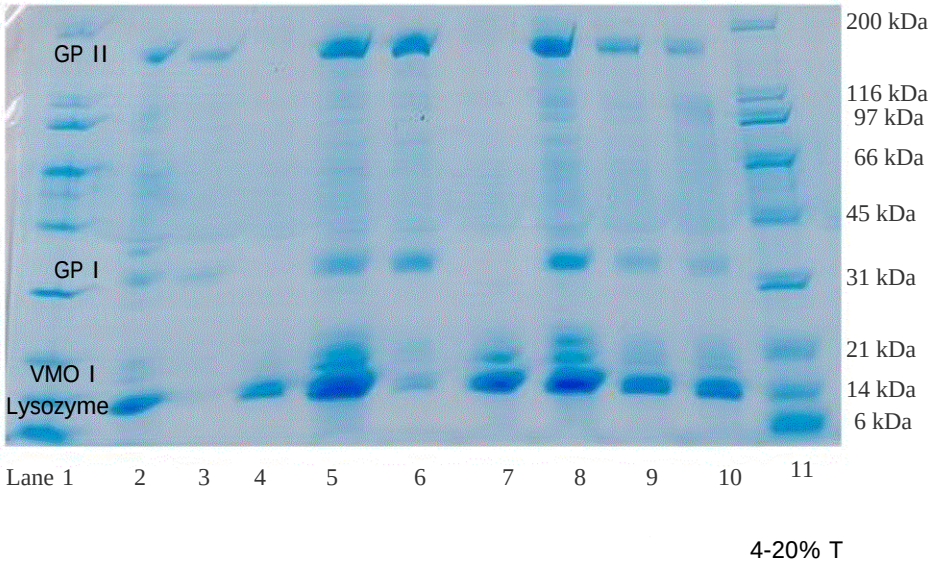

Figure A-35. Day 1 gel of vitelline membrane proteins from 74 wk molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

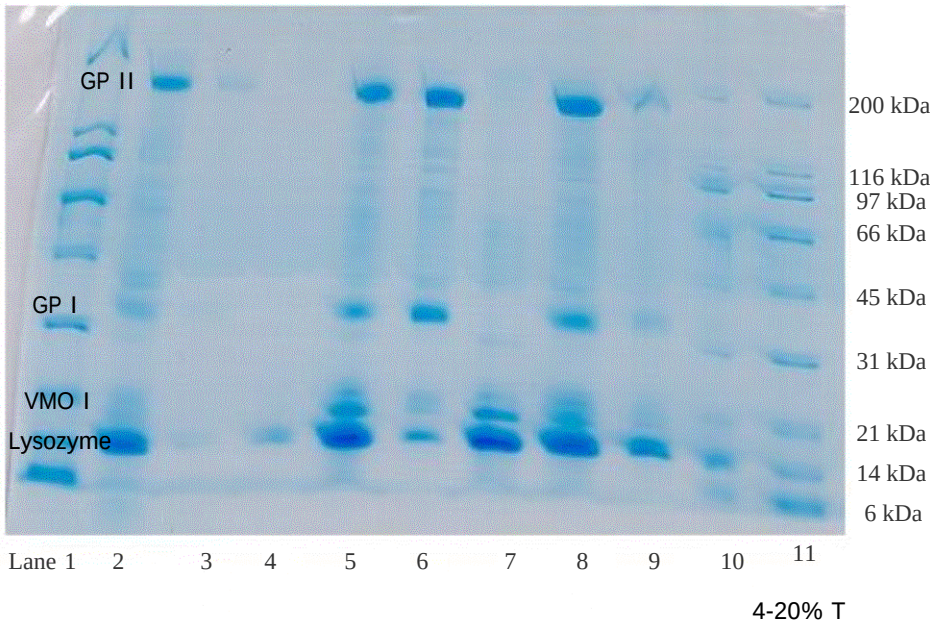

Figure A-36. Day 7 gel of vitelline membrane proteins from 74 wk molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

