POULTRY CRC LTD

FINAL REPORT

Sub-Project No: 3.2.4

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Sub-Project Title: Proteomic measures of albumen degradation as indicators of egg freshness

DATE OF COMPLETION: September 2016

ISBN 1 921010 96 7

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In submitting this report, the researcher has agreed to the Poultry CRC publishing this material in its edited form.

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Published in 2017

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Executive Summary

Immediately after lay the egg components will begin to deteriorate. The gross changes that take place after an egg is laid include a decrease in water and CO_2 content, an increase in air cell space, osmotic changes between yolk and albumen and weakening of the vitelline membrane. While there are many proteins in egg albumen the main ones are ovalbumin, ovotrasferrin, α- and β-ovomucin, lysozyme and clusterin. Changes in the albumen especially the viscosity of the thick albumen is used as a measure of freshness. It has been tradition to measure albumen quality in terms of Haugh Units (HU), a method based on measuring the albumen height and egg weight. Questions have been asked about the use of HU as a quality measure because of the effects of genotype, storage time and strain. It is the modification in the proteins of the albumen that determines the rate of thinning. Recent proteomic studies have attempted to identify the range of specific proteins in albumen and changes in the abundance of specific proteins during storage. The measurement of protein changes is likely to provide an accurate determination of freshness.

A core part of the current project was to use Two Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) to separate and isolate albumen proteins and using this procedure to identify a protein bio-marker that could be used to develop an assay to measure egg albumen deterioration and by association egg age.

There is a range of factors that affect egg quality and the ones that have received extensive evaluation are storage temperature, storage time and atmospheric conditions, genotype, hen age and production system.

The objectives of the project were:

1. Provide an understanding of the proteome changes in egg albumen associated with a deterioration in egg freshness.

2. Evaluate the feasibility of developing a rapid field test (biomarker) for egg freshness based on changes in the egg albumen proteome.

3. Utilise understanding of these egg albumen proteome changes to consider feasibility of developing practice or protocols to enhance egg shelf life or persistence of egg freshness.

4. Develop an understanding of how stress influences egg quality and freshness.

5. Define a set of limits on hen age, ambient temperature and stress which could then be used to evaluate the quality of eggs from different production systems. Unless the influence that these factors have on egg quality can be removed from the evaluation then an assessment of egg quality from different production systems would not be valid.

Methodology

The first study generated suitable samples for the 2D-PAGE analysis. It is well known that storage temperature and storage time have significant effects on albumen deterioration. Egg were collected from conventionally caged hens and stored in a refrigerator (4.2 ± 0.1ºC), a cool room (15.9 ± 0.1ºC) or at room temperature (21.8 ± 0.1ºC) over 29 days

Eggs were sampled on the day of lay and then at 7, 14, 21 and 29 days after storage. At each sampling time conventional measures of internal egg quality were made on 15-20 eggs. The albumen was collected and used in the 2D-PAGE analysis.

The 2D-PAGE analysis was based on published procedures but these needed to be refined to better meet the needs of the current project. This required extensive effort and, by the end of the project, the protocol provided protein profiles that were consistent with there being differences in some protein spots associated with deterioration in egg albumen. Having experienced the difficulties associated with the 2D-PAGE analysis, an alternative protein identification procedure was used. This was Terminal Amine Isotopic Labelling of Substrates (N-TAILS), a method used to identify differences in abundance of specific proteins in a sample.

An early objective of the project was to evaluate egg quality from different production systems. In retrospect, such an evaluation would have little relevance if some of the other factors such as hen age, ambient temperature and acute stress, could not be accounted for. So, three experiments were conducted to determine, the effects that hen age, housing temperature and acute stress had on egg quality at lay and during storage.

In two experiments the effect of acute stress on egg quality was investigated. In the first experiment, hens were exposed to relocation and the stressor. Hens individually housed in conventional cages were transferred to new cages and housed in groups of five. Eggs were collected before and after relocation and then stored for 29 days in a refrigerator, a cool room or at a room temperature. In the second experiment, hens housed in individual conventional cages were removed and placed into crates (6 birds) and then transported on the open road for approximately 20 min and on return, transferred to group cages (4 birds/cage) that were modified to contain a perch and nest box. Eggs were collected before being moved and then after the acute stress treatment. Eggs were stored in either a refrigerator, in a cool room or at room temperature for 28 days. For both studies egg quality using conventional measures were determined at the time of lay, and then at 7, 14, 21 and 28 (29) days after the start of storage. The albumen was collected and stored at -80ºC for later analysis of corticosterone concentration and any proteomic analysis in the further.

While the effect of hen age on egg quality has been investigated extensively, it is not clear if is there any period in the production cycle where hen age has no effect on egg quality. To address this issue, eggs were collected from a barn facility where different aged flocks were housed in a single shed. All hens were the Isa Brown strain and supplied from the one pullet rearing farm, having being moved to the layer facility at 16 weeks of age. Different aged flocks were housed in the individual units of this one shed. Eggs were collected on the same day from 6 different flocks, these being 21, 30, 50, 63, 83 and 90 weeks of age. The flocks at 21, 30, 50 and 63 weeks of age had been in continuous uninterrupted production but the 83 and 90 week old flocks had undergone a moult at 67 and 70 weeks of age, respectively. Twenty eggs were sampled at the time of lay and then at 7, 14, 21 and 28 days of storage, 15-20 eggs were sampled. Conventional measures of egg quality were made at each on these sampling times. The albumen was collected and stored at -80ºC for any proteomic analysis in the further.

In any comparison between the effects of production systems on egg quality the effect of shed temperature needs to be eliminated from having any impact on the comparison. To address this question, Isa brown hens were housed in individual cages. The layer shed ambient temperature and humidity was recorded using monitors at 15 min intervals over

the months of January to June. Eggs were collected on 7 days (T1-T7) covering a range of shed temperatures, with three in summer, one in autumn and three in winter. After collection eggs were stored in either a refrigerator, a cool room or at room temperature. Twenty eggs were sampled on the day of lay (control time) and then at 7, 14, 21 and 28 days of storage. Conventional measures of egg quality were made at each on these sampling times. The albumen was collected and stored at -80ºC for any proteomic analysis in the further

Results and Discussion

Using the 2D-PAGE procedures, the most obvious changes occurred when eggs were stored at room temperature and was in line with the more produced changes observed in conventional measures of egg quality. The changes in albumen protein were obvious after just 7 days storage at room temperature.

After refinement of the 2D-PAGE procedures, protein spots were isolated from stored albumen samples. By comparing the location of these spots, based on pH and molecular weight with published data, it appeared that the abundance of ovoinhibitor and clusterin proteins had changed during storage. This is the current status of the 2D-PAGE analysis and the future step is to remove these protein spots and have them identified.

Because of the difficulties experienced in using the 2D-PAGE procedures to identify albumen proteins, it was decided to investigate other potential methods. While this decision was made in the later stages of the project, it has provided evidence that the 2D-PAGE procedures might not have been the best option. Terminal amine isotopic labelling of substrates (N-TAILS) was used to identify the proteins and peptides in albumen from freshly laid eggs and comparing these to those in albumen stored in a refrigerator or at room temperature after 14 and 21 days. There was good consistency between all of the runs, and it appears that there has been excellent coverage of the egg white proteome. The N-TAILS approach was instigated late in the current project and so the analysis is in its earlier stages but does provide encouragement that it could achieve our initial objective of identifying a suitable biomarker of albumen quality and indirectly egg aging.

Those eggs having normal shell morphology and laid the day after the acute stress was applied had similar egg quality at lay and during storage as eggs lain the day before the stress and the changes in egg quality during storage were similar.

Hens aged between 50 and 63 weeks had similar measures of egg quality and the changes during storage were similar and so independent of hen age at this time in the production cycle.

When the housing temperature ranged from 15 to 26 ^{0}C, the temperature had no effect on egg quality. However, to eliminate the effect of temperature in any assessment of egg quality under commercial conditions it would be ideal to collected eggs during periods when the housing temperature was between 15 and 20ºC.

Implications

1. Storage of eggs in a cool room or at room temperature for storage 14 and 21 days resulted in significant deterioration in egg albumen quality and provided samples suitable for use in the proteomic analysis of egg aging.

2. Hens aged between 50 and 63 weeks had similar measures of egg quality and the changes during storage were similar and so egg quality was independent of hen age at this time in the production cycle.

3. In the current work when hens were faced with summer shed temperatures and the daily average prior to lay was less than 26ºC, the shed temperature had no effect on egg quality at the time of lay or during storage. In winter temperatures when the daily average temperature was between 15-20ºC, the shed temperature had no effect on egg quality.

4. Using short duration transport and pen relocation to induce acute stress resulted in approximately one third of hens to lay eggs with some form of shell abnormality with the main condition being the 'target' shell morphology. The 'target' eggs had lower HU measures at the time of lay but after this storage temperature and time had similar effects as seen for eggs laid with normal shell morphology. When eggs with normal shell morphology from acutely stressed hens are compared to similar normally shelled eggs before the stress, there was no difference in egg quality.

5. The N-TAILS approach to identifying bio-markers of albumen deterioration and aging is likely to be a better strategy that using the 2D-PAGE analysis because the predominance of ovalbumin in egg albumen makes separation difficult using the 2D-PAGE procedure.

Recommendations

1. In an effort to identify bio-markers of egg albumen deterioration and aging the N-Terminal Amine Isotopic Labelling of albumen proteins would appear to be a superior approach compared to 2D-PAGE analysis.

2. The effect of production system on egg quality should be determined using Isa Brown laying hens aged between 53-60 weeks of age, when the daily shed temperature is less than 20ºC (winter) and using only egg with normal shell morphology.

Introduction

The gross changes that take place after an egg is laid include a decrease in water and CO_2 content, increase in air cell space, osmotic changes between yolk and albumen and weakening of the vitelline membrane (Stadelman and Corterill, 1995). As time progresses, a thinning of the albumen will be noticeable (Reviews in: Roberts, 2004; Ahmadi and Rahimi, 2011).

Egg albumen is 84-89% water with 90% of the remaining dry matter being protein with six proteins making up 85% of the total. The main proteins are ovalbumin, ovotrasferrin, α and β ovomucin, lysozyme and clusterin (Johnson, 2000). The albumen proteins are continuously synthesised in the magnum under the influence of the sex hormones (Muramatsu *et al.,* 1991). Immediately after lay the egg components will begin to deteriorate. It is the changes in albumen, especially the viscosity of the thick albumen that has been used as measure of freshness (Reviews: Roberts, 2004; Ahmadi and Rahimi, 2011). Thinning is thought to be a sign that albumen has lost some of its quality (Silversides and Budgell, 2004). The viscosity is a measure of the tendency to flow, and the viscosity of the thick albumen is linked to its functional characteristics (whipping, emulsifying and gelling properties) (Kemps *et al.,* 2010). It has been tradition to measure albumen quality as Haugh Units (HU) (Williams, 1992), based on measuring the albumen height and egg weight (Haugh, 1937). Questions have been asked about the use of HU as a quality measure because of the effects of genotype, storage time and strain (Silversides, 1994). Some measures of albumen quality are made using albumen index, albumen ratio, yolk ratio according to specific formulae (Ahmadi and Rahimi, 2011). All these are essentially based on physical measures and not chemical changes which are more aligned with nutritional quality. As eggs age, CO_2 is lost and pH increases as a result (Benton and Blake, 2000; Caner, 2005), with changes in pH having been fundamental to quality evaluation in many studies. Storing eggs in a CO_2 enriched atmosphere reduces the rate of loss in egg freshness with storage time (Rocculi *et al.,* 2009). Kemp *et al.* (2010) tried to measure viscosity on intact albumen using a rate controlled rheometer but this was not successful. The sample had to be mixed but this physically disrupted the thick albumen matrix. The pre-preparation needed has a major influence on the viscosity measurement and so really measuring viscosity has limitations in evaluating freshness.

Very early on it was reported that thick albumen consisted of a series of translucent bands separating a transparent phase (Romanoff and Romanoff, 1949). The transparent phase was similar to thin albumen and this was held together by the translucent bands of protein. It is the modification in the proteins of the albumen that determines the viscosity. While the changes that occur when albumen become less viscous are not completely determined, it has been reported to involve changes in ovalbumin and ovomucin (Robinson and Monsey, 1972; Kato *et al*., 1979; Li-Chan and Nakai, 1989; Toussant and Latshaw, 1999; Nys and Guyot, 2011). Kato *et al*. (1988) indicated that the interactions between lysozyme and ovomucin were involved in the highly viscous nature of thick albumen. Ovalbumin is the most abundant protein in albumen and during storage is converted to S-albumin (Smith and Back, 1962; Masaaki, 2005). Ovalbumin can convert to the heat stable S-albumin under prolonged storage with the suggestion that this occurs because of the increase in pH during storage (Kitabatake *et al*., 1988). This conversion involves a limited secondary conformational modification (Huntington *et al*., 1995). A rapid increase in S-ovalbumin was observed after storage at 25°C for 27 days, increasing from 14.1% to 91.9%, and when stored at 37°C, it increased from 14.1% to 91.2% after 12 days (Huang *et al*., 2012). There was a highly significant correlation between S-ovalbumin and storage time ($P < 0.001$).

Recently, proteomic studies have attempted to identify the range of specific proteins in albumen (Gurein-Dubiard *et al*., 2006; Mann, 2007; D'Ambrosio *et al*., 2008; Mann & Mann 2011; Omana *et al*., 2011) and albumen has been well characterised at this level with most recent reports identifying up to 158 different proteins. Omana *et al*. (2011) evaluated the change in protein concentrations at 10 day intervals in eggs using two-dimensional-electrophoresis (2D-PAGE) during storage at 22˚C for 40 days. At 10 days of storage, eight proteins were found to significantly increase in abundance and one decreased. Protein spots that increased aligned with ovalbumin, clusterin, ovoinhibitor and ovotrasnferrin while the decreased protein aligned with ovalbumin. After 20 days albumen thinning was observed and after this the changes in abundance of protein was minimal. After 30 days the only decrease in protein abundance was seen for prostaglandin D2 synthase.

Similar experiments, conducted by Qiu *et al*. (2012) identified that eggs stored for 15 days at 4°C, 20°C and 37°C utilising 2D-PAGE and MALDI-TOF MS/MS similarly exhibited changes in albumen proteome, with 32 protein spots derived from 8 proteins identified as altered due to storage conditions with ovalbumin exhibiting increased rate of degradation with increasing temperature. Ovalbumin complexes were shown to increase while other proteins (eg. lipocalins, including prostaglandin D2 synthetase) decreased in concentration at higher temperature. Clusterin increased significantly after 10 days but continued to increase up to 40 days of storage. Clusterin acts to stabilised unfolded or partly folded proteins and could help stabilised proteins in albumen after an initial period of degradation (Gurein-Dubiard *et al*., 2006). In other studies however, clusterin declined in concentration under high temperature storage conditions (Qiu *et al*., 2012). The measurement of protein changes is likely to give an accurate determination of freshness.

Factors affecting egg quality

Factors affecting egg quality have been reviewed by Roberts, (2004) and Ahmadi and Rahimi, (2011). Those that have received the most intensive investigation are storage temperature, storage time and atmospheric conditions, genotype, hen age and to a lesser extent, production system. The measures of freshness used have been largely the HU, albumen height and albumen and yolk pH. Eggs stored at room temperature will lose 1-15 HU in the first few days and 30 units at 30 days of storage (Okeudo *et al*., 2003). As hens age the HU drops 1-1.5 units each month (Kato *et al*., 1970). Eggs laid at peak production lose quality when stored at 21°C or 29°C for 10 days (Jin *et al*., 2011). The changes were not seen when eggs were stored at 5˚C (Jin *et al.*, 2011).

The effects of genotype on egg quality have to a large extent concentrated on differences between white and brown strains of laying hens (Suto *et al*., 1997; Ledur *et al*., 2002; Singh *et al*., 2009; Tumova, *et al*., 2007; 2011). A significant relationship between housing system and genotype for HU has been established (Wall and Tauson, 2002; Vits *et al*., 2005). This was supported by the interactions seen between brown layer lines and the conventional cage (CC), furnished cage (FC) and floor litter housing systems as reported by Tumova *et al*., (2011). The egg quality was lower in the litter systems and this was attributed to ammonia production from the litter and was similar to the suggestion made by Singh *et al*., (2009). Selection of an appropriate genotype for a particular housing system may be a requirement for best egg quality (Tumova, *et al*., 2011). Interactions between housing system and albumen height and pH were described by Brand *et al*., (2004) with there being more variation in free range hens. When eggs from 22 and 50 week old free-

range hens were evaluated for effects of storage time (0-14 days) and temperature (4ºC vs 20ºC) on egg quality, it was found that the egg weight loss, HU, yolk and albumen indexes were worse with age while yolk and albumen pH increased as hens aged and the storage time and temperature increased (Akyurek and Okur, 2009). As hens aged the storage temperature and time will cause a more rapid deterioration in albumen quality (Akyurek and Okur, 2009). These hen age effects are supported by reports from Silversides and Scott (2001) and Tona *et al*. (2004).

During storage, CO_2 diffuses from the egg and this causes an increase in pH (Keener *et al*., 2001). The albumen pH has been reported to change from 7.95 to 9.25 during 14 days of storage for eggs from hens 22 and 50 weeks of age (Akyurek and Okur, 2009).

Involvement of stress in egg quality

The role stress has on internal egg quality has not received much attention but there is some indirect evidence that indicates that it has a role. Internal egg quality was evaluated for eggs from hens, 56-62 weeks of age after being graded as normal (82%) or abnormal (18%) based on shell characteristics (HeuiSoo *et al*., 2012). When eggs of the same age were compared, normal eggs had a higher HU and as the degree of abnormality increased the HU value decreased. This could indicate that factors responsible for abnormal egg shells also affect quality.

Stressors imposed on hens can result in delayed oviposition (Hughes *et al*., 1986; Mills *et al*., 1991; Reynard and Savory, 1999). It was predicted that stress induced increases in circulating adrenalin affects uterine motility and shell gland contraction, resulting in poor shell quality and other egg deformities (Hughes and Black, 1976). Moving hens housed in groups of 4, 3 or 2 in floor pens to cages caused a delay in oviposition with some hens retaining the egg overnight (Hughes *et al*., 1986). Over the first 6-8 days after moving, egg production decreased from around 90% to 50% and then gradually increased after this. A higher percentage of the eggs laid were abnormal, especially during the first 6 days after the move.

Injection of adrenocorticotropic hormone ACTH causes a dose-dependent increase in number of abnormal eggs laid (Flickinger, 1966). There is evidence indicating that corticosterone can also affect oviposition time and the incidence of abnormal eggs. In hens 66-74 weeks of age, plasma corticosterone concentration was higher (increased by approximately 1.0 ng/ml) in hens laying soft-shelled or membranous eggs compared to hens laying normal hard shelled eggs (Klingensmith *et al*., 1984). Roland (1978) found that the percentage of misshapen eggs was related to group size and increased or decreased by changing the number of hens in the cage. Walker and Hughes (1998) reported various effects on egg shell quality, when they compared conventional cages and furnished cages. Engimaierova and Tumova, (2009) compared eggs from conventional cages and litter housing over 21 days of storage. Storage time had highly significant effect on quality. At lay, egg shell quality and albumen quality were higher in the litter system but there was greater deterioration in albumen quality characteristics in eggs from the litter system. The stressors hens are exposed will differ between production systems but the conditions on a particular farm are likely to be more influential than the production system per se (Downing 2012).