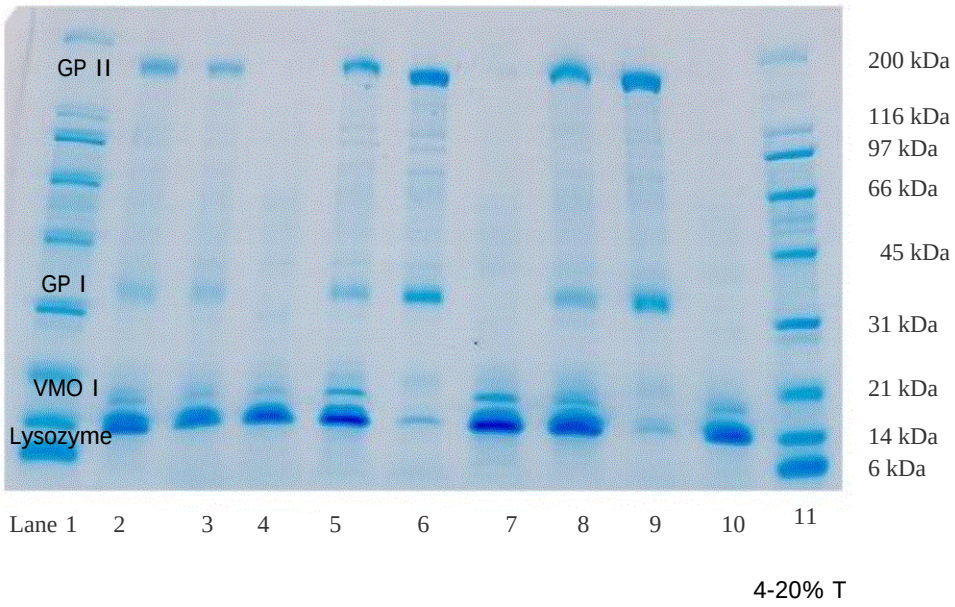

Figure A-37. Day 14 gel of vitelline membrane proteins from 74 wk molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

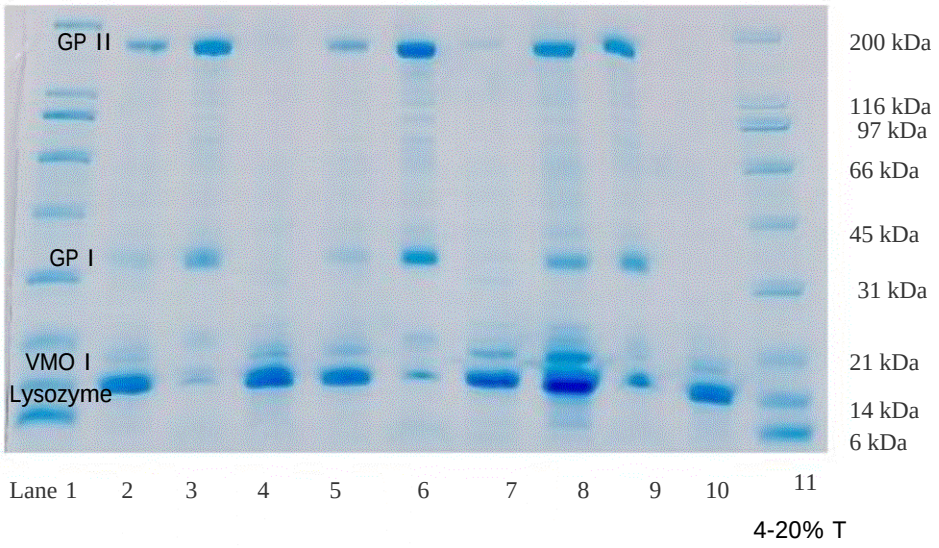

Figure A-38. Day 21 gel of vitelline membrane proteins from 74 molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