Egg quality was measured from two strains of hens, maintained in conventional cages or on litter and housed at 26°C or 35°C. Egg quality was found to be lower at the high

temperature and was poorer for caged birds. Based on the respiratory frequency it appeared that caged birds were under greater stress (Barbosa Filho *et al.,* 2006).

High ambient temperature causes decreased egg weight (Emery *et al*., 1984; Balnave and Muheereza, 1997; Samara *et al*., 1996; Mashaly *et al*., 2004), inferior shell and albumen quality (Emery *et al*., 1984; Odom *et al*., 1985; Sauver and Picard, 1987; Mahmoud *et al*., 1996; Mashaly *et al*., 2004) and increased egg breakage (Tanor *et al*, 1984; Roland, 1988). Depending on the severity, heat stress is potentially going to influence egg quality and protein changes in albumen. The role that stress has on internal egg quality and the rate of albumen deterioration needs to be investigated.

Objectives

1. Provide an understanding of the proteome changes in egg albumen associated with a deterioration in egg freshness.

2. Evaluate the feasibility of developing a rapid field test (biomarker) for egg freshness based on changes in the egg albumen proteome.

3. Utilise understanding of these egg albumen proteome changes to consider feasibility of developing practice or protocols to enhance egg shelf life or persistence of egg freshness.

4. Develop an understanding of how stress influences egg quality and freshness.

5. Define a set of limits on hen age, ambient temperature and stress which could then be used to evaluate the quality of eggs from different production systems. Unless the influence that these factors have on egg quality can be removed from the evaluation then an assessment of egg quality from different production systems would not be valid.

Methodology

Experiment 1. **A model for generating suitable samples for detecting proteomic changes in egg protein during aging: The effects of storage temperature and time on egg albumen quality.**

Study design

After hens had been housed in individual conventional cages or 6 weeks, eggs laid on one single day were collected and stored in a refrigerator with a mean (± SEM) temperature of 4.2 ± 0.1ºC, in a cool room with a mean (± SEM) temperature of 15.9 ± 0.1ºC, or in a room temperature with a mean (± SEM) temperature of 21.8 ± 0.1ºC during a storage period of 29 days.

Eggs were sampled on the day of lay and then at 7, 14, 21 and 29 days after storage. At each sampling time the following conventional measures of internal egg quality were made on 15-20 eggs:

1. Egg weight
2. Albumen height
3. Haugh units (HU) – using $HU = 100 \times \log (H - 1.7W^{0.37} + 7.57)$
4. Thick albumen diameter
5. Albumen index (AI) determined as albumen height/albumen diameter
6. Yolk height
7. Yolk diameter
8. Yolk index (YI) (AI) determined as albumen height/albumen diameter

The albumen from three sample eggs was collected at these times and stored at -80°C for 2D-PAGE analysis.

Statistical Analysis

Data were stored in Microsoft Excel® and the statistical analysis was conducted using the linear mixed model function (REML) of Genstat® 17th edition. The data were first tested for equality of variance. Where significance for main effects was detected, the least significant difference (LSD), equal to two times the standard error of differences (SED), was used to make pairwise comparisons between means.

Experiment 2. Determination of candidate proteomic biomarkers for egg freshness

2D-PAGE methodology

The methodology has two stages, first extraction of albumen proteins and then their separation using 2D-PAGE gel electrophoresis.

The initial extraction procedure was based on the publication of Omana *et al.* (2011). An initial problem associated with this published procedure was the low protein content in the sample at the end of the extraction. A deal of time was committed to improving the extraction procedure to increase the protein yield.

The modified protein extraction protocol used was:

Reagents

Solution 1. A 13.3% trichloroacetic acid (W/V) solution in acetone (13.3 g TCA in 100 mL acetone). The solution was stored in a brown bottle at -20ºC. Just before use, dithiothreitol (DTT) was added at a rate of 2 mg/mL (0.2% W/V).

Solution 2. Neat acetone was stored at -20˚C and just before being used, DTT was added at 2 mg/mL (0.2% W/V).

Re-suspension solution. The re-suspension solution consisted of 7 M urea, 2 M thiourea, 4% CHAPS and 0.0002% bromophenol blue. Before use amplolytes 3-10 (0.05%) and DTT (0.4%) were added.

Protein extraction procedure

1. Albumen was collected by cracking open the egg and manually separating the albumen from the yolk. The albumen was then gently agitated on a magnetic stirrer for 30 min to homogenise the thick albumen. The albumen was stored in a 50 mL sterile plastic tube at -80ºC until extracted.

2. To one volume of albumen protein three volumes of solution 1 were added to a 1.7 mL centrifuge tube and then the contents were mixed using a plastic stirrer (a plunger from a 0.5 mL syringe pipettor is suitable for this purpose).

3. The contents were incubated for 1.5 h at -20ºC.

4. After incubation the contents were centrifuged at 16,000 X *g* for 10 min at 4ºC. The supernatant was then discarded and the centrifugation repeated and any further supernatant discarded.

5. To the precipitate, 1 mL of solution 2 was added and the pellet was broken up with a plunger from a 0.5 mL syringe pipettor in an effort to help redissolve the precipitate. The contents were stored on ice for 1 h and at each 20 minutes mixed again. The contents were then centrifuged as described in step 4.

6. Step 5 was then repeated.

7. After the final centrifugation the precipitate was air dried in a fume hood for 15-20 min.

8. To the precipitated pellet 550 µL of re-suspension solution was added and the contents mixed with the plunger from a 0.5 mL syringe pipettor. The contents were not vortexed as this would act to help oxide the proteins. The contents were left on ice for 2 h and mixed every 30 min.

9. If the precipitate appeared to be in solution the contents were centrifuged at 18,000 x *g* for 10 min at 4ºC.The supernatant was then transferred to another centrifuge tube and a 30 uL subsample was removed for analysis of protein content with the remainder stored at -80ºC. The protein content of the supernatant was analysed using a Bradford assay using bovine serum albumin (BSA) as the reference standard (Bradford, 1976).

The 2D-PAGE Gel Analysis

Preparation and rehydration of the IPG strips.

The thawed protein was centrifuged at 4ºC for 1 min to remove any solid material. The protein sample was equilibrated in a buffer containing, 50 mM Tris (pH 8.8), 6 M urea, 30% glycerol (V/V), 2% SDS (w/v) and 0.002% bromopheol blue with the total volume being 250 µL. Rehydration of the IPG strip, 13 cm 'Immobiline Dry Strip' with a pH range 4-7 (GE Healthcare, Uppsala, Sweden) was performed overnight. The protein was loaded into a 'DryStrip' rehydration re-swelling tray and the IPG laid gel side down and then covered with paraffin oil. While all efforts were made to run the strip in the first phase immediately after rehydration, if this was not possible they were stored at -80ºC.

First dimension isoelectric focusing

Separation in the first dimension was performed using a LKB model 2117 Multiphor II Electrophoresis attached to a Amersham MultTemp 111 cooling system running at 10ºC, and a Amersham Pharmacia Bioteck Electrophoresis power supply, model EPS 3501 X. The power supply was set at 2 mA and 5 W and run at 300 V for 0.5 h, 700 V for 0.5 h 1,500 V for 1.5 h and 3,500 V for > 20 h.

Second dimension - SDS-Page separation

The second dimension was run in a SE 600 Ruby Standard Dual Cooled Vertical Unit (GE Healthcare Australia Pty. Ltd. Parramatta, Australia) with a two plate capacity. Single phase gels were prepared in the laboratory by mixing:

- 25.13 mL high quality water
- 18.75 mL of 0.5 M Tris buffer
- 30 mL of 30% acrylamide/bis-acrylamide (Sigma-Aldrich, Castle Hill, Australia)
- 750 µL of 10% SDS
- 375 µL of 10% ammonium persulfate (Sigma-Aldrich, Castle Hill, Australia)
- And 25 µL tetrimethyl ethylenediamine (TEMED: Sigma-Aldrich, Castle Hill, Australia) was added just prior to casting the gel.

The IGP strips were equilibrated in 10 mL of equilibration buffer with DTT added at 10 mg/mL and for 15 min and then 10 mL of equilibration buffer with iodacetmide (25 mg/mL) for 15 min.

The strip was then placed on top of the cast gel and to the left side of the gel a protein ladder was loaded as a molecular weight reference standard (Page Ruler Unstained Protein Ladder (10-250 kDa; Thermo Fisher Scientific, North Ryde. Australia). Once loaded the strip was sealed on the upper side by dispensing 0.5% agarose solution (100 mL SDS electrophoresis running buffer plus 0.5 g agarose and 200 µL Bromphenol blue).

The gels were loaded into the SE 600 Ruby Standard Dual Cooled Vertical Unit and SDS running buffer was added to the holding tank and the upper sealing tank and before placing on the lid, the system was checked for potential leakage.

The SDS running buffer was prepared by dissolving 60.4 g Tris, 20 g SDS and 288 g glycine in 2 L of nanopure water. When needed, the stock was diluted 10:1 with nanopure water before being used. A two phase electrical input was used. Phase 1 used 200 V, 50 mA and 2 W/gel and operated for 45 min. Phase 2 used 200 V, 50 mA and 17 W/gel and operated until the bromophenol dye had washed off the bottom of the gel (approximately 4 h).

After the completion of the second phase the gels were removed and placed in an osculating Commassie blue staining bath overnight. The Commassie stain was prepared by dissolving 0.625 g Commassie blue (Brilliant red - Sigma-Aldrich, Castle Hill, Australia), 225 mL methanol and 50 mL acetic acid in 225 mL nanopure water.

The following day the excess strain was removed by washing the gels in a detaining solution prepared by dissolving 250 mL methanol and 100 mL acetic acid in 650 mL nanopure water and after this in pure nanopure water. The gel was transferred to a flat top scanner and images recorded using Image Master 2D Platinum Software (V5 – Amersham Biosciences, Castle Hill. Australia.).

Further refinement of the 2D-PAGE Gel Analysis

From the early gel images (see Results), it was apparent that the second dimension conditions were adequate for the objectives of the work but there was a clear need for conditions to be modified to improve the protein separation in the first dimension of the 2-D PAGE procedure. To achieve this objective the work was coordinated with staff at The University of Sydney's Charles Perkins Centre where the most up to date equipment was available.

To improve the protein separation the flowing approaches were taken at different stages in the project

1. Increasing the voltage hours applied in the first dimension

Using equipment at the Charles Perkins Centre the kilo volts hours applied during the first dimension of the 2D-PAGE analysis was increased in an effort to improve the protein separation in this dimension.

2. Changing the amount of protein loaded onto the IGP strip

After identifying the overestimation of the protein content in the extracted albumen supernatant when using the Bradford assay (see Results), gels were run with 50 or 10 µg of protein loaded onto the IGP strips and then the 2D-PAGE analysis completed as discussed above.

3. Comparing using neat (un-extracted) protein loaded onto the IGP strip with the conventional extracted protein loading.

While the extraction of albumen protein has been the normal method of preparation the use of neat albumen rather than extraction was an option evaluated here. All procedures for the 2D-PAGE electrophoresis were the same as for the extracted samples.

4. Using cup-loading to disperse the albumen extract onto the IPG strip during the rehydration step.

Cup loading, where the protein is loaded onto the IGF strip from one end of the strip during the rehydration step rather than laying the protein horizontally in the re-swelling tray was evaluated.

5. Using a gradient gel in place of a single phase gel in the second dimension phase.

Gels layered using a fixed acrylamide percentage are useful when separating proteins over a narrow molecular weight range while gels layered using a gradient of changing acrylamide percentages are useful for separating proteins over a wide molecular weight range and give better replication of the protein separation.

Two acrylamide mixtures, one of 10% and one of 18% were prepared using the same method described previously. The acrylamide mixtures were placed in a gradient mixer and while being stirred pumped into the space between the glass plates. The rehydrated IGF strips were placed on the top of the gel and second phase operated in the same manner as described previously.

6. Using a IGP strip with the a pH range of 5-8

The proteins of interest appear at the higher pH values (pH 6-7) during the first dimension of the 2D-PAGE analysis. The bulk of the albumen protein is ovalbumin which is located at around pH 4-5. In an effort to improve the protein separation the extracted albumen was loaded onto a 11 cm pH 5-8 Immobiline Dry Strip (GE Healthcare, Uppsala, Sweden) using the procedures described for strip rehydration.

<u>Protein separation using terminal amine isotopic labelling of albumen proteins (N-TAILS)</u>

The objective was to use resin free N-TAILS to look for novel protein cleavage products associated with aging of egg albumen.

The procedure is an intensive laboratory commitment made over a number of days. The samples used were albumen from freshly laid eggs and those stored at room temperature or in the refrigerator for 14 and 21 days (5 samples in total). The albumen from 5 eggs taken at each collection, were added to a beaker and the contents gently stirred at 4ºC

until a homogenous albumen sample was obtained. The samples were then stored at -80ºC until assayed.

Sample preparation

Four samples of the albumen from the freshly laid eggs (control sample) and one each of the albumen from the stored eggs were prepared using the following protocols.

Day 1

1. A 100 µL subsample of each thawed egg albumen sample was added to 900 µL 0.2 M HEPES buffer (pH 8).
2. A 100 µL subsample of the mixture was taken (10 µg) and then 100 µL of 8 M guanidine hydrochloride was added.
3. A 2 µL of 1 M DTT was added as a reducing agent and the mixture incubated for 1 hr at 55ºC.
4. Then 10 µL of 0.5 M of the alkylating agent Indole-3-acetic acid (IAA) was added and the contents incubated in the dark at room temperature for 30 minutes.
5. To stop the reaction the contents were quenched by adding 2µL 1M DTT and incubating the mixture for 30 min.

Sample labelling

6. Then 4.2 µL of 2 M formaldehyde (5.96 µL in 20 µL water) was added to the control albumen samples with 2.1 µL of 2 M formaldehyde (3.24 µL in 20 µL water) added to the stored albumen samples.
7. Then 4.2 µL of 1 M cyanoborohydride (2.5 mg in 40 µL water) was added to each control and albumen sample and the pH checked to be between 7.0-7.3 and the contents incubated overnight at 37^0C.

Day 2

8. The excess formaldehyde in the mixture was quenched by adding 12 µL ammonium bicarbonate and incubating the content for 90 min at room temperature
9. The extracted protein in the supernatant was precipitated using an ice cold chloroform and methanol.
    - To 200 µL aliquots of protein supernatant 800 µL of ice cold methanol was added and then 200 µL ice cold chloroform added followed by a further 600 µL chloroform of cold high quality water.
    - The mixture was centrifuged at 9000 x *g* for 2 min.
    - The supernatant was removed and the precipitated washed with ice cold methanol and then centrifuged at 9000 x *g* for 1 min. The precipitate was saved.
10. The protein pellet was solubilised in 100 µL of 8 M guanidine hydrochloride made up in 0.2M HEPES buffer by sonicating and vortexing the content.
11. A further 500 µL L 0.2M HEPES buffer and the protein content quantified using the QUBIT protein assay kit (Thermo Fisher Scientific Australia Pty Ltd., Scoresby. Australia).
12. Trypsin was added to the samples at the rate of 1 µg for each 100 µg of extracted protein and the mixture allowed overnight to digest.

Day 3

13. The mixture was cleaned up using Hydrophilic-Lipophilic-Balance reverse-phases separation (HLB) as follows:-
    - The column was activated using 1 mL of 100% methanol and then 1 mL of 100% acetonitrile and then washed with 0.1% trifluoroacetic acid (TFA).
    - The sample was slowly loaded onto the column
    - The column was then washed with 6 mL of 0.1% TFA
    - The peptides were eluted using 1.2 mL of 50% acetonitrile and 0.1% TFA
    - The peptide mixture was dried overnight.

Day 4

14. The samples were resuspended in 100 µL of 50 nM sodium hydroxide and then 900 µL of 100 nM HEPES buffer, 10 µL of 2 M sodium cyanoborohydride and 20 µL of 1 M 4-formyl-3-hydroxybenzoic acid were added and the pH adjusted to 7 using 20 µL of 1 M hydrochloric acid.
15. The mixture was incubated at 37^0C for 1 h.
16. A further 10 µL of 2 M sodium cyanoborohydride and 20 µL of 1 M 4-formyl-3-hydroxybenzoic acid were added and the mixture was incubated at 37^0C for 1 h.
17. Another 10 µL of 2 M sodium cyanoborohydride and 20 µL of 1 M 4-formyl-3-hydroxybenzoic acid were added and the mixture was incubated overnight at 37^0C.

Day 5

18. The next morning, 100 µL of 1 M ammonium bicarbonate was added and the pH adjusted to 7.0 by adding 40 µL of 1 M hydrochloric acid.
19. The contents were incubated for 4.5 h at 37ºC.
20. The sample was cleaned using HLB as described above and then dried down overnight.

Day 6

21. The dried precipitates were reconstituted in 200 µL of a mixture of 30% acetonitrile and 1% acetic acid and the pH was checked to ensure it was between 2.5 and 3.0.
22. The protein content was determined by Qubit quantification.

<u>Strong Cation Exchange Chromatography</u>

Purification of the samples was made using strong cation-exchange chromatography (ICAT strong CATION Exchange Kit – Applied Biosystems). The process was as follows:-

1. The column was washed with 2 mL of clean loading buffer, then 2 mL of a 30% acetonitrile plus 1% acetic acid solution.
2. The prepared samples were loaded slowly and then washed with 2 mL of the loading buffer.
3. The protein was eluted using 500 µL of 0.5 M potassium chloride constituted in loading buffer.

4. The eluted sample was dried down overnight.
5. The next day a salt clean up step was undertaken. Using 200uL stage tips the peptide mixture was loaded onto C8 material column and then eluted using 50 µL of 50% acetonitrile and 0.1% formic acid.
6. The eluted mixture was dried down and then reconstituted in 50 µL liquid chromatography loading buffer (3% acetonitrile and 0.1% formic acid).

Liquid chromatography–mass spectrometry (LC-MS)

The proteins were than separated using LC-MS which combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. A 3 µL of sample was loaded onto the LC-MS.

Experiment 3. The effect of acute stress on egg quality during storage at different temperatures

Experiment 3a. The effect of acute relocation stress on egg albumen quality

Study design

Two hundred and twenty laying hens were housed in individual conventional cages for 6 weeks. Then on day 1 of the experiment, eggs were collected from these hens as the control treatment. On day 2, the hens were then moved to new cages and housed in groups of five. Eggs were collected from these hens on the following day (day 3) after being relocation (Stress treatment). Eggs from the control and relocation treatments were stored in a refrigerator with a mean (± SEM) of 4.1 ± 0.06ºC, in a cool room with a mean (± SEM) temperature of 15.9 ± 0.1ºC, or in a room temperature with a mean (± SEM) temperature of 21.8 ± 0.1ºC during the storage period of 29 days.

Eggs were sampled on the day of lay and then on days 7, 14, 21, 28 and 36 days after the start of storage. At each sampling time the following conventional measures of internal egg quality were made:

1. Egg weight
2. Albumen height
3. Haugh units (HU) – using HU = 100×log (H-1.7$W^{0.37}$+7.57)
4. Thick albumen diameter
5. Albumen index (AI) determined as albumen height/albumen diameter
6. Yolk height
7. Yolk diameter
8. Yolk index (YI) (AI) determined as albumen height/albumen diameter

Following the quality measurements the albumen was collected and stored at -20ºC until analysed for corticosterone concentration following the method of Downing and Bryden (2008).

Statistical Analysis

Data were stored in Microsoft Excel® and the statistical analysis was conducted using the REML linear mixed model function of Genstat® 17th edition. The data were first tested for

equality of variance using residual plots. When the equality of variance could be improved by using a log transformation, the data were transformed. The fixed model included the effects of treatment, storage day and storage temperature, with all interactions between fixed effects included in the model. Significance testing of fixed effects was conducted using Wald tests with a significance threshold of $P < 0.05$. Any non-significant interactions were removed from the model. The predicted means for all significant fixed effects were copied to Microsoft Excel®, as well as the standard errors, which were used to calculate the standard error of the mean (SEM). The least significant difference (LSD), which is equal to two times the standard error of differences (SED), was used to make pairwise comparisons between means when significance was detected.

Experiment 3b. The effect of transport and relocation acute stress on egg quality

Study design

Two hundred 55 week old Isa Brown hens that had been housed in individual conventional cages for 6 weeks, were used. On day 1 (S1), all eggs laid were collected from these hens (control treatment). On the following morning (day 2) starting at 05:00 h, hens were removed from the cages into crates (6 birds) and then transported on the open road for approximately 20 min and on return, transferred to group cages (4 birds/cage) that were modified to contain a perch and nest box. Any eggs laid on day 2 were discarded because the albumen would have been deposited before the acute stress was applied. On day 3 (S3), all eggs laid were collected and similarly on day 4 (S4) all eggs laid were again collected.

At the time of collection (days 1, 3 and 4) a sample of 20 freshly laid eggs were evaluated for quality, using the conventional measures of Haugh unit (HU), albumen index (AI) and yolk index (YI).

The remaining eggs (approximately 160-180) from each collection day were stored at one of three temperature conditions. One third of eggs were stored in either a refrigerator (temperature: mean ± SEM; 4.3 ± 0.1ºC), in a cool room (temperature: mean ± SEM; 16.1 ± 0.1ºC) or at room temperature (temperature: mean ± SEM; 21.2 ± 0.1ºC) for 28 days. After 7, 14, 21 and 28 days after the start of storage, 15-20 eggs were processed from each treatment temperature using the above mentioned conventional measures of egg quality. For all eggs sampled, the albumen was collected stored at -80ºC for later analysis of corticosterone concentration and any future proteomic analysis in the further.

All sampled eggs had the shell morphology evaluated before the internal quality was measured. The following classifications were used to assess shell morphology:

1. White banded eggs (see Figure 1) – These are often referred to as target eggs. This occurs when oviposition is delayed and a fully formed egg is held in the oviduct and extra calcium is deposited over and already formed shell except where it is in contact with a new egg entering the oviduct or maybe the oviduct wall.
2. Misshapen eggs (see Figure 1) – these differ from the normal shape and/or size. The modification can be very minimal or excessively malformed.
3. Soft-shelled – these are eggs are laid with an incomplete shell development and often crack when laid.

4. Slab sided or flat sided eggs – this is caused by two eggs being in the shell gland at the same time with an egg side flattened due to contact with another egg.
5. Calcium coated eggs – these eggs have an extra powdery layer of calcium deposited over the shell. The area covered varies.

While there can be other types of egg shell defects these were not observed in the current study. An initial evaluation of the quality of defective shelled eggs was made to determine the influence they would have on egg quality during storage.

It was only after the application of the acute stress on day 3 that eggs with defective shells were found in abundance. So in the first instance, the egg quality was determined in normal shelled eggs and compared to defective shelled eggs at the time of lay on day 3 (S3). It was only possible to carry targeted eggs through the 28 day storage period as soft shelled eggs were not compatible with being stored.

Statistical analysis

Statistical analysis was conducted using the REML linear mixed model function as described for experiment 3a.

Figure 1. Photos of target and soft-shelled eggs collected on day 3.

Experiment 4. The effect of hen age and storage temperature and storage time on egg quality

Introduction

While the effect of hen age on egg quality has been investigated extensively what isn't clear, is if there any periods in the production cycle where hen age has no effect on egg

quality. In any comparison between the effects of production systems on egg quality the effect of hen age needs to be eliminated from having any impact on the comparison.

Study Design

The eggs were collected from a barn facility where different aged flocks were housed in a single shed. The shed area had been divided into 8 individual housing units. All hens were Isa Brown strain and supplied from the one pullet rearing farm, having being moved to the layer facility at 16 weeks of age. Different aged flocks were housed in the individual units of this one shed. Eggs were collected on the same day from 6 different flocks, these being 21, 30, 50, 63, 83 and 90 weeks of age. The flocks at 21, 30, 50 and 63 weeks of age had been in continuous uninterrupted production but the 83 and 90 week old flocks had undergone a moult at 67 and 70 weeks of age, respectively. After collection eggs were stored in either a refrigerator at a temperature of (mean ± SEM) 4.20 ± 0.05ºC, in a cool room at a temperature of (mean ± SEM) 15.87 ± 0.07ºC, or in an open room at a temperature of (mean ± SEM) 21.8 ± 0.06ºC. Twenty eggs were sampled at the time of lay and then at 7, 14, 21 and 28 days of storage, 15-20 eggs were sampled. The following conventional measures of egg quality were made at each on these sampling times:

1. Egg weight
2. Haugh units (HU) – using $HU = 100 \times \log (H - 1.7W^{0.37} + 7.57)$
3. Albumen index (AI) determined as albumen height/albumen diameter
4. Yolk index (YI) (AI) determined as albumen height/albumen diameter

At each sampling time the albumen was collected from 3 eggs and stored at -80ºC for later potential assessment of albumen protein degradation using markers that might result from other aspects of this project.

Statistical analysis

The statistical analysis of hen age, the storage time and the storage temperature effects on egg quality were limited to those flocks that had an uninterrupted production cycle (21, 30, 50 and 63 weeks of age). The quality measures for the moulted flock at 83 and 90 weeks of age are included in the results but no statistical analysis was made for these two flocks.

Data were stored in Microsoft Excel® and the statistical analysis was conducted using the REML linear mixed model function of Genstat® 17th edition. The data were first tested for equality of variance. The fixed model included hen age, day of storage, storage temperature and all interaction between these. Significance was determined using Wald tests and non-significant effects were dropped from the model. When significance ($P < 0.05$) for main effects was detected, the least significant difference (LSD), equal to two times the standard error of differences (SED), was used to make pairwise comparisons between means.

Experiment 5. The effect of layer house ambient temperature on egg quality during storage under three different temperatures

Introduction

The effect of ambient temperature on egg quality has been investigated using various experimental designs where fixed or cyclic temperature patterns have been used. In any comparison of egg quality from different production systems, the effect of housing temperature needs to be eliminated for the comparison to be valid. The objective of this experiment was to determine the effect of housing temperature on egg quality, and identify a range in temperature where there is no effect on egg quality.

Study design

Isa brown hens were housed in individual cages. The layer shed ambient temperature and humidity was recorded using monitors at 15 min intervals over the months of January to June. Eggs were collected on 7 days (T1-T7) covering a range of shed temperatures, with three in summer, one in autumn and three in winter.

After collection eggs were stored in either a refrigerator (temperature mean ± SEM: 4.12 ± 0.05ºC), a cool room (temperature mean ± SEM: 15.80 ± 0.06ºC), or at room temperature (temperature mean ± SEM: 21.5 ± 0.05ºC). Twenty eggs were sampled on the day of lay (control time) and then at 7, 14, 21 and 28 days of storage. The following conventional measures of egg quality were made at each on these sampling times:

1. Egg weight
2. Haugh units (HU) – using $HU = 100 \times \log (H - 1.7W^{0.37} + 7.57)$
3. Albumen index (AI) determined as albumen height/albumen diameter
4. Yolk index (YI) (AI) determined as albumen height/albumen diameter

Statistical Analysis

Data were stored in Microsoft Excel® and the statistical analysis was conducted using the REML linear mixed model function of Genstat® 17th edition. The data were first tested for equality of variance. The fixed model included average shed temperature, day of storage, storage temperature and all interaction between these. Significance was determined using Wald tests and when significance ($P < 0.05$) for main effects was detected, the least significant difference (LSD), equal to two times the standard error of differences (SED), was used to make pairwise comparisons between means.

Results

Experiment 1. **A model for generating suitable samples for detecting proteomic changes in egg albumen protein during aging: The effects of storage temperature and time on egg albumen quality.**

Egg weight

The effects of storage temperature and time on egg weight are shown in Table 1.1. Both temperature and time had significant effects on egg weight but these changed over time as the temperature x day interaction was significant ($P = 0.015$).

Egg weight did not change over time when stored in the refrigerator or cool room. When eggs were stored at room temperature, egg weight was progressively lower from day 14 (P

< 0.05). On day 21 and 29 egg weight was lower when eggs were stored at room temperature compared to other storage temperatures (P < 0.05).

Table 1.1. The effect of storage time and storage temperature on the weight of eggs (g) stored at three different temperatures regimes.

Storage day	Storage temperature			SEM
	Refrigerator	Cool room	Room	
0	65.7	65.7	65.7_{A}	1.06
7	66.4^{a}	64.2^{b}	$66.1_{A}{}^{a}$	
14	65.0	64.9	64.3_{B}	0.61
21	64.8^{a}	64.5^{a}	$62.5_{C}{}^{b}$	
29	64.7^{a}	64.3^{a}	$59.6_{D}{}^{b}$	
SEM		0.53		

[a-b] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different subscripts are significantly different (P < 0.05)

Haugh unit measurement (HU)

The effects of storage temperature and storage time on HU measurements are shown in Table 1.2. Both temperature and time had significant effects but these changed over time as the temperature x day interaction was significant (P < 0.001).

At all storage days, the HU measurement was higher when eggs were stored in the refrigerator compared to other storage temperatures, while the values for eggs stored in the cool room were higher when compared to eggs stored at room temperature (all, P < 0.05).

There was a significant decrease in HU measures from point of lay to day 7 of storage for all temperature treatments (P < 0.05). The HU measures at day 29 were significantly lower than at day 7 but the rate of change varied for the different storage temperatures. The decrease was substantially less when eggs were stored in the refrigerator. Storage in the cool room saw significant differences in HU measures on all days (P < 0.05). The decrease in HU measure was more extreme when eggs were stored at room temperature with significant decreases on all storage days except for values on days 21 and 29 which were similar but were at their lowest (P < 0.05).

Table 1.2. The effect of storage time on the HU of eggs stored at three different temperatures regimes.

Storage day	Storage temperature			SEM
	Fridge	Cool room	Room	
0	86.7_{A}	86.7_{A}	86.7_{A}	2.53
7	74.9_{C}^{a}	70.4_{B}^{b}	65.2_{B}^{c}	
14	79.6_{B}^{a}	66.1_{C}^{b}	48.1_{C}^{c}	1.46
21	76.1_{BC}^{a}	60.7_{D}^{b}	37.3_{D}^{c}	
29	70.7_{D}^{a}	50.3_{E}^{b}	32.4_{D}^{c}	
SEM		1.26		

[a-c] Within a row values with different superscripts are significantly different ($P < 0.05$)
[A-D] Within a column values with different subscripts are significantly different ($P < 0.05$)

The albumen index (AI)

The effects of storage temperature and storage day on AI are shown in Table 1.3. The effects of both temperature and storage day on albumen index were significant but these changed over time as the interaction between the two was significant ($P < 0.001$).

On all storage days, the AI was higher when eggs were stored in the refrigerator than when stored in the cool room ($P < 0.05$) and, in turn, the AI for the eggs stored in the cool room were higher than those stored at room temperature ($P < 0.05$).

There was a significant decrease in the AI from point of lay to day 7 of storage for all temperature treatments ($P < 0.05$). The AI at day 29 was significantly lower than at day 7 but the rate of change differed at the different storage temperatures. The decrease was substantially less when eggs were stored in the refrigerator and it was only at day 29 of storage that the value was lower than it was at day 7 ($P < 0.05$). Storage in the cool room saw significant differences in AI at all storage days ($P < 0.05$). The decrease in AI measure was more extreme when eggs were stored at room temperature with significant decreases on all storage days except for values on days 21 and 29 when they were at their lowest ($P < 0.05$).

Table 1.3. The effect of storage time on the AI of eggs stored at three different temperatures regimes.

Storage day	Storage temperature			SEM
	Fridge	Cool room	Room	
0	0.100_{A}	0.100_{A}	0.100_{A}	
7	0.073_{BC}^{a}	0.062_{B}^{b}	0.051_{B}^{c}	
14	0.081_{B}^{a}	0.056_{B}^{b}	0.031_{C}^{c}	0.033
21	0.071_{C}^{a}	0.044_{C}^{b}	0.021_{D}^{c}	
29	0.066_{C}^{a}	0.035_{D}^{b}	0.017_{D}^{c}	
SEM		0.003		

[a-c] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different subscripts are significantly different (P < 0.05)

Yolk index (YI)

The effects of storage temperature and storage day on YI are shown in Table 1.4. The effects of both temperature and storage day on YI were significant but these changed over time as the interaction between the two was significant (P < 0.001).

From day 14 and onwards the YI was higher for eggs stored in the refrigerator than those stored in the cool room (P < 0.05), which in turn were higher than when egg were stored at room temperature (P < 0.05).

There was a no significant decrease in the YI from day of lay to day 7 of storage for all temperature treatments. As for HU and AI, the YI at day 29 of storage was significantly lower than at day 7 but again the rate of change differed for the different storage temperatures. When eggs were stored in the refrigerator, the YI on days 14, 21 and 29 were similar but lower than the value on day 7 (P < 0.05). When eggs were stored in the cool room the YI was lower on day 14 than day 7, lower on day 14 than day 21 (all, P < 0.05) but similar on days 21 and 29. At room temperature there was progressively lower YI as storage time increased after day 7 (P < 0.05).

Table 1.4. The effect of storage time on the YI of eggs stored at three different temperatures regimes.

Day	Storage Temperature			SEM
	Fridge	Cool room	Room	
0	0.400_{A}	0.400_{A}	0.400_{A}	0.008
7	0.395_{A}	0.382_{A}	0.398_{A}	
14	0.364_{B}^{a}	0.344_{B}^{b}	0.318_{B}^{c}	0.005
21	0.357_{B}^{a}	0.311_{C}^{b}	0.275_{C}^{c}	
29	0.365_{B}^{a}	0.315_{C}^{b}	0.255_{D}^{c}	
SEM		0.004		

[a-c] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different superscripts are significantly different (P < 0.05)

Experiment 2. Determination of candidate proteomic biomarkers of egg aging

Initial results of the 2D-PAGE Gel Analysis

As a standard model in the development of the 2D-PAGE procedures, the protein changes occurring in albumen when eggs were stored at different temperatures were examined. Eggs were processed at the time of lay then after 7, 14, 21 and 28 days after lay when stored at room temperature, in a cool room or refrigerator. The most obvious changes occurred when eggs were stored at room temperature and this was in line with the more produced changes observed in conventional measures of egg quality reported in

experiment 1. The 2D-PAGE gel profiles, of albumen protein from a fresh and eggs and stored for 7 days at room temperature are shown in Figure 2.1 and 2.2, respectively.

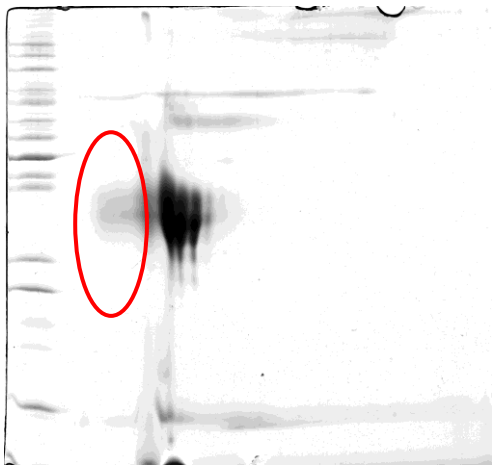


Figure 2.1. The 2D-PAGE gel profile of albumen proteins from a freshly lain egg. The standard protein ladder is included to the left of the gel profile.

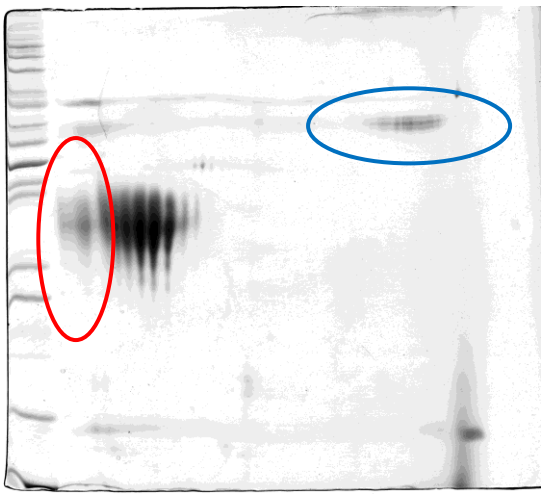


Figure 2.2. The 2D-PAGE gel protein profile of an egg stored at room temperature for 7 days.

There were distinct changes in the protein profile after 7 days of storage at room temperature. The two areas of interest are identified on Figures 2.1 and 2.2. Compared to the fresh egg profile the file of an egg stored at room temperature for 7 days suggested that there had been a shift and an increase in intensity of proteins identified by the red oval marking and by the presence of protein bands identified in Figure 2.2 by the oval blue marking. This second area was also seen in the sample at 14 days storage at room temperature (see Figure 2.3)

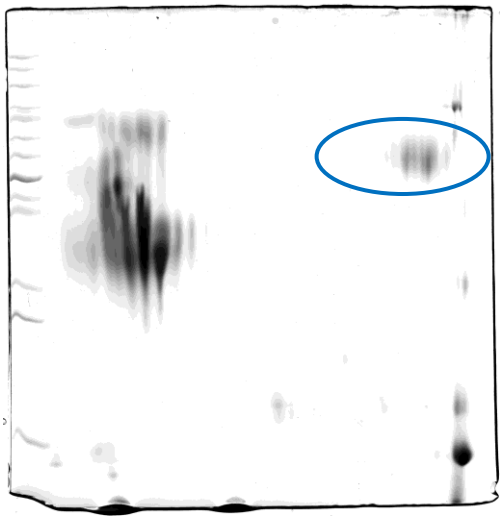


Figure 2.3. The 2D-PAGE gel protein profile of an egg stored at room temperature for 14 days.

Further samples of the gel profiles are given in Figure 2.4 (A-H) for different storage temperatures and storage times

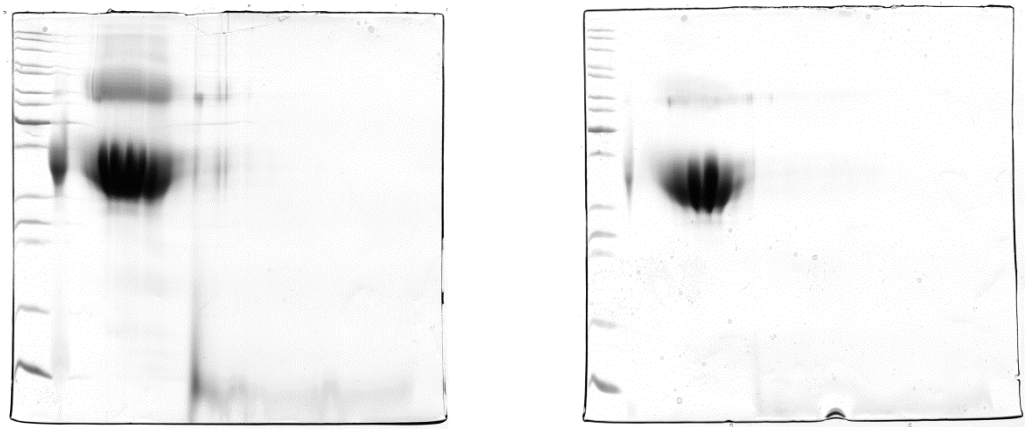


Figure 2.4 A and B. The 2D-PAGE protein profile for eggs stored in the refrigerator for 7 days (left) or cool room for 7 days (right).