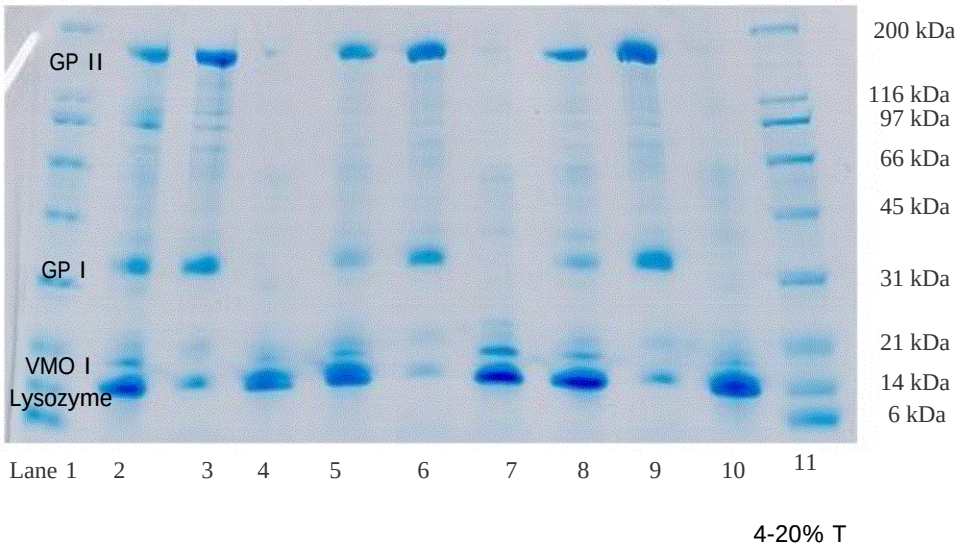

Figure A-39. Day 28 gel of vitelline membrane proteins from 74 wk molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

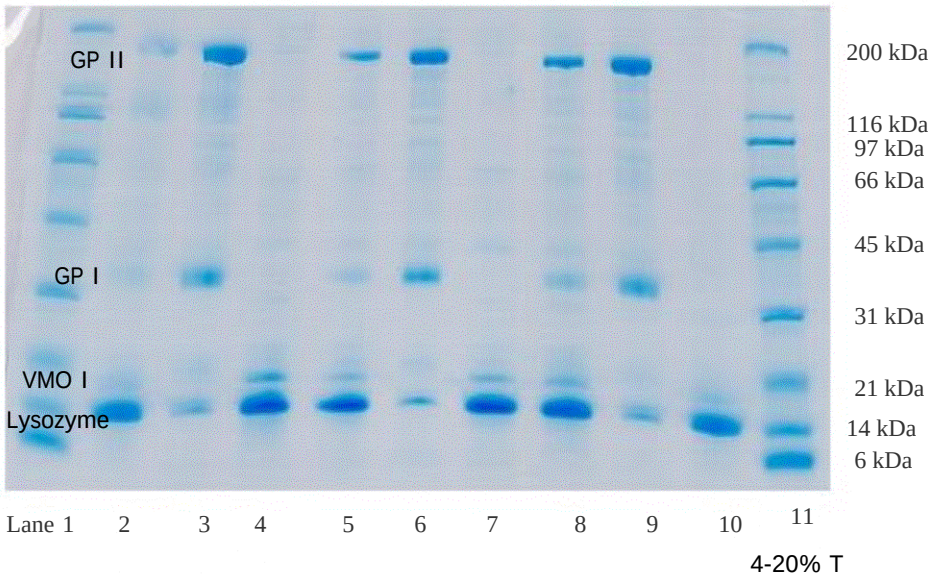

Figure A-40. Day 35 gel of vitelline membrane proteins from 74 wk molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

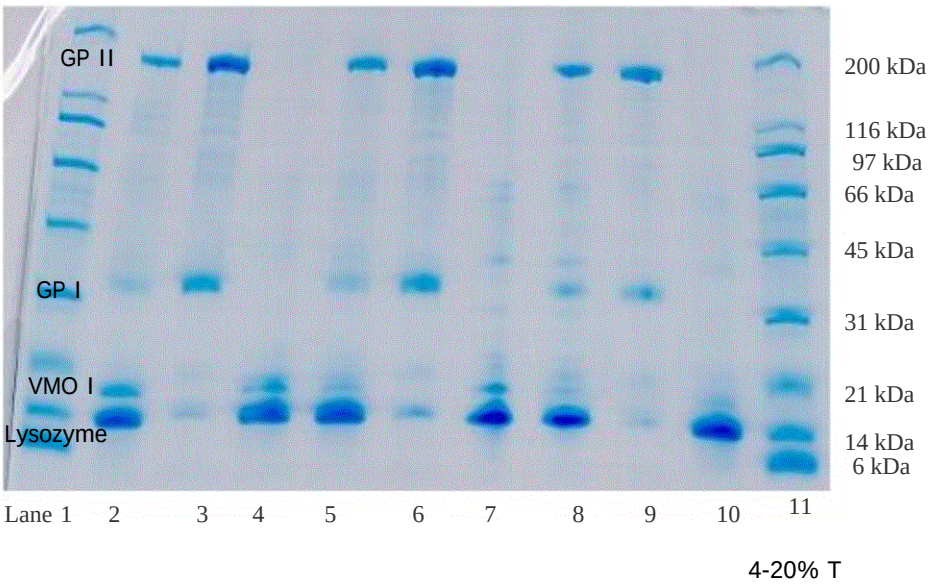

Figure A-41. Day 42 gel of vitelline membrane proteins from 74 wk molted hens egg; Experiment 1. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

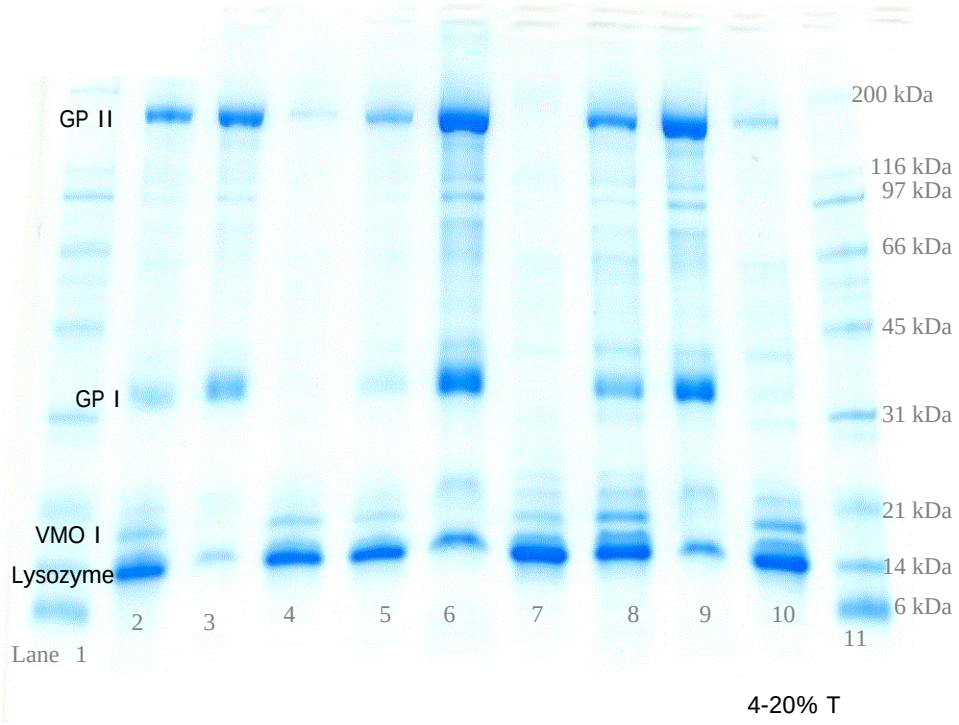

Figure A-42. Day 1 gel of vitelline membrane proteins from 82 wk molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