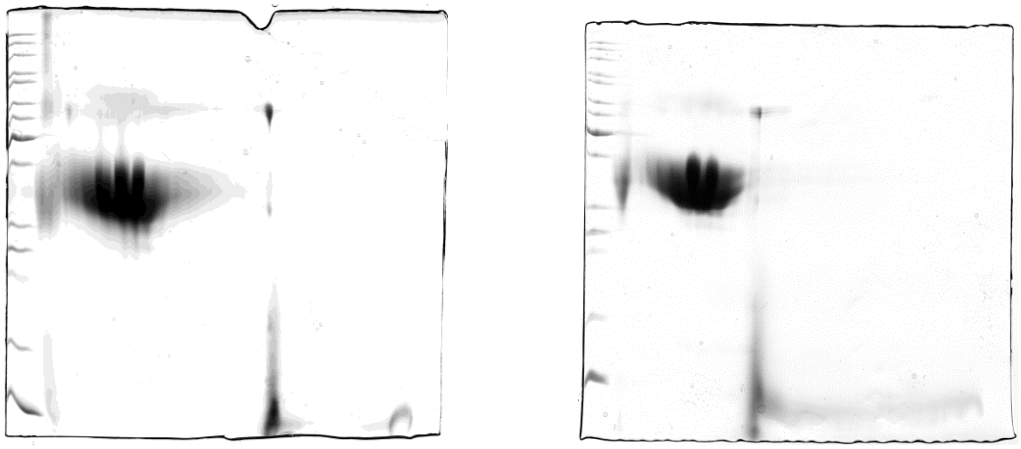


Figure 2.4. C and D. The 2D-PAGE gel protein profile for eggs stored in the refrigerator for 14 days (left) or cool room for 14 days (right).

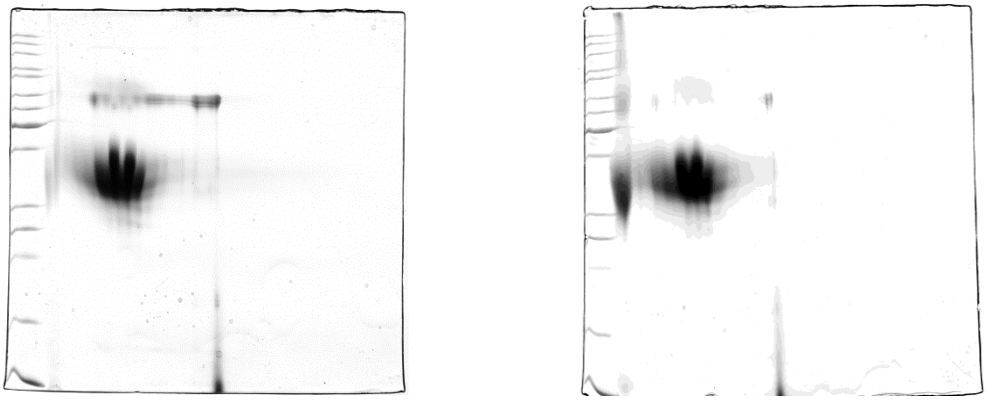


Figure 2.4. E and F. The 2D-PAGE gel protein profile for eggs stored at room temperature for 14 days (left) or the refrigerator for 21 days (right).

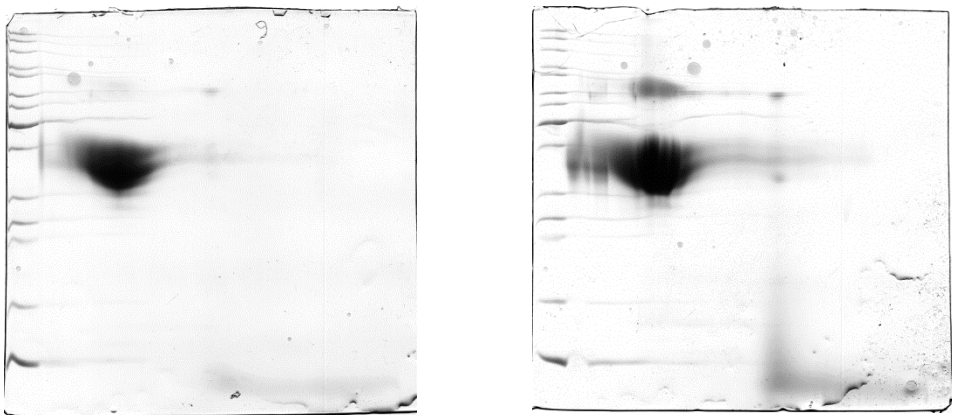


Figure 2.4. G and H. The 2D-PAGE gel protein profile for eggs stored in the cool room for 21 days (left) or at room temperature for 21 days (right).

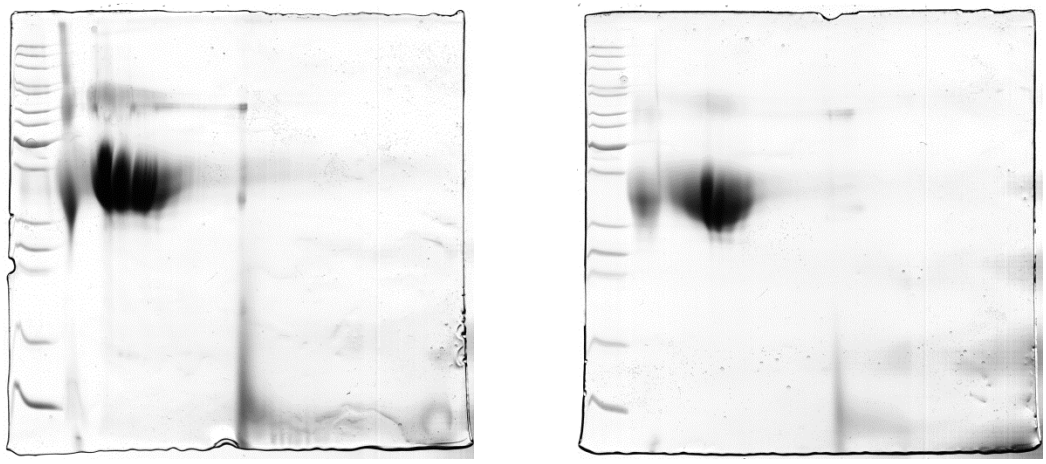


Figure 2.4. I and J. The 2D-PAGE gel protein profile for eggs stored in the refrigerator for 29 days (left) or at room temperature for 29 days (right).

In the above profiles, the protein density was too intense for any potential identification of a specific protein marker of egg aging. The 2D-PAGE procedures needed refinement. The amount of protein loaded onto the IPG strips was estimated to be 100 µg and was the quantity suggested as appropriate by Omana et al. (2011). However, in the current work this amount seemed to be excessive. After further investigation, we determined that the use of the Bradford assay to determine the protein content was underestimating the amount of protein in our extracted samples. This problem is highlighted in Figure 2.5. The Bradford assay used bovine serum albumin (BSA) as the references standard. When ovalbumin (the main protein in egg albumen) was used as the reference standard much higher concentrations are needed in the standard to given the same optical density reading as BSA. To our knowledge and after an investigation of the literature, this issue has not been previously identified as a problem. On finding this, the 2D-PAGE analysis was conducted using the refined Bradford assay to determine protein concentrations.

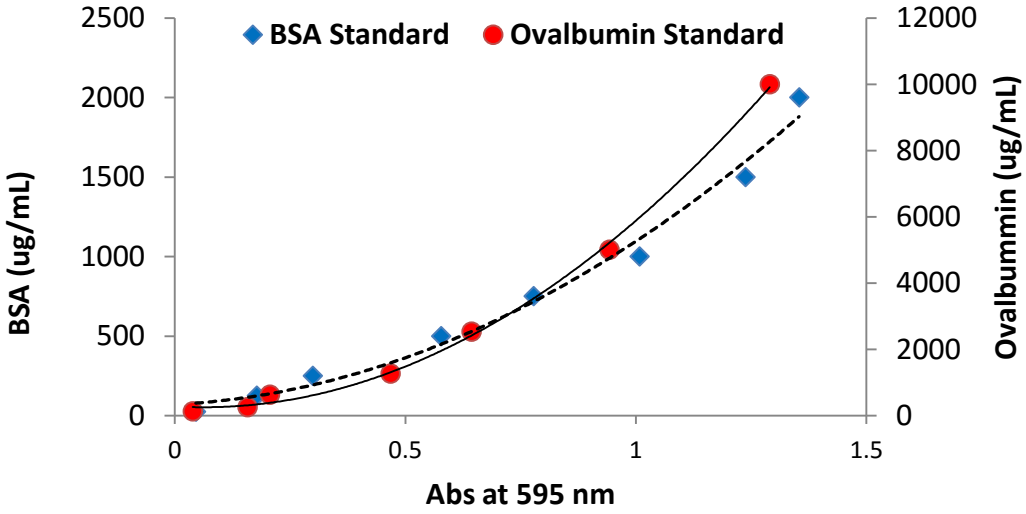


Figure 2.5. The protein curves generated when using either bovine serum albumin (BSA) or ovalbumin as the reference standard with the optical density (OD) measured at 595 nm.

Further refinement of the 2D-PAGE Gel Analysis

It was obvious after running many gels that the procedures provided by Omana et al., (2011) needed refining if suitable protein markers of egg aging were to be identified using 2D-PAGE analysis.

Increasing the voltage hours applied in the first dimension of the 2D-PAGE analysis

For the remainder of the protein work, the voltage potential shown in Table 2.1 was applied in the first dimension of the 2D-PAGE analysis. The program was applied in six steps with the current set at 2 mA and 17 W per gel. The purpose was to improve the separation of the proteins in the first dimension.

Table 2.1. The six step voltage protocol applied in the first phase of the 2D-PAGE analysis.

Step	Volts (V)	Time (h)	Total voltage (V)
1	200	1	200
2	500	1	500
3	1000	1	100
4	3000	1	3000
5	8000	18.75	150000
6	100	24	2400

Changing the amount of protein loaded onto the IPG strips

After identifying the over estimation of protein content in the extracted albumen supernatants, gels were run with different protein amounts loaded on to the IPG strips and then run in the first dimension using the extended voltage protocol described above. The protein profiles using 50 µg or 10 µg of extracted albumen protein are shown in Figure 2.6. While the 10 µg protein loading gave slightly better separation in the area of the gel enclosed by the red markings it was decided that the amount of protein present would not be sufficient for any protein identification. All further work used 50 µg of extracted protein as the loading amount.

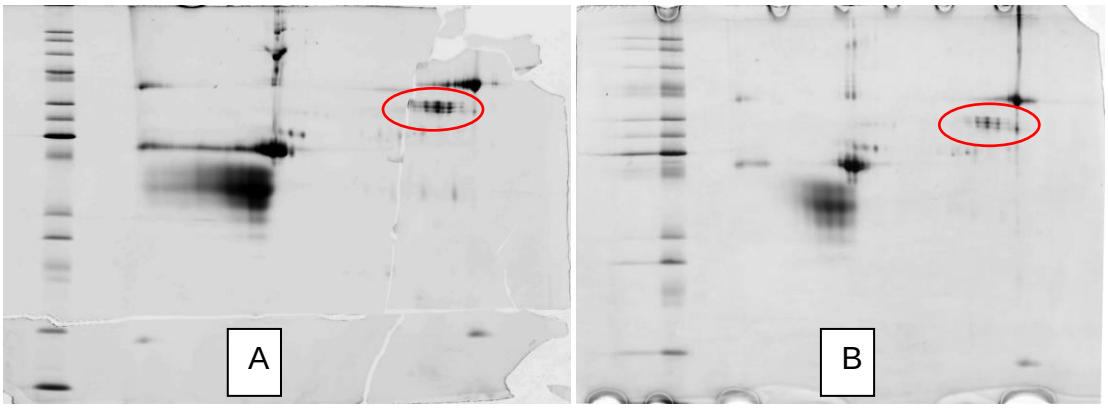


Figure 2.6. The 2D-PAGE gel protein profiles for albumen extracted form a freshly laid eggs and loaded onto a 13 cm (pH 4-7) IPG strip at 50 ug (A) or 10 ug (b).

Comparing neat (unextracted) albumen protein loaded onto the IPG strip with extracted protein.

The protein gel profiles for neat (unextracted) egg albumen and extracted albumen protein analysed using 2D-PAGE electrophoresis is given in Figure 2.7. Extracting the albumen protein provided no better protein separation than using neat unextracted albumen. In all further work unextracted protein was used.

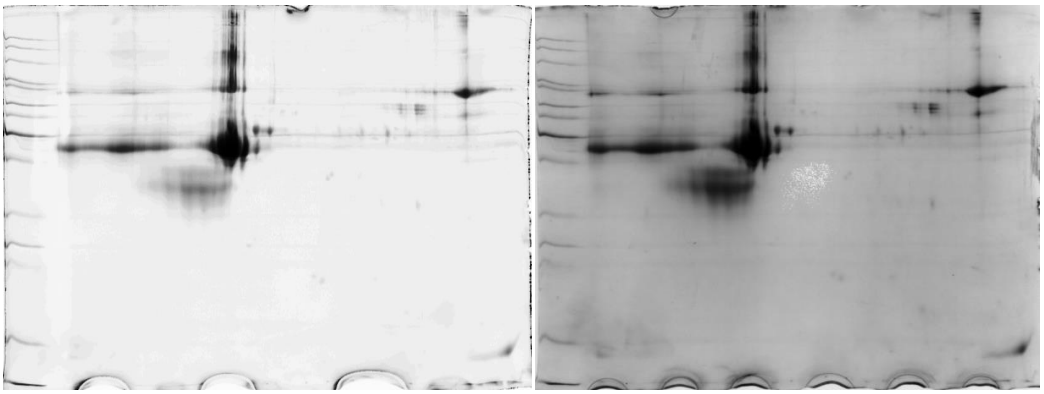


Figure 2.7. The 2D-PAGE gel protein profiles for 50 µg neat (unextracted) albumen from an egg stored for 21 days at room temperature and loaded onto a 13 cm (pH 4-7) IGF strip and using two different staining methods. `

Using a cup-loading to disperse the albumen extract onto the IPG strip during the rehydration step

The albumen sample was taken for eggs stored at room temperature for 21 days using the cup loading technique after the IPG strip was hydrated overnight in buffer only. Four of the gel profiles are given in Figure 2.8. Those parts of the gels circled in red are areas that tended to show better separation using the cup loading. The profiles provided a good

reason to concentrate on this area and to use a different IPG strip with a pH range that better covers this part of the gel.

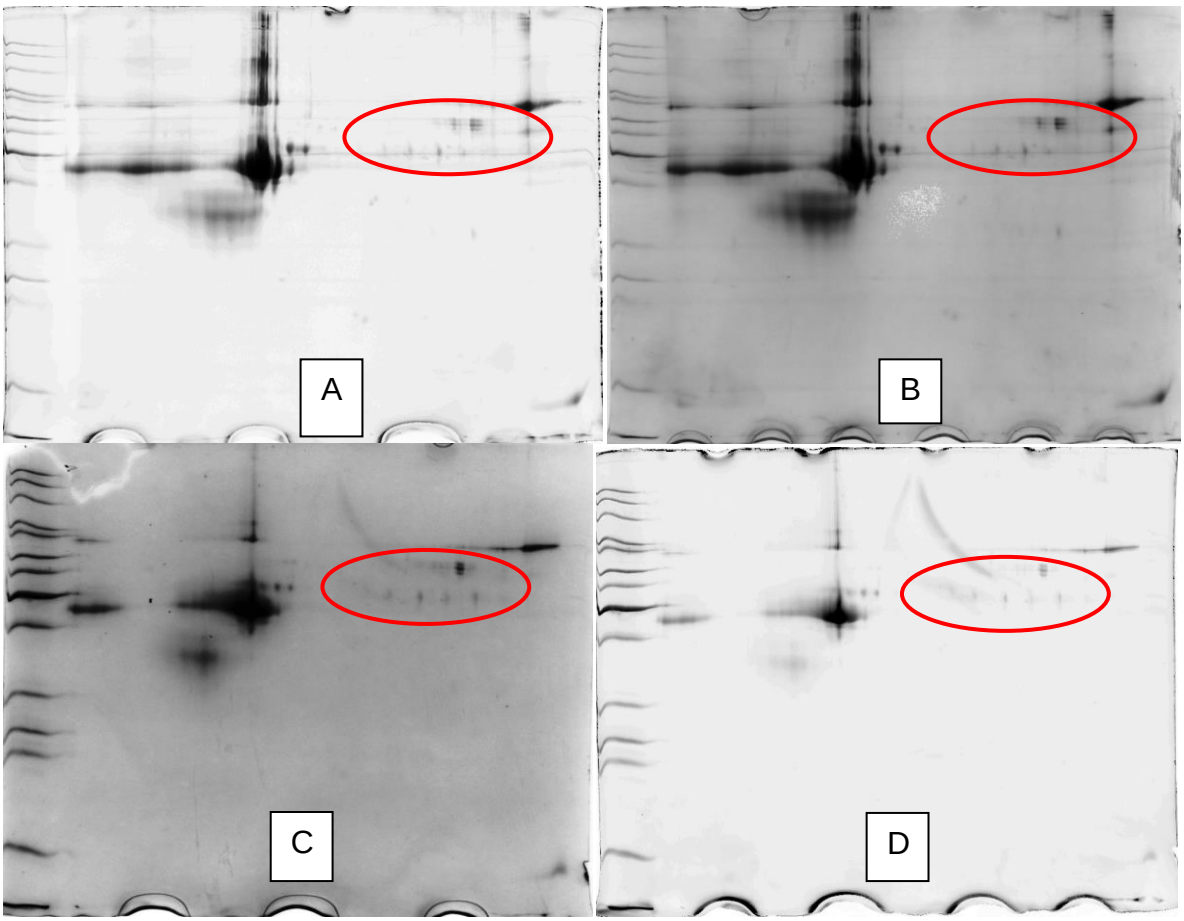


Figure 2.8 A-D. The 2D-PAGE gel protein profiles for albumen from an egg stored at room temperature for 21 days. Two 50 µg aliquiots of unextracted protein (A and B) or two 50 µg aliquiots of extracted protein (C and D) were diluted in 100 µL of buffer and rehydrated onto a 13 cm (pH 4-7) IPG strip using the cup loading technique and using two different Commassie stains. Those parts of the gels circled in red are areas that tended to show better separation using the cup loading technique

Using a gradient gel in place of a single phase gel in the second dimension of the 2D-PAGE analysis

The protein profiles for albumen from an egg stored at room temperature for 14 days and run on a 10-18% gradient gel are shown in Figure 2.9. Using a gradient gel provided no advantage to the protein separation and it was decided that the single gradient gel was the better option.