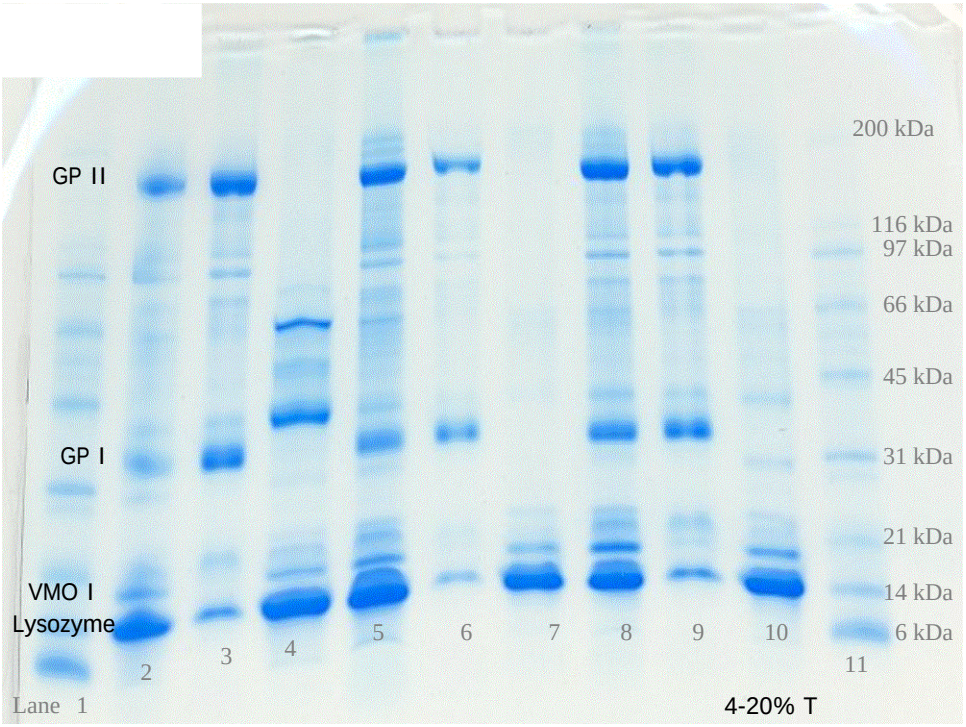

Figure A-43. Day 7 gel of vitelline membrane proteins from 82 wk molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

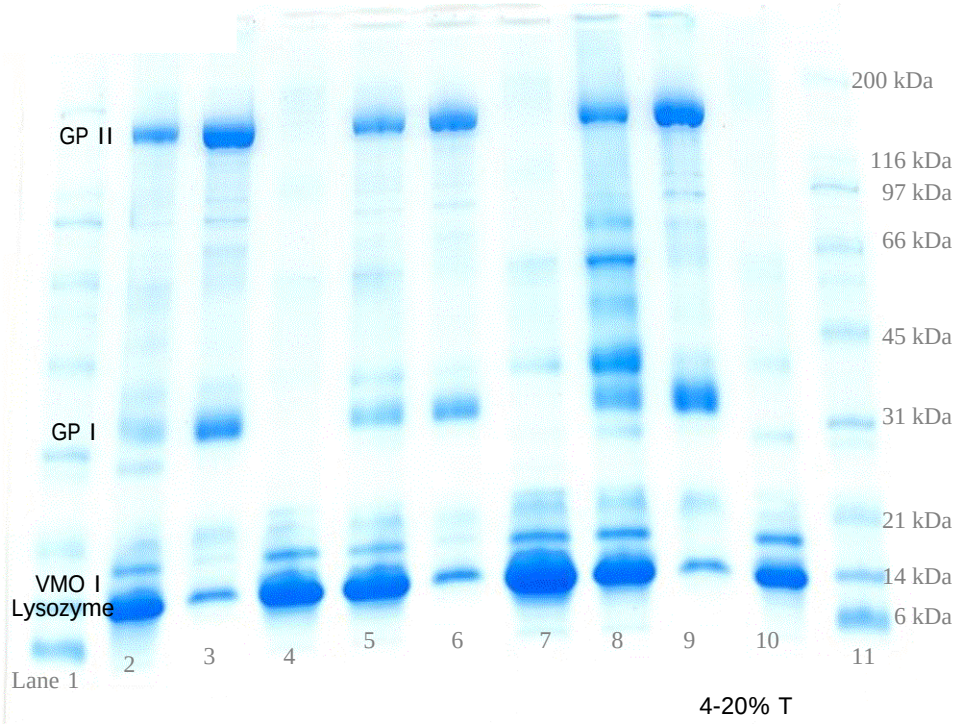

Figure A-44. Day 14 gel of vitelline membrane proteins from 82 wk molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