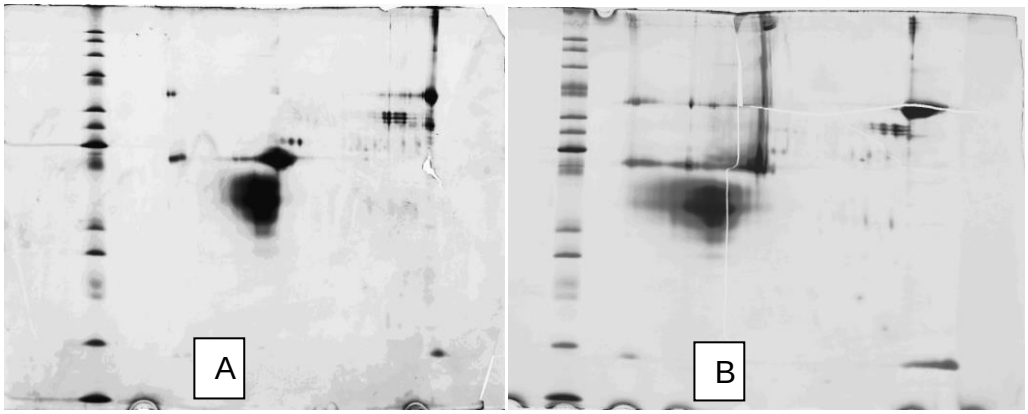


Figure 2.9. The 2D-PAGE gel protein profiles for albumen extracted form an egg stored for 14 days at room temperature and run on a gradient gel (10-18% acrylamide) at 10 µg protein (A) and 50 µg protein (B).

Using a IGP strip with a pH range of 5-8

The protein profile for albumen extracted from a freshly laid egg separated using 2D-PAGE electrophoresis is shown in Figure 2.10 (2 dimensional presentation) and Figure 2.11 (3-dimensional presentation). A molecular weight grid is overlain on the 2-dimensional gels in Figure 2.12. The protein profile for albumen extracted from an egg stored for 21 days at room temperature using 2D-PAGE electrophoresis is shown in Figure 2.13 (2 dimensional presentation) and Figure 2.14 (3-deimensiol presentation). A molecular weight grid is overlain on the 2-dimensional gel is given in Figure 2.15

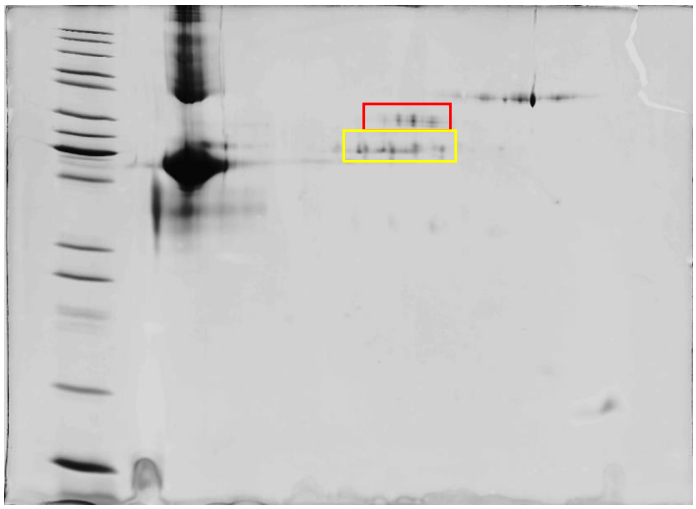


Figure 2.10. The two dimesional protein gel profile for 50 µg albumen extracted form a freshly laid egg and loaded onto a 11 cm IGF strip with a pH range of 5-8.

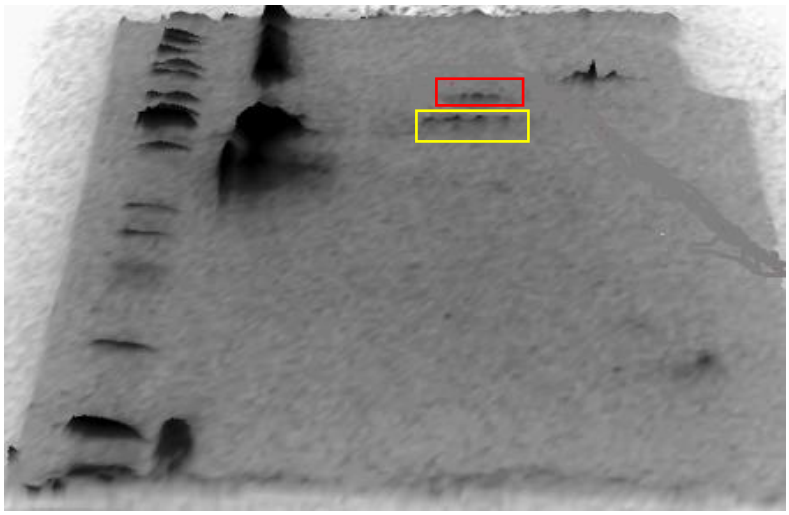


Figure 2.11. The three dimesional protein gel profile for 50 µg albumen extracted form a freshly laid egg and loaded onto a 11 cm IGF strip with a pH range of 5-8.

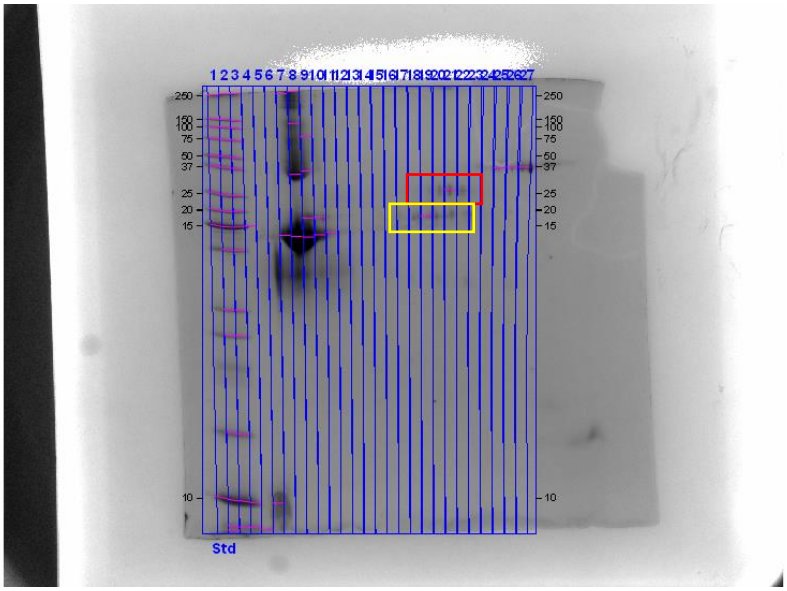


Figure 2.12. The two dimesional protein gel profile for 50 µg albumen extracted form a freshly laid egg and loaded onto a 11 cm IGF strip with a pH range of 5-8 with the molecular weight grid overlain.

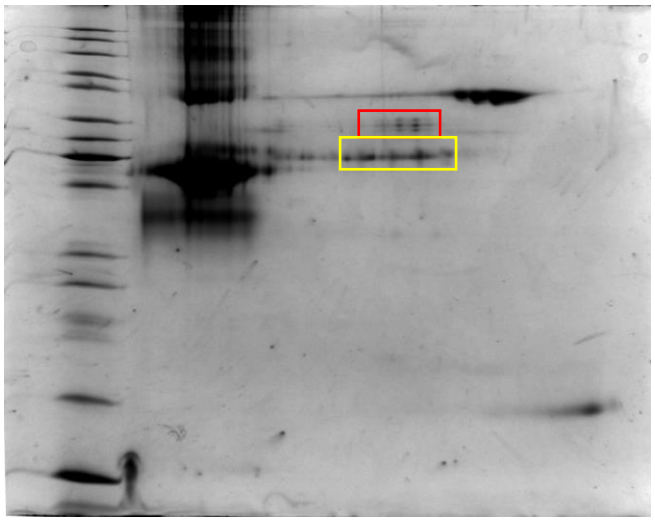


Figure 2.13. The two dimesional protein gel profile for 50 µg albumen extracted form a egg stored for 21 days at room temperature and loaded onto a 11 cm IGF strip with a pH range of 5-8.

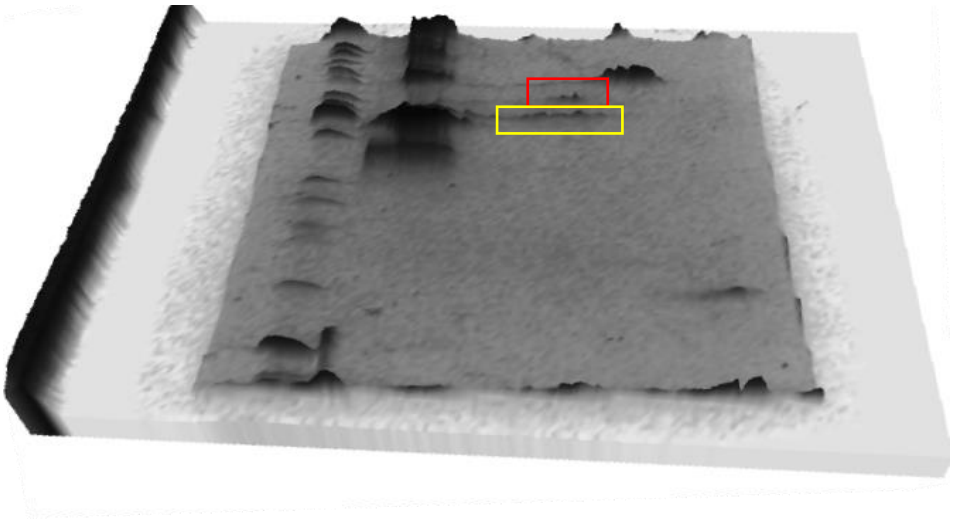


Figure 2.14. The three dimesional protein gel profile for 50 µg albumen extracted form a egg stored for 21 days at room temperature and loaded onto a 11 cm IGF strip with a pH range of 5-8.

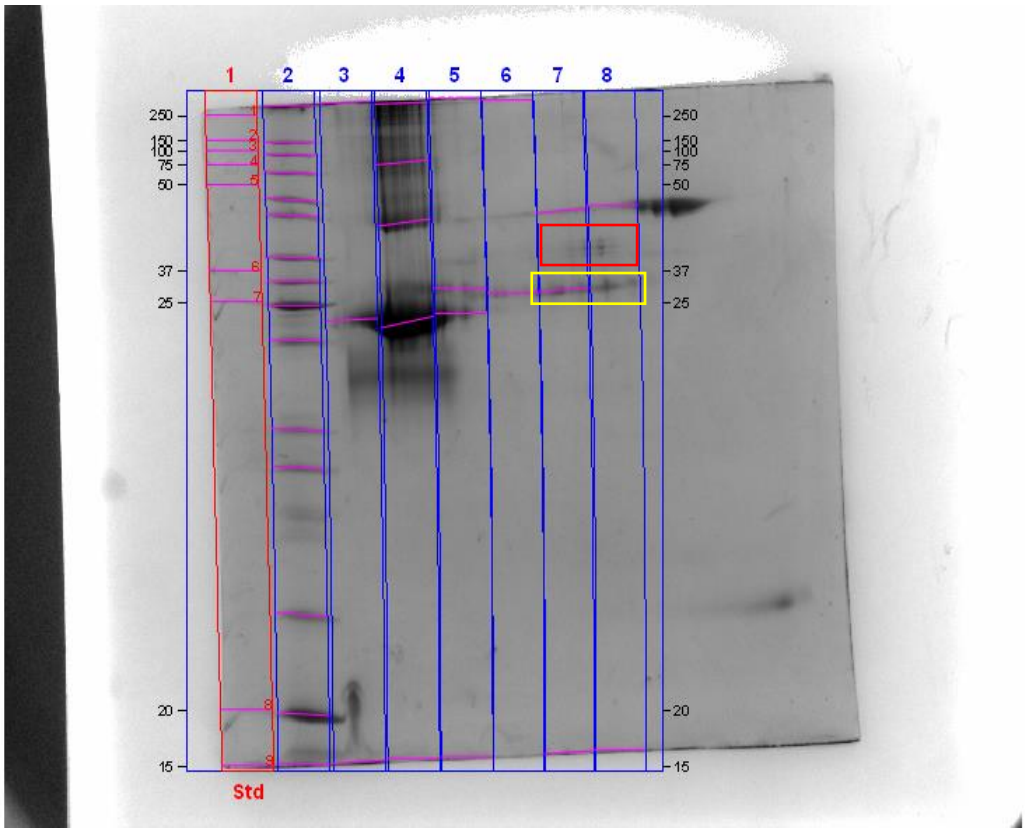


Figure 2.15. The three dimesional protein gel profile for 50 µg albumen extracted form a egg stored for 21 days at room temperature and loaded onto a 11 cm IGF strip with a pH range of 5-8 with the molecular weight grip overlain.

The areas of the profile that are of interest are marked in red and yellow boxes. At this point there has only been a visual appraisal of the protein profiles and comparison to published proteomic analysis by Wang et al. (2011) and Omana et al. (2011).

Protein separation using terminal amine isotopic labelling of albumen proteins (N-**TAILS**)

Terminal amine isotopic labelling of substrates (N-TAILS) is a method used to identify the protein content of samples based on the N-terminal fragments of proteins and can determine differences in abundance of specific proteins in a sample. TAILS uses negative selection and amine labelling-based quantification of proteins (Lai et al., 2015). The assay uses trypsin to break proteins into fragments and separates the N-terminal peptides (the fragments containing the N-termini of the original proteins) from the other fragments (internal tryptic peptides).

The LC-MS profile for albumen bulked from 5 eggs and stored in the refrigerator for 14 days is shown in Figure 2.16 and when stored for 21 days is shown in Figure 2.17 The LC-MS profile for albumen bulked from 5 eggs stored at room temperature for 14 days is shown in Figure 2.18 and when stored for 21 days is shown in Figure 2.19.

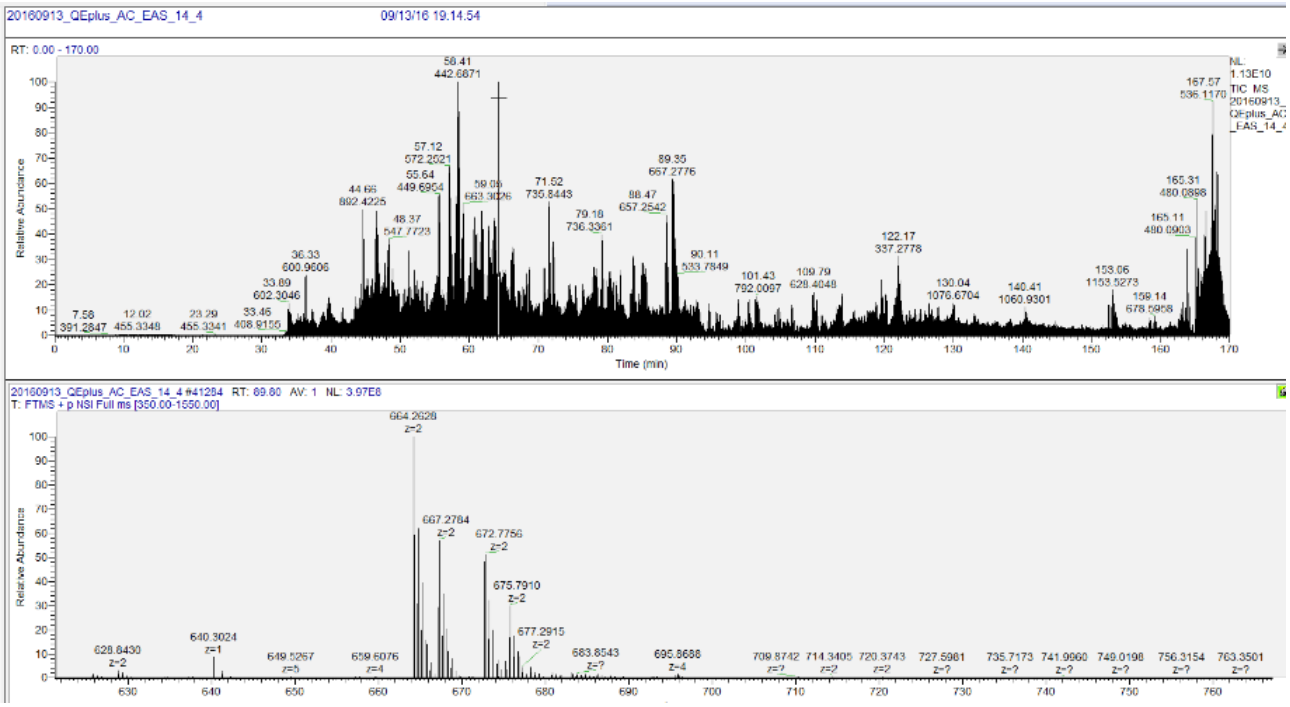


Figure 2.16. The LC/MS profile for albumen collected from bulked freshly lain eggs and eggs bulked stored in the refrigerator for 14 days.

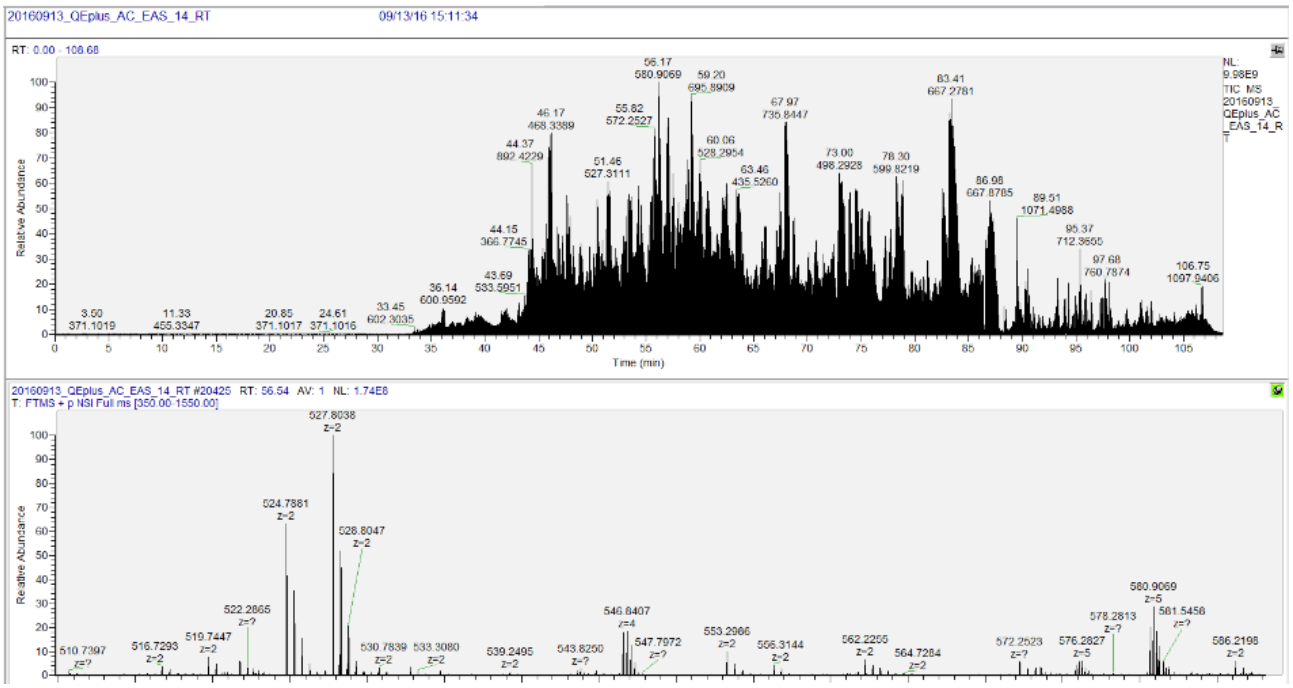


Figure 2.17. The LC/MS profile for the albumen collected from bulked freshly lain eggs and eggs stored at room temperature for 14 days.

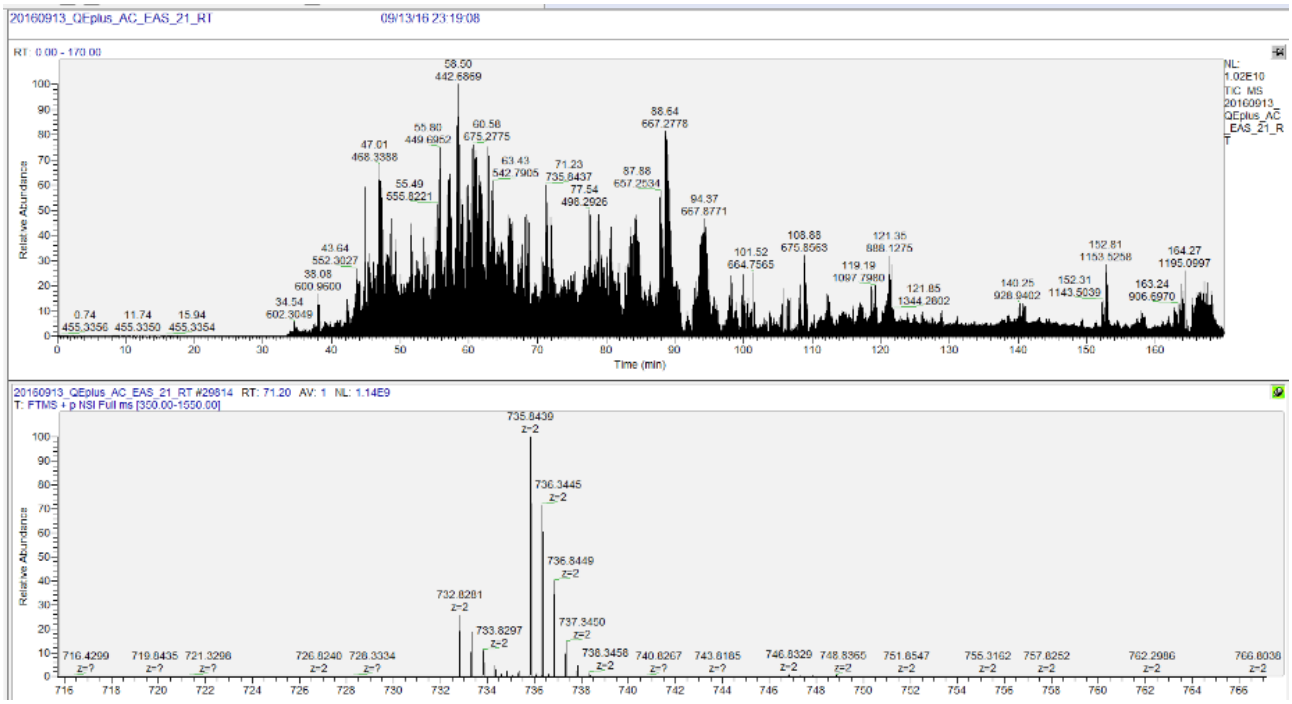


Figure 2.18. The LC/MS profile for the albumen collected from bulked freshly lain eggs and eggs store at room temperature for 21 days.

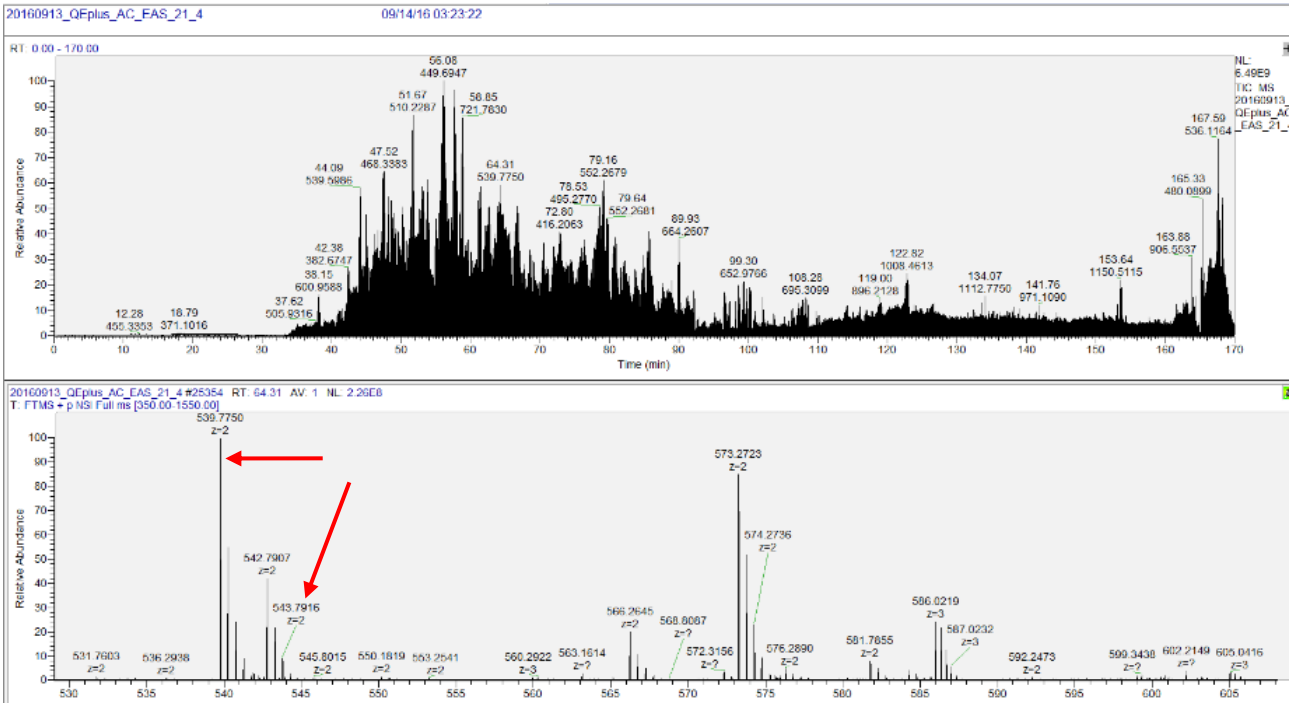


Figure 2.19. The LC/MS profile for the albumen collected from bulked freshly lain eggs and eggs stored in the refrigerator for 21 days.

Each of the LC-MS profiles consist of the peptides from the stored samples and that from the control freshly lain eggs. In the profiles the same peptide in the control and stored samples will elute at slightly different times because during the preparation phase the control peptides are tagged with ‘heavy formaldehyde’ and the peptides from the stored samples tagged with ‘light formaldehyde’. This results in the same peptides in the fresh

and stored samples having a weight differential of 6 Daltons when eluted. The upper panel in each figure is the peptide profile over the full elution time period with each peak being a peptide detected by the mass spectrometer. The lower panel of each figure shows a portion of the spectra when zoomed in on at a one particular time point. For example in Figure 2.19 two peaks are identified by the red arrows. One peptide is identified at 539 (m/z; mass/ion) and the other at 542 (m/z) which is mass difference of 6 daltons and most likely a dimethyl pair with one peptide from the stored albumen sample and the same peptide from the control albumen sample.

The number of proteins and peptides matched in each of the four LC-MS runs (see Table 2.2). The column ‘protein groups’, shows the number of unique proteins detected. The term “peptide spectral matches” (PSMs) indicates the total peptide matches with some peptides being detected multiple times. The number of unique peptides detected is listed in the ‘peptide groups’ column. The MS/MS column is the number of product ion scans collected during the analysis. There is good consistency between all of the runs, and it appears that there has been excellent coverage of the egg white proteome.

Table 2.2. A summary of the LC-MS peptide analysis of the egg albumen samples

LC-MS Run	Proteins	Protein Groups	PSMs	Peptide Groups	MS/MS
Control and day 21 room stored sample	2,125	2,076	36,702	17,035	156,468
Control and day 14 room stored sample	2,133	2,083	34,749	17,231	147,560
Control and day 21 refrigerator stored sample	2,137	2,102	37,468	20,669	150,560
Control and day 14 refrigerator stored sample	2,134	2,085	37,610	17,587	155,486

The data set generated is very large and indicates that there is a reasonable enrichment of new N-termini peptides observed in the stored albumen samples. The work is progressing to filter the data set and identify peptides associated with storage at each of the temperatures. An example of this process is shown Figure 2.20. The light and heavy versions of specific peptides are linked by the dotted lines.

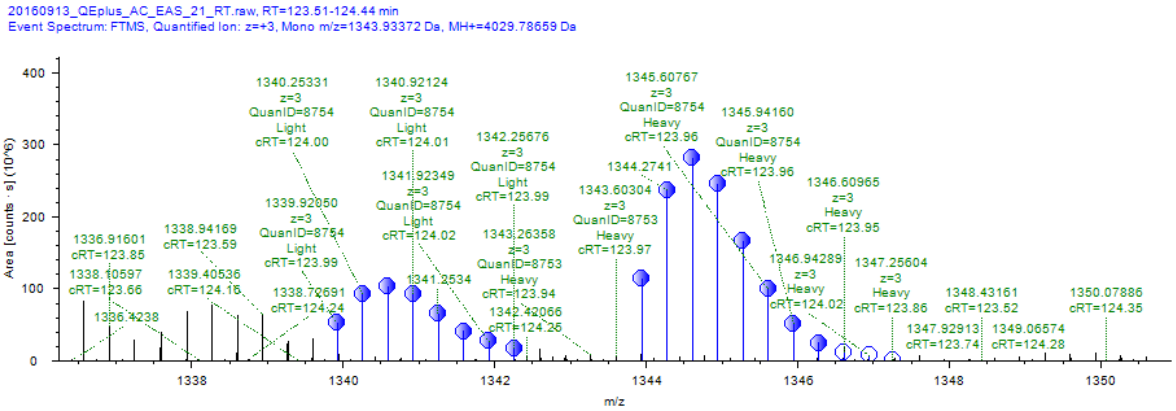


Figure 2.20. An example of the software analysis of the LC-MS profiles identifying the light and heavy versions of specific peptides.

Experiment 3. The effect of acute stress on egg quality during storage at different temperatures

Experiment 3a. The effect of acute relocation stress on egg albumen quality

Storage temperatures

The mean (± SEM) temperature for the refrigeration storage was of 4.2 ± 0.1ºC, for the cool room storage was 15.9 ± 0.1ºC, and storage in the room was 21.8 ± 0.1ºC.

Egg weight

The effects of stress and storage day on egg weight are shown in Table 3.1. Neither treatment (P = 0.545) nor storage day (P = 0.502) had an effect on egg weight. Storage temperature had a significant effect (P = 0.001). When eggs were stored at room temperature the weight (62.2 ± 0.4 g) was lower (P < 0.05), than when stored in the refrigerator (63.7 ± 0.3 g), in the cool room (64.1 ± 0.4 g) or at the point of lay (63.7 ± 03 g).

Table 3.1. The average (± SEM) weight (g) of eggs from hens housed in individual cages (Control) and then relocated (Relocation) to group cages and housed at 5 birds per cage.

Day	Treatment	
	Control	Relocation
0	63.5 ± 1.1	63.9 ± 1.0
7	63.7 ± 0.7	63.6 ± 0.7
14	63.5 ± 0.7	63.6 ± 0.7
21	65.1 ± 0.7	62.3 ± 0.7
28	62.1 ± 0.7	62.8 ± 0.7
36	63.2 ± 0.8	63.5 ± 0.7

Haugh Unit (HU)

Relocation had no effect on HU measurements (P = 0.758). There was a significant effect of storage temperature (see Table 3.2) but this changed with storage time as the interaction between the two was significant (P < 0.001). For all storage temperatures there was a significant decrease in HU from the day of lay until day 7 of storage (P < 0.05). On all storage days except day 14, the HU for eggs stored in the refrigerator was higher than for eggs stored in the cool room (P < 0.05). After day 14 eggs stored in the cool room had higher HU measures than those stored at room temperature (P < 0.05).

The HU measure remained the same over days 7 to 28 when storage was in the refrigerator but it was lower on day 36 than other days (P < 0.05). When stored in the cool room, the HU on day 7 was similar to that on days 14 and 21 but the difference between day 14 and 21 was significant (P < 0.50) as was the difference between days 14 and 28 (P < 0.05). The HU measure on day 36 was lower than all previous storage days (P < 0.05). When eggs were stored at room temperature the HU measures were similar on days 7 and 14 but higher than on day 21 (P < 0.05) which was in turn was higher than on days 28 and 36 (P < 0.05).

Table 3.2. The HU measures of eggs collected from hens housed in individual cages and then relocated to group cages and housed at 5 birds per cage. The eggs were stored in a refrigerator, in a cool room or at room temperature for 36 days after lay.

	Temperature treatment			
Day	Refrigerator	Cool room	Room	SEM
Day of lay	92.6_{A}	92.6_{A}	92.6_{A}	1.36
7	77.7_{B}^{a}	71.7_{BC}^{b}	70.2_{B}^{b}	
14	77.6_{B}^{a}	74.2_{B}^{ab}	71.4_{B}^{b}	
21	77.1_{B}^{a}	67.5_{C}^{b}	60.7_{C}^{c}	1.17
28	73.7_{B}^{a}	62.8_{C}^{b}	52.5_{D}^{c}	
36	68.9_{C}^{a}	56.4_{D}^{b}	50.7_{D}^{c}	
SEM		1.69		

[a-b] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different subscripts are significantly different (P < 0.05)

Albumen index (AI)

Relocation had no effect on the AI (P = 0.565). There was a significant effect of storage temperature (see Table 3.3) but this changed with storage time as the interaction between the two was significant (P = 0.012). For all storage temperatures there was a significant decrease in the AI from the day of lay until day 7 of storage (P < 0.05). On all storage days, except for day 14, were the AI was higher when eggs were stored in the refrigerator than in the cool room. After day 14, eggs stored in the cool room had higher AI than those stored at room temperature (P < 0.05).

The AI remained the same over days 7 to 28 when eggs were stored in the refrigerator but it was lower on day 36 (P < 0.05). When stored in the cool room the AI was similar on days

7 and 14 but higher on day 14 than day 21 ($P < 0.05$). The AI was similar on days 28 and 36 but lower on these days than on other days ($P < 0.05$). When stored at room temperature the AI was similar on days 7 and 14, but higher than the value at day 21 ($P < 0.05$). At day 28 and 36 the AI was similar and lower than the values on the previous days ($P < 0.05$).

Table 3.3. The mean (± SEM) AI of eggs collected from hens housed in individual cages and then relocated to group cages and housed at 5 birds per cage. The eggs were stored in a refrigerator, a cool room or at room temperature for 36 days after being laid.

Day	Temperature treatment			SEM
	Fridge	Cool room	Room	
0	0.112_{A}	0.112_{A}	0.112_{A}^{a}	1.36
7	0.075_{B}^{a}	0.60_{BC}^{b}	0.062_{B}^{b}	
14	0.072_{B}^{a}	0.66_{B}^{ab}	0.059_{B}^{b}	
21	0.071_{B}^{a}	0.056_{C}^{b}	0.045_{C}^{c}	0.003
28	0.070_{B}^{a}	0.045_{D}^{b}	0.034_{D}^{c}	
36	0.55_{C}^{a}	0.038_{D}^{b}	0.031_{D}^{b}	
SEM		0.003		

[a-b] Within a row values with different superscripts are significantly different ($P < 0.05$)
[A-D] Within a column values with different subscripts are significantly different ($P < 0.05$)

Yolk index (YI)

Treatment, storage day and storage temperature had significant effects on YI but there was a significant three-way interaction between these factors ($P = 0.006$). The effects of relocation stress on YI are given in Table 3.4.

Storage in the refrigerator. For control eggs stored in the refrigerator, the YI was lower on day 21 than on days 7, 28 and the day of lay ($P < 0.05$), but not the other days. For eggs collected after relocation the only difference was the higher YI on day 36 compared to day 14 of storage ($P < 0.05$). On storage days 21 and 36 eggs collected after relocation had a higher YI than eggs from the control treatment ($P < 0.05$).

Storage in the cool room. No differences in YI were seen when the eggs were stored in the cool room.

Storage at room temperature. When storage was at room temperature, the YI was higher on day 28 for eggs collected after relocation ($P < 0.05$). The YI for control eggs was lower on days 28 and 36 compared to day 7 and the day of lay ($P < 0.05$). For the eggs collected after relocation the YI on day 28 and was higher than on other days except for the day of lay ($P < 0.05$).

Table 3.4. The mean (± SEM) YI of eggs collected from hens housed in individual cages and then relocated to group cages and housed at 5 birds per cage. The eggs were stored in a refrigerator, a cool room or at room temperature for 36 days after being laid.

Treatments	Sampling day 0	7	14	21	28	36	SEM
Stored in the refrigerator							
Control	0.360^{a}	0.351^{a}	0.339^{ab}	$0.325_{B}{}^{b}$	0.357^{a}	$0.345_{B}{}^{ab}$	0.009
Relocation	0.354^{ab}	0.355^{ab}	0.340^{b}	$0.361_{A}{}^{ab}$	0.358^{ab}	$0.379_{A}{}^{a}$	
Stored in the cool room							
Control	0.360	0.338	0.336	0.340	0.341	0.354	0.009
Relocation	0.354	0.333	0.344	0.348	0.333	0.347	
Stored in the room							
Control	0.360^{a}	0.345^{ab}	0.333^{bc}	0.334^{bc}	$0.316_{B}{}^{c}$	0.315^{c}	0.009
Relocation	0.354^{ab}	0.330^{bc}	0.331^{bc}	0.322^{c}	$0.364_{A}{}^{a}$	0.333^{bc}	

$^{a-b}$ Within a row values with different superscripts are significantly different ($P < 0.05$)
$_{A-D}$ Within a column values with different subscripts are significantly different ($P < 0.05$)

Albumen corticosterone concentration

The effects of hen relocation on egg albumen corticosterone concentrations are given in Table 3.5. The treatment (control or relocation), storage day and storage temperature all had significant effects on egg albumen corticosterone concentrations. However, there was a significant interaction between the three factors ($P < 0.001$). On the day of lay, the egg albumen corticosterone concentrations were similar for both treatments. At all storage temperatures there was a gradual increase the corticosterone concentration over the period of storage.

Storage in the refrigerator. When eggs were stored in the refrigerator, the corticosterone concentration was higher in eggs from the relocated hens on day 21 and 36 compared to other days ($P < 0.05$). When the control eggs were stored in the refrigerator the corticosterone concentrations were similar on the day of lay and day 7, and lower than they were on days 14 and 21, were again they were similar but lower than on days on days 28 and 36, and independently on days 28 and 36 different to all other days (all, $P < 0.05$).

Storage in the cool room. When stored in the cool room, the eggs collected after relocation had higher corticosterone concentrations on all storage days ($P < 0.05$).

When the control eggs were stored in the cool room the corticosterone concentrations were similar on day of lay and day 7, and then higher on day 14 and then again higher on day 21 but thereafter remained similar (all, P< 0.05). For eggs collected after relocation the corticosterone concentrations were progressively higher as storage time increased (all, P < 0.05).

Storage at room temperature. When stored at room temperature, the eggs collected after relocation had higher corticosterone concentrations on days 14, 28 and 36 compared to control eggs (P < 0.05).