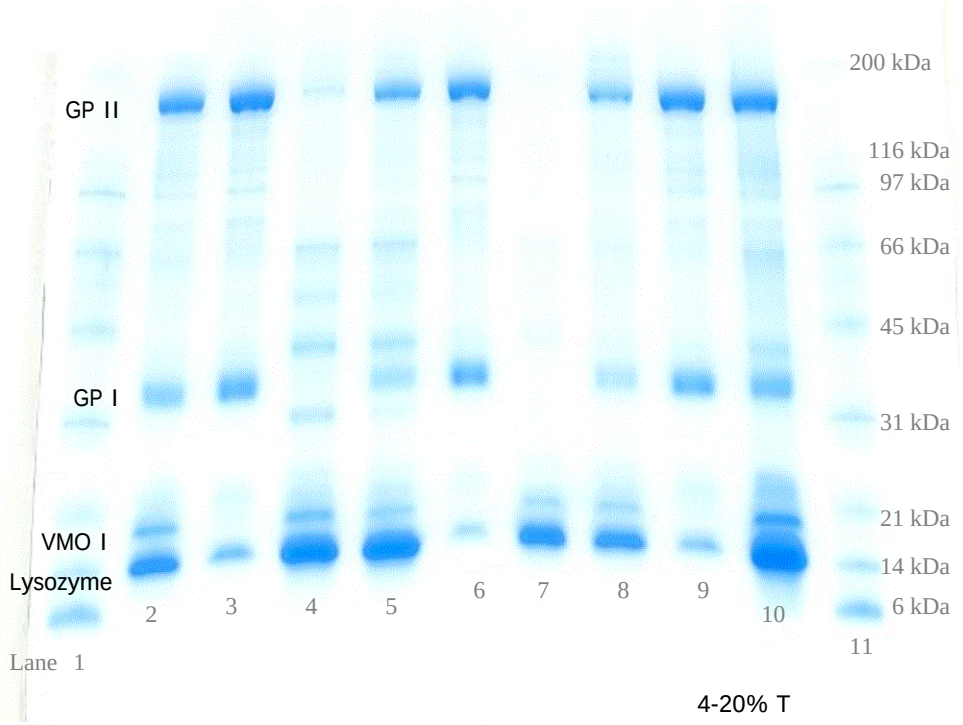

Figure A-45. Day 21 gel of vitelline membrane proteins from 82 wk molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

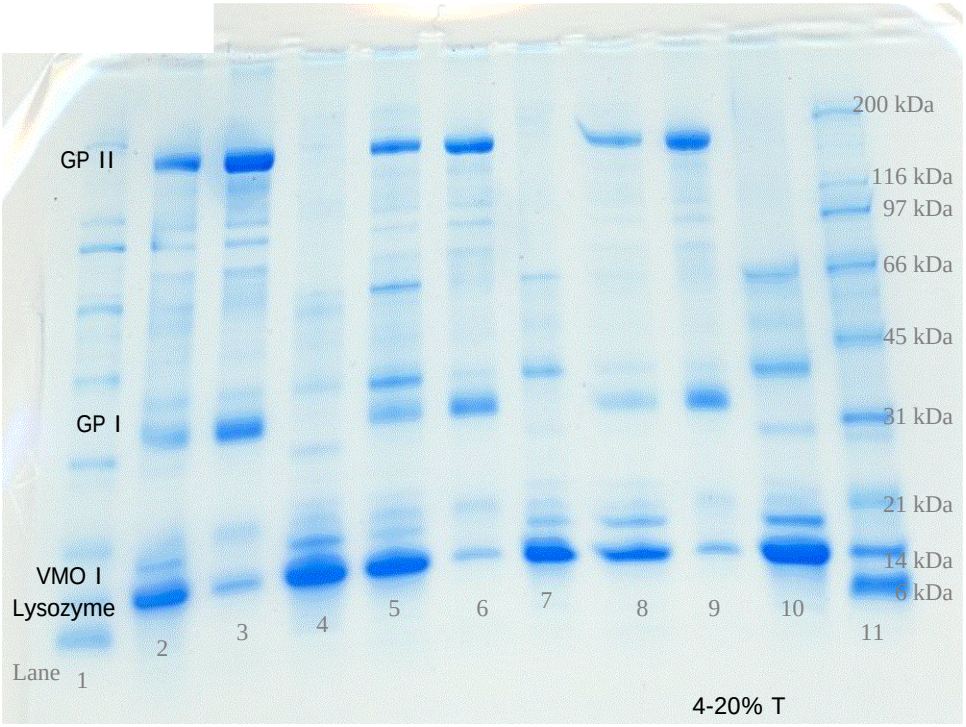

Figure A-46. Day 28 gel of vitelline membrane proteins from 82 wk molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