For the control eggs the corticosterone concentrations was higher on day 7 than the day of lay (P < 0.05), were similar of days 7 and 14, but then higher again on the days thereafter compared to the earlier storage days (all, P < 0.05). For eggs collected after relocation, the corticosterone concentrations were similar on the day of lay and day 7, then higher on days 14 and 21 where they were similar and then higher on day 28 and again then higher on day 36 (all, P < 0.05).

Table 3.5. The albumen corticosterone concentrations of eggs collected from hens housed in individual cages and then relocated to group cages and housed at 5 birds per cage. The eggs were stored in a refrigerator, a cool room or at room temperature for 36 days after being laid.

Treatments	Sampling day						SEM
	0	7	14	21	28	36	
Stored in refrigerator							
Control	0.33^{d}	0.55^{d}	1.11^{c}	$1.28_{B}{}^{c}$	1.83^{b}	$2.30_{B}{}^{a}$	0.10
Relocation	0.48^{d}	0.65^{d}	1.02^{c}	$1.78_{A}{}^{b}$	2.04^{b}	$2.75_{A}{}^{a}$	
Stored in cool room							
Control	0.33^{c}	$0.35_{B}{}^{c}$	$0.63_{B}{}^{b}$	$1.23_{B}{}^{a}$	$1.24_{B}{}^{a}$	$1.45_{B}{}^{a}$	0.10
Relocation	0.48^{f}	$0.75_{A}{}^{e}$	$1.11_{A}{}^{d}$	$1.50_{A}{}^{c}$	$1.87_{A}{}^{b}$	$2.09_{A}{}^{a}$	
Stored in the room							
Control	0.33^{e}	0.72^{d}	$0.65_{B}{}^{d}$	0.99^{c}	$1.36_{B}{}^{b}$	$1.24_{B}{}^{a}$	0.11
Relocation	0.48^{d}	0.63^{d}	$1.04_{A}{}^{c}$	1.22^{c}	$1.75_{A}{}^{b}$	$2.40_{A}{}^{a}$	

$^{a-b}$ Within a row values with different superscripts are significantly different (P < 0.05)
$_{A-D}$ Within a column values with different subscripts are significantly different (P < 0.05)

The mean (± SEM) pattern of change in corticosterone concentrations for control eggs and those collected after relocation and stored at different temperatures for 36 days after lay is given in Figure 3.1. There was an accumulative increase in the albumen corticosterone concentration as storage time increased and was higher in albumen of eggs collected after relocation from day 14 of storage.

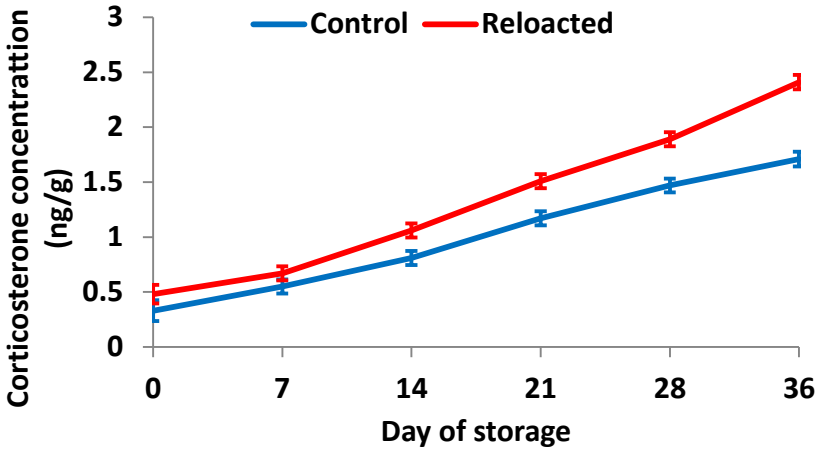


Figure 3.1. The pattern of albumen corticosterone concentrations in eggs collected from hens housed in individual cages and then relocated to group cages and housed at 5 birds per cage. The eggs were stored in a refrigerator, a cool room or at room temperature for 36 days after being laid.

Experiment 3b. The effect of transport and relocation acute stress on egg quality

Housing temperature during the experimental period

The temperature and humidity recorded in the layer housing during the trial is given in Figure 3.2. The average daily temperature and humidity are given in Table 3.6. The average daily temperature was similar for all days. The average humidity varied to a larger degree but the differences were not excessive.

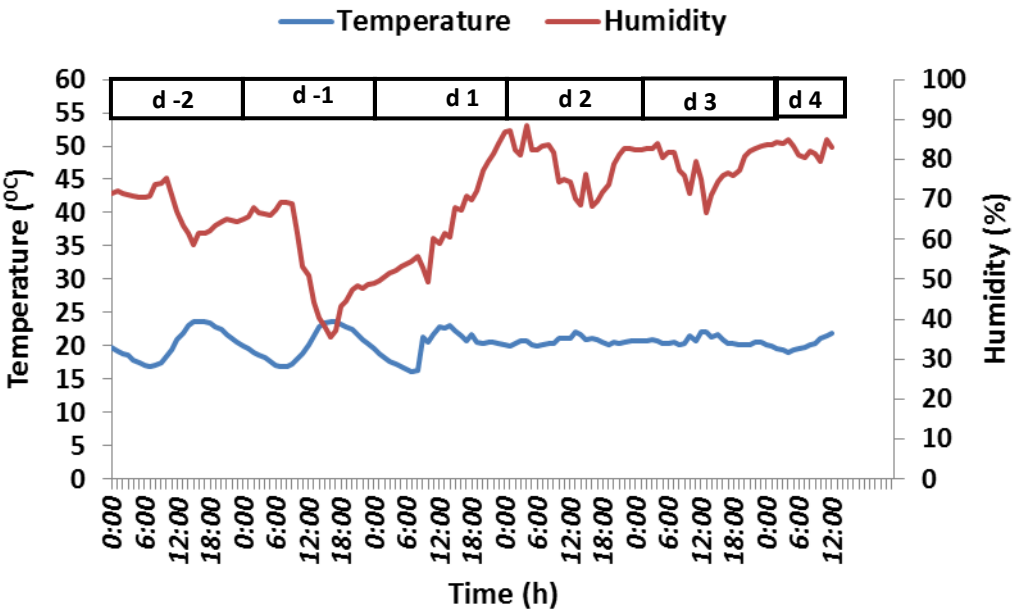


Figure 3.2. The layer housing temperature and humidity during the experimental period. The recordings were made for 2 days (d -2 and d -1) before the start of egg collections. Eggs were collected on day 1 (S1) and then on day 2 (d 2) the hens were exposed to transport and relocation stress. Eggs were collected on the day following the stress, day 3 (S3), and also the on day 4 (S4).

Table 3.6. The average (± SEM) daily temperatures and humidity's during the experimental period. The values were made for the 2 days (d -2 and d-1) before the initial egg collection on day 1 (S1) and then the day 2 when the acute stress as applied, and then on day 3 and day 4, being the first and second days after the acute stress was applied.

Day	Average temperature (^{0}C)	Average relative humidity (%)
Day -2	20.3 ± 0.5	67.5 ± 1.0
Day -1	20.2 ± 0.5	54.0 ± 2.4
Day 1	19.9 ± 0.4	61.8 ± 2.2
Day 2	20.7 ± 0.5	78.6 ± 1.2
Day 3	20.7 ± 0.1	78.5 ± 0.9
Day 4	20.1 ± 0.2	74.7 ± 4.4

Haugh unit (HU) measurements for normal and defective shelled eggs

Of the eggs laid on day 3, 67.5% had normal shell morphology, 22.8% had the target shell morphology and 9.7% were misshapen or soft-shelled. The effect of shell morphology on HU measures is given in Figure 3.3. Misshapen and soft-shelled eggs had similar HU measures as the eggs with normal shells but the targeted eggs had a lower HU than the other egg shell categories ($P < 0.05$). Because the target eggs had lower HU measures at the point of lay they were discarded from the overall comparison of eggs stored at different temperatures and collected on individual days 1, 3 and 4. However, a comparison of the effect of storage temperature on the targeted eggs in comparison with normal shelled eggs was made on day 3 collections as an independent analysis.

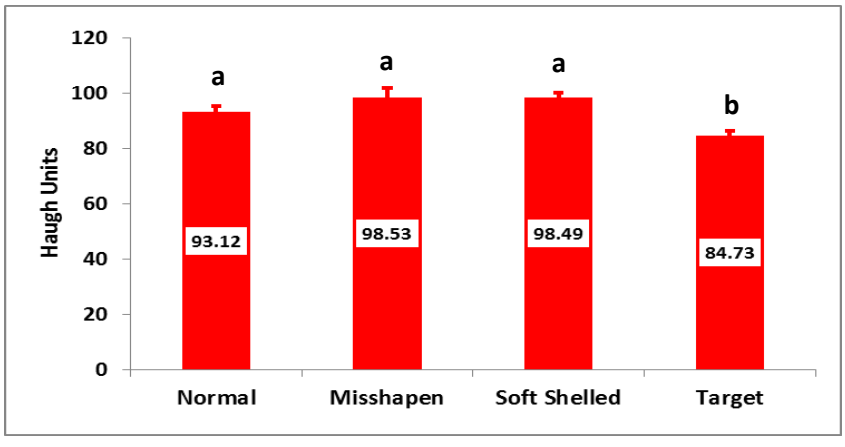


Figure 3.3. The HU measures for egg collected after hens were acutely stressed by transporting them for 20 min and then rehousing them in groups of four in modified cages (containing a perch and nest box). The eggs were collected on the morning after the stress was applied. Values with different letters (a-b) are significantly different ($P < 0.05$).

The effects of shell morphology on egg quality during storage

Haugh unit (HU) measurements during storage

There was no significant effect of shell morphology on HU during storage (P = 0.176) but there was a significant (P= 0.022) interaction between egg shell morphology and storage temperature on HU (see, Table 3.7). There was also a significant interaction between storage day and storage time on HU (P < 0.001).

As stated previously the eggs laid on the day (day 3), and classified as being targeted had lower HU measures compared to the eggs having normal shells (P < 0.05). This early difference was not seen during the 28 days of storage at any of the storage temperatures. For both normal and targeted shelled eggs there was a significant differences in HU at all storage temperatures (P < 0.05), with it being highest when storage was in the refrigerator, followed by storage in the cool room and the lowest when eggs were stored at room temperature. So while there was a difference in HU at lay, the targeted eggs had similar measures at all storage temperatures over the 28 day storage period.

Table 3.7. The effect of storage conditions on mean (± SEM) HU for eggs sampled over 28 days while being stored at room temperature, in a cool room or refrigerator and having been collected from hens acutely stressed by transporting them for 20 min and then rehousing them in groups of four in modified cages (containing a perch and nest box). The eggs were collected on the day after applying the acute stress and identified as having normal shell morphology or those with excess calcium deposited on the shell to give a targeted appearance.

Egg shell morphology	Storage conditions			
	Day of lay	Refrigerator	Cool room	Room
Normal	$93.6 \pm 3.1^{a}{}_{A}$	81.4 ± 1.5^{b}	67.1 ± 1.52^{c}	54.1 ± 1.5^{d}
Targeted	$81.6 \pm 4.3^{a}{}_{B}$	78.7 ± 2.8^{a}	71.6 ± 2.4^{b}	58.3 ± 2.7^{c}

$^{a-d}$ Values in a row without similar superscripts are significantly different (P < 0.05)
$_{AB}$ Values in a column for specific storage temperature (refrigerator, cool room or room) without similar subscripts are significantly different (P < 0.05)

The effects of storage conditions and storage time on the HU measures for eggs collected from acutely stressed hens with normal and targeted shells are given Table 3.8. From day 7, the HU measures remained the same when eggs were stored in the refrigerator for the entire storage period. When eggs were stored in the cool room, the HU measures were similar on days 14 and 21 and lower than they were on day 7 (P< 0.05) and the HU at day 28 was lower than on all other days (P< 0.05). This same pattern was evident for eggs stored at room temperature.

After day 7, eggs stored in the refrigerator had higher HU measures compared to other storage temperatures (P < 0.05) and those in the cool room had higher HU than those stored at room temperature at all times (P < 0.05).

Table 3.8. The effect of sampling day and storage temperature on mean (± SEM) HU for eggs sampled over 28 days while being stored at room temperature, in a cool room or refrigerator and having been collected from hens acutely stressed by transporting them for 20 min and then rehousing them in groups of four in modified cages (containing a perch and nest box). The eggs were collected on the morning after applying the acute stress and identified as having normal shell morphology or those with excess calcium deposited on the shell to give a targeted appearance.

	Storage conditions		
Storage day	Refrigerator	Cool room	Room
Day of lay	89.4_{A}	89.4_{A}	89.4_{A}
7	$80.2^{a}{}_{B}$	$76.1^{a}{}_{B}$	$66.0^{b}{}_{B}$
14	$80.7^{a}{}_{B}$	$70.8^{b}{}_{C}$	$55.6^{c}{}_{C}$
21	$82.7^{a}{}_{B}$	$70.2^{b}{}_{C}$	$50.8^{c}{}_{C}$
28	$78.6^{a}{}_{B}$	$53.5^{b}{}_{D}$	$45.8^{c}{}_{D}$
SEM		2.2	

$^{a-d}$ Values in a row without similar superscripts are significantly different ($P < 0.05$)
$_{AB}$ Values in a column for specific storage temperature (refrigerator, cool room or room) without similar subscripts are significantly different ($P < 0.05$)

Albumen index (AI)

Shell morphology had an effect on AI (see, Table 3.9) but this changed with storage temperature as the interaction between shell morphology and storage time was significant ($P < 0.001$). On the day of lay, eggs classified as being targeted had lower AI compared to normal shelled eggs ($P < 0.05$). This early difference was not seen during the 28 days of storage at any of the storage temperatures. For both the normal and targeted eggs the AI was highest when eggs were stored in the refrigerator than at other temperatures and the AI was higher when storage was in the cool room compared to storage at room temperature (all, $P < 0.05$).

Table 3.9. The effect of storage conditions on mean (± SEM) AI for eggs sampled over 28 days while being stored at room temperature, in a cool room or refrigerator and having been collected from hens acutely stressed by transporting them for 20 min and then rehousing them in groups of four in modified cages (containing a perch and nest box). The eggs were collected on the morning after applying the acute stress and identified as having normal shell morphology or those with excess calcium deposited on the shell to give a targeted appearance.

		Storage conditions		
Egg shell morphology	Day of lay	Refrigerator	Cool room	Room
Normal	$0.118^{a}{}_{A}$	0.080^{b}	0.055^{c}	0.033^{d}
Targeted	$0.079^{a}{}_{B}$	0.072^{a}	0.060^{b}	0.041^{c}
		0.004		

SEM	

[a-d] Values in a row without similar superscripts are significantly different (P < 0.05)
[AB] Values in a column for specific storage temperature (refrigerator, cool room or room) without similar subscripts are significantly different (P < 0.05)

The effects of storage temperature and storage day on the AI are given in Table 3.10. There was a significant interaction between storage temperature and storage time (P = 0.003). The AI remained the same when eggs were stored in the refrigerator for the entire period and it was a similar situation when stored in the cool room except at 28 days when it was lower (P< 0.05). Storage at room temperature resulted in lower AI at 21 and 28 days compared to days 7 and 14 (P < 0.05).

After day 7 eggs stored in the refrigerator had higher AI compared to other storage temperatures (P < 0.05) and those in the cool room had higher index than those stored at room temperature at all times (P < 0.05).

Table 3.10. The effect of storage temperature and storage conditions on mean (± SEM) AI for eggs sampled over 28 days while being stored at room temperature, in a cool room or refrigerator and having been collected from hens acutely stressed by transporting them for 20 min and then rehousing them in groups of four in modified cages (containing a perch and nest box). The eggs were collected on the morning after applying the acute stress and identified as having normal shell morphology or those with excess calcium deposited on the shell to give a targeted appearance.

	Storage conditions		
Storage day	Refrigerator	Cool room	Room
Day of lay	0.098_{A}	0.098_{A}	0.098_{A}
7	$0.73^{a}{}_{B}$	$0.65^{a}{}_{B}$	$0.52^{b}{}_{B}$
14	$0.77^{a}{}_{B}$	$0.61^{b}{}_{B}$	$0.47^{c}{}_{B}$
21	$0.83^{a}{}_{B}$	$0.59^{b}{}_{B}$	$0.32^{c}{}_{C}$
28	$0.71^{a}{}_{B}$	$0.32^{b}{}_{C}$	$0.23^{b}{}_{C}$
SEM		0.005	

[a-d] Values in a row without similar superscripts are significantly different (P < 0.05)
[AB] Values in a column for specific storage temperature (refrigerator, cool room or room) without similar subscripts are significantly different (P < 0.05)

Yolk Index (YI)

There was no effect of egg shell morphology on the YI (P = 0.185). The effects of storage conditions and storage time on the YI are given Table 3.11. The interaction between storage day and storage temperature was considered too be significant with P = 0.052. The YI remained the same when eggs were stored in the refrigerator for the entire period and it was a similar situation when stored in the cool room except at 28 days when it was lower compared to days 7 and 14 (P < 0.05). Storage at room temperature resulted in lower YI at 21 and 28 days compared to days 7 and 14 (P < 0.05).

After day 14 eggs stored in the refrigerator had higher yolk YI compared to other storage temperatures ($P < 0.05$) and those in the cool room had higher YI than those stored at room temperature after day 7 ($P < 0.05$).

Table 3.11. The effect of storage day and storage conditions on mean (± SEM) YI for eggs sampled over 28 days while being stored at room temperature, in a cool room or refrigerator and having been collected from hens acutely stressed by transporting them for 20 min and then rehousing them in groups of four in modified cages (containing a perch and nest box). The eggs were collected on the morning after applying the acute stress and identified as having normal shell morphology or those with excess calcium deposited on the shell to give a targeted appearance.

	Storage conditions		
Sampling day	Refrigerator	Cool room	Room
Day of lay	0.497	0.497_{A}	0.497_{A}
7	0.462	0.422_{B}	0.408_{B}
14	0.457^{a}	$0.433^{a}{}_{AB}$	$0.389^{b}{}_{B}$
21	0.456^{a}	$0.380^{b}{}_{BC}$	$0.316^{c}{}_{C}$
28	0.458^{a}	$0.363^{b}{}_{C}$	$0.314^{c}{}_{C}$
SEM		0.021	

$^{a-d}$ Values in a row without similar superscripts are significantly different ($P < 0.05$)
$_{AB}$ Values in a column for specific temperature treatments (refrigerator, cool room or room) without similar subscripts are significantly different ($P < 0.05$)

The effect of acute stress on egg quality in eggs having normal shell morphology

Based on the differences in egg quality between targeted eggs and normally shelled eggs on the day of lay and the fact, that they would not be included in those eggs which reach the retail market they were removed from the following analysis. So the comparisons here are only between normal shelled eggs collected on treatment days 1 (S1), 3 (S3) and 4 (S4) of the study.

Haugh units (HU)

The effect of the acute stress on HU measures is given in Table 3.12. There was a significant interaction between treatment day x storage condition x storage time ($P = 0.003$). For all treatment days and at all storage temperatures there was significant decrease in HU from the day of lay and day 7 of storage ($P < 0.05$).

Storage in the refrigerator. When stored in the refrigerator, the HU measure for treatment S1 on day 7 was similar to day 14 but different to those on day 21 and 28 ($P < 0.05$). The values on days 14, 21 and 28 were similar. For treatments S3 and S4, HU measures were similar on all storage days.