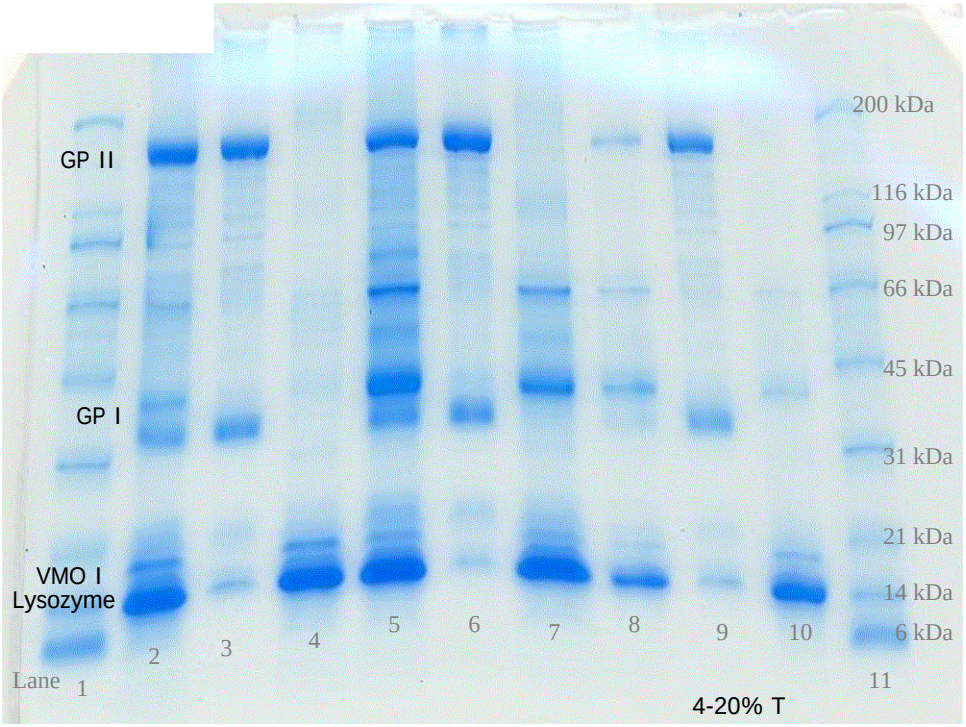

Figure A-47. Day 35 gel of vitelline membrane proteins from 82 wk molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

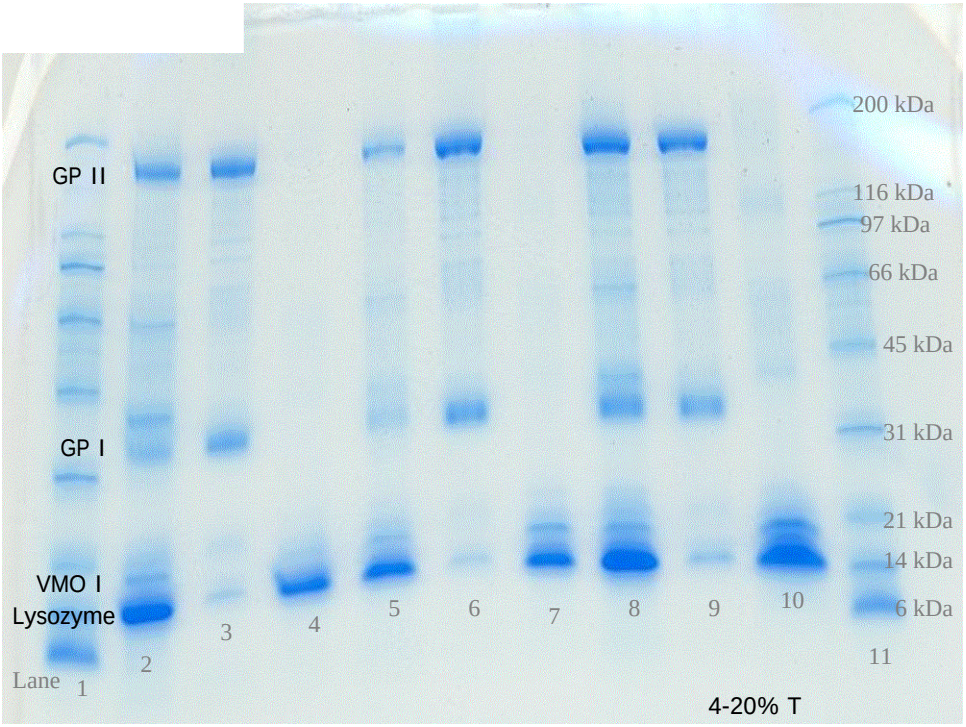

Figure A-48. Day 42 gel of vitelline membrane proteins from 82 wk molted hens egg; Experiment 2. Pre-cast gels used, 4-20% gradient. Lines 1 and 11 represent the standard; and lines 2, 5, and 8 are the whole membranes; lines 3, 6, and 9 are the inner layers; and lines 4, 7, and 10 are the outer layers.

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