On days 0, 7 and 14 all treatment groups had similar HU measures. On days 21 and 28, the HU value for treatment S1 was lower than that for S3 and S4 ($P < 0.05$).

Storage in the cool room. The differences here were more varied than when the storage was in the refrigerator. For treatment S1, the HU measures differed between storage days except for the comparison between day 21 and 28 ($P < 0.05$). For treatment day S3, HU was similar for days 7, 14 and 21 but lower on day 28 than the previous days ($P < 0.05$). For treatment S4, the differences in HU were similar to that seen for S1 with the lowest values being on days 21 and 28.

No differences between groups was observed until day 21 of storage when the HU for treatment S3 was higher than for treatment S1 ($P < 0.05$) but similar to S4. However, on day 28 the HU measure was lower for treatment S3 than other treatments ($P < 0.05$).

Storage at room temperature. Again the differences here were more varied than when the storage was in the refrigerator. For all treatment groups, the HU measures were higher on day 7 then day 14 ($P < 0.05$). For treatment S1 the HU measures on day 21 and 28 were similar and the value on day 14 was lower than day 28 ($P < 0.05$). For treatment S3 the HU measure on day 21 was similar to day 14 and day 28 but the day 28 measure was lower than day 14 ($P < 0.05$). For treatment S4 the HU measures on day 21 and 28 were similar but lower when compared to earlier storage days.

On day 14 and 21 the HU measure was higher for treatments S3 and S4 compared to treatment S1 ($P < 0.05$). On day 28 the HU measure for treatment S3 was higher than for other treatments ($P < 0.0$).

Table 3.12. The mean (± SEM) HU for eggs sampled over 28 days while being stored at room temperature, in a cool room or refrigerator and having been collected from hens housed in individual cages (S1 - being day 1) and then acutely stressed by transporting them for 20 min and then rehousing in groups of four in modified cages (containing a perch and nest box), with eggs collected on the next day (S3 - being day 3) and then again on the following day (S4 - being day 4).

Treatments	Sampling day 0	7	14	21	28	SEM
			Stored in refrigerator			
S1	94.1^{a}	85.6^{b}	83.1^{bc}	76.6^{c}_{B}	77.3^{c}_{B}	2.72
S3	93.6^{a}	81.2^{b}	80.1^{b}	84.1^{ab}_{A}	79.8^{b}_{A}	
S4	98.5^{a}	84.4^{b}	79.3^{b}	82.4^{b}_{A}	77.5^{b}_{A}	
			Stored in cool room			
S1	94.1^{a}	78.2^{b}	69.6^{c}	60.7^{d}_{B}	60.3^{d}_{A}	2.72
S3	93.6^{a}	77.4^{b}	70.4^{b}	70.4^{b}_{A}	44.9^{c}_{B}	
S4	98.5^{a}	74.9^{b}	69.6^{bc}	65.0^{cd}_{AB}	61.5^{d}_{A}	
			Stored in the room			
S1	93.9^{a}	65.1^{b}	45.2^{c}_{B}	38.6^{cd}_{B}	37.7^{d}_{B}	2.75
S3	89.5^{a}	63.5^{b}	55.6^{c}_{A}	48.1^{cd}_{A}	45.4^{d}_{A}	
S4	98.3^{a}	68.1^{b}	55.9^{c}_{A}	46.3^{d}_{A}	38.9^{d}_{B}	
SEM			2.73			

$^{a-d}$ Values in a row without similar superscripts are significantly different ($P < 0.05$)
$_{AB}$ Values in a column for specific temperature treatments (refrigerator, cool room or room) without similar subscripts are significantly different ($P < 0.05$)

Albumen index (AI)

Treatment day had no effect on AI ($P = 0.312$). Storage temperature had a significant effect on AI but it was influenced by day as the storage temperature x day interaction was significant ($P < 0.001$). The temperature and day effects are given in Table 3.13. There was also a marginally non-significant interaction ($P = 0.077$) between storage temperature and treatment day (See Table 3.14).

On all storage days the eggs stored in the refrigerator had higher AI compared to other storage temperatures ($P < 0.05$) and those eggs stored in the cool room had higher AI than eggs stored at the room temperature ($P < 0.05$).

At all storage temperatures there was a significant decrease in AI from day of lay and 7 days of storage ($P < 0.05$). When stored in the refrigerator AI was lower on day 28 compared to day 7 ($P < 0.05$) with the AI on days 14 and 21 being intermediate between these values. Storage in the cool room resulted in a progressively lower AI on all storage days (all, $P < 0.05$). This was similar when storage was at room temperature except the values of day 21 and 28 were similar (all, $P < 0.05$).

Table 3.13. The mean AI for eggs sampled over 28 days while being stored at room temperature, in a cool room or refrigerator having been collected from hens housed in individual cages (S1 - being day 1) and then acutely stressed by transporting them for 20 min and then rehousing in groups of four in modified cages (containing a perch and nest box), with eggs collected on the next day (S3 - being day 3) and then again on the following day (S4 - being day 4).

	Storage conditions		
Sampling day	Refrigerator	Cool room	Room
Day of lay	0.113_{A}	0.113_{A}	0.113_{A}
7	$0.083^{a}{}_{B}$	$0.066^{b}{}_{B}$	$0.047^{c}{}_{B}$
14	$0.077^{a}{}_{BC}$	$0.055^{b}{}_{C}$	$0.030^{c}{}_{C}$
21	$0.077^{a}{}_{BC}$	$0.048^{b}{}_{D}$	$0.022^{c}{}_{D}$
28	$0.070^{a}{}_{C}$	$0.037^{b}{}_{E}$	$0.018^{c}{}_{D}$
SEM		0.002	

$^{a-d}$ Values in a row without similar superscripts are significantly different ($P < 0.05$)
$_{AB}$ Values in a column for specific temperature treatments (refrigerator, cool room or room) without similar subscripts are significantly different ($P < 0.05$)

At all storage temperatures there was no difference in AI for different treatment days. On the day of lay there was a tendency for the AI to be lower for eggs collected on the morning after the acute stress.

Table 3.14. The mean AI for eggs stored at room temperature or in a cool room or refrigerator having been collected from hens housed in individual cages (S1 - being day 1) and then acutely stressed by transporting them for 20 min and then rehousing in groups of four in modified cages (containing a perch and nest box), with eggs collected on the next day (S3 - being day 3) and then again on the following day (S4 - being day 4).

Treatment	Storage conditions			
	Day of lay	Refrigerator	Cool room	Room
S1	0.112	0.077	0.050	0.025
S3	0.106	0.078	0.048	0.030
S4	0.124	0.077	0.051	0.029
SEM		0.002		

<u>Yolk index (YI)</u>

The storage temperature effects on YI are given in Table 3.15. There was a significant interaction between treatment day and storage temperature ($P < 0.001$). On the day of lay

the YI was higher for treatment day S3 compared to other treatment days ($P < 0.05$). At all storage temperatures there was no effect of treatment day on the YI.

The YI of eggs stored in the refrigerator was higher than for eggs stored at room temperature ($P < 0.05$), with the values of eggs stored in the cool room being intermediate.

Storage day had a significant effect on YI ($P < 0.001$). The YI on the day of lay (0.565 ± 0.03) was higher than other days ($P < 0.05$), but after this the YI on all storage days was similar, with it the same for day 7 and 14 at 0.415 ± 0.02; for day 21 it was 0.374 ± 0.02 and for day 28 it was 0.381 ± 0.02).

Table 3.15. The mean YI for eggs stored at room temperature, in a cool room or refrigerator having been collected from hens housed in individual cages (S1 - being day 1) and then acutely stressed by transporting them for 20 min and then rehousing in groups of four in modified cages (containing a perch and nest box), with eggs collected on the next day (S3 - being day 3) and then again on the following day (S4 - being day 4).

	Storage conditions			
Treatment	Day of lay	Refrigerator	Cool room	Room
S1	$0.471^{a}{}_{B}$	0.452^{a}	0.400^{ab}	0.352^{b}
S3	$0.877^{a}{}_{A}$	0.456^{b}	0.407^{bc}	0.341^{c}
S4	$0.462^{a}{}_{B}$	0.430^{a}	0.381^{ab}	0.341^{b}
SEM	0.002			

$^{a-d}$ Values in a row without similar superscripts are significantly different ($P < 0.05$)
$_{AB}$ Values in a column for specific temperature treatments (refrigerator, cool room or room) without similar subscripts are significantly different ($P < 0.05$)

Albumen corticosterone concentrations

Treatment day had a significant effect on the egg albumen corticosterone concentration but it was influenced by storage day and storage temperature as the three-way interaction, treatment day x storage time x storage temperature was significant ($P < 0.001$). The effect of treatment day on albumen corticosterone concentrations are shown in Table 3.16. On the day of lay the albumen corticosterone concentration was lower for treatment S4 compared to treatments S1 and S3 ($P < 0.05$).

Storage in the refrigerator. For treatment S1, the corticosterone concentration on day 28 of storage was higher than on the day of collection ($P < 0.05$), with the values on other days being intermediate to these values. For treatment S3, the corticosterone concentration was lower on the day of lay than other days ($P < 0.05$), while the values on day 7, 14 and 21 were similar, but lower than on day 28 ($P < 0.05$). For treatment S4, the corticosterone concentration was lower on the day of lay than other days ($P < 0.05$). The concentrations were similar on days 21 and 28 and higher on these days are compared to days 7 and 14 ($P < 0.05$).

On storage days 7 and 14 the albumen corticosterone concentration was higher for treatment day S3 than other treatment days ($P < 0.05$). On day 21 and 28 the albumen corticosterone concentration was lower on treatment day S1 than other days ($P < 0.05$), and on day 28 the albumen corticosterone concentration was lower on treatment day S4 than day S3 ($P < 0.05$).

Storage in the cool room. For treatment day S1 the corticosterone concentrations on the day of lay, days 7 and 14 were similar, but lower than on day 28 ($P < 0.05$). While the concentration on day 21 was similar to day 7 it was higher than on the day of lay and day 14 of storage ($P < 0.05$). For treatment S3, the corticosterone concentration was lower on the day of lay than other days ($P < 0.05$), and the concentrations on days 21 and 28 were higher than on day 7 ($P < 0.05$), but similar to day 14. For treatment S4, the corticosterone concentration was higher on day 21 than other days and lower on day of lay than other days (all, $P < 0.05$). The concentration was similar on days 7 and 14 but lower on these days than day 28 ($P < 0.05$).

On all storage days the albumen corticosterone concentration was higher for treatment day S3 than S1 ($P < 0.05$). On storage days 21 and 28 the albumen corticosterone concentration was higher for treatment day S4 than S1 ($P < 0.05$), while on these days the albumen corticosterone concentration was similar for collection days S3 and S4. However, the albumen corticosterone concentration on days 7 and 14 were higher for treatment day S3 than S4 ($P < 0.05$).

Storage at room temperature. For treatment S1 and S4, the corticosterone concentration was lower on the day of lay than other days ($P < 0.05$). For these same treatment days the corticosterone concentrations on day 7 and 14 were similar and lower when compared to day 21 and 28 ($P < 0.05$) where again they were similar to one another. For treatment S3, the corticosterone concentration was lower on the day of lay than other days ($P < 0.05$). The albumen corticosterone concentration were similar on day 7 and 14, but lower on these days than day 21 which in turn was lower than the concentration on day 28 (all, $P < 0.05$).

After day 7 of storage, the albumen corticosterone concentrations for treatment day S3 were higher than on treatment day S1 ($P < 0.05$). The albumen corticosterone concentrations for treatment S4 were higher compared to treatment day S1 on storage days 21 and 28 ($P < 0.05$) but not day 7 and 14. The albumen corticosterone concentrations were higher for treatment day S3 than day S4 on storage days 14 and 28 ($P < 0.05$) but not day 21.

Table 3.16. Egg albumen corticosterone concentration for eggs stored at room temperature or in a cool room or refrigerator having been collected from hens housed in individual cages (S1 - being day 1) and then acutely stressed by transporting them for 20 min and then rehousing in groups of four in modified cages (containing a perch and nest box), with eggs collected on the next day (S3 - being day 3) and then again on the following day (S4 - being day 4).

Treatments	Sampling day					SEM
	0	7	14	21	28	
			Stored in refrigerator			
S1	1.80_{A}^{b}	2.28_{B}^{ab}	2.11_{B}^{ab}	2.34_{B}^{ab}	2.52_{C}^{a}	
S3	1.33_{A}^{c}	3.52_{A}^{b}	3.59_{A}^{b}	3.98_{A}^{b}	4.66_{A}^{a}	1.97
S4	1.09_{B}^{c}	2.48_{B}^{b}	2.61_{B}^{b}	3.69_{A}^{a}	3.39_{B}^{a}	
			Stored in cool room			
S1	1.80_{A}^{c}	2.07_{B}^{bc}	1.91_{B}^{c}	2.55_{B}^{ab}	2.79_{B}^{a}	
S3	1.33_{A}^{c}	3.02_{A}^{b}	3.55_{A}^{ab}	3.98_{A}^{a}	3.78_{A}^{a}	2.03
S4	1.09_{B}^{d}	2.45_{B}^{c}	2.06_{B}^{c}	4.40_{A}^{a}	3.68_{A}^{b}	
			Stored in the room			
S1	1.80_{A}^{c}	2.48^{b}	2.61_{B}^{b}	3.69_{B}^{a}	3.39_{C}^{a}	
S3	1.33_{A}^{d}	2.77^{c}	3.20_{A}^{c}	3.98_{A}^{b}	5.11_{A}^{a}	1.96
S4	1.09_{B}^{c}	2.31^{b}	2.63_{B}^{b}	4.29_{A}^{a}	4.07_{B}^{a}	
SEM			1.99			

[a-d] Values in a row without similar superscripts are significantly different ($P < 0.05$)
[AB] Values in a column for specific temperature treatments (refrigerator, cool room or room) without similar subscripts are significantly different ($P < 0.05$)

The pattern of corticosterone concentration in albumen for treatment days S1, S3 and S4 from the eggs stored at different temperatures over 28 days is shown in Figure 3.5.

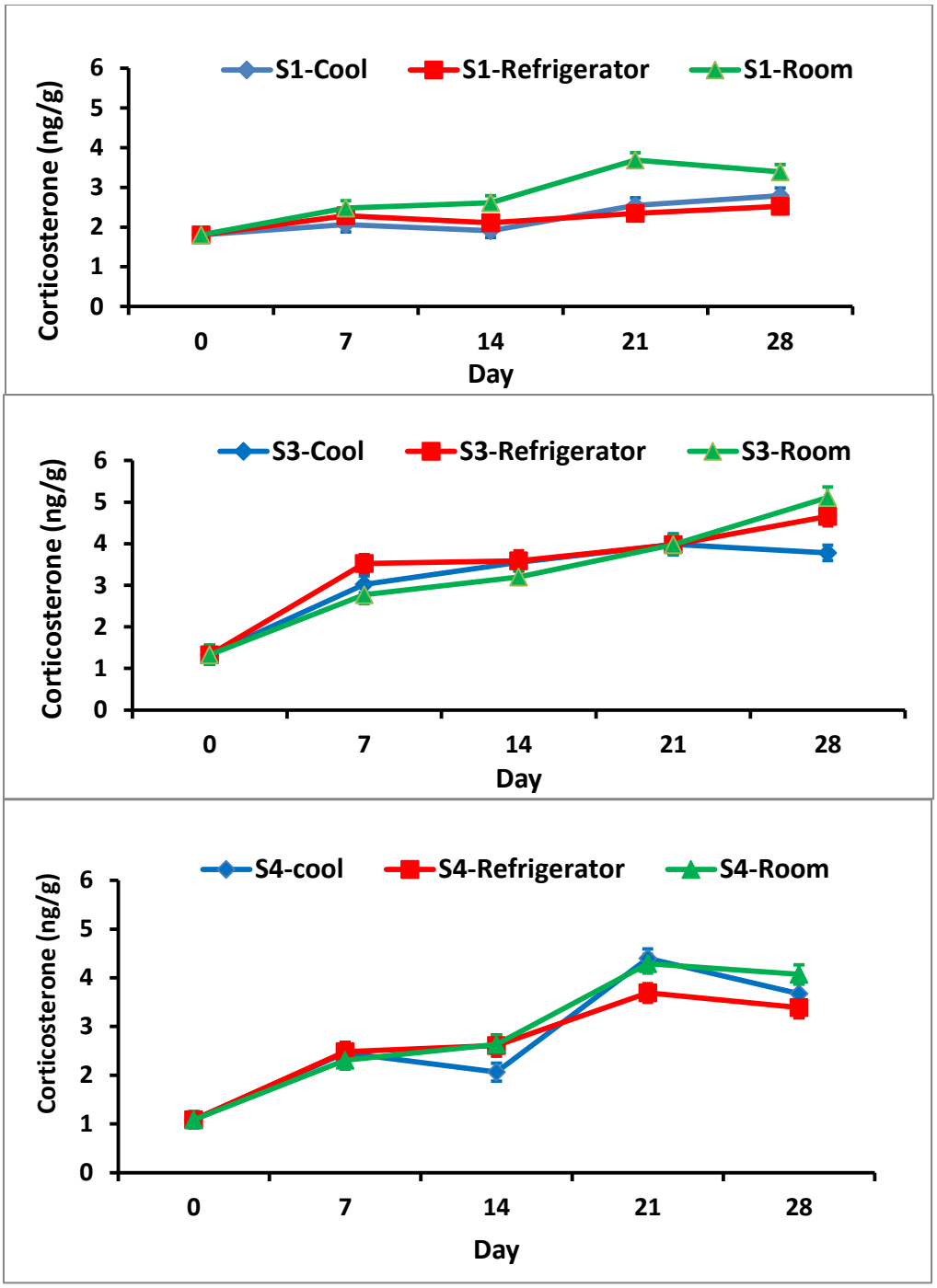


Figure 3.5. The pattern of albumen corticosterone concentration for eggs stored at room temperature, in a cool room or refrigerator and having been collected from hens housed in individual cages (S1 - being day 1: upper panel) and then acutely stressed by transporting them for 20 min and then rehousing in groups of four in modified cages (containing a perch and nest box), with eggs collected on the next day (S3 - being day 3: middle panel) and then again on the following day (S4 - being day 4: lower panel).

Experiment 4. The effect of hen age and storage temperature and storage time on egg quality

Egg weight

Hen age had a significant effect on egg weight with the interaction with storage temperature marginally non-significant (P < 0.079: see Table 4.1). Egg weight at 50 (63.6 ± 0.4 g) and 63 weeks of age (65.0 ± 0.4 g) were similar, but heavier compared to egg weight at 30 weeks (58.9 ± 0.4 g; P < 0.05) and in turn heavier at 30 weeks than 21 weeks of age (52.2 ± 0.4 g; P < 0.05).

Table 4.1. The effect of hen age and storage temperature on the mean (± SEM) weight (g) of eggs collected on the same day from different aged flocks and stored at room temperature, in a cool room or refrigerator for 28 days.

Hen Age (weeks)	Storage conditions				SEM
	Day of lay	Fridge	Cool Room	Room	
21	53.9	52.2	51.3	51.2	
30	60.5	59.8	59.5	57.8	0.90
50	62.7	64.3	64.5	63.1	
63	64.9	63.9	66.9	64.1	
SEM	1.32		0.79		
			Moulted flocks		
82	66.9	66.3	66.9	64.9	
90	62.5	62.1	63.6	60.3	

Storage day had a significant effect on egg weight but this was influenced by storage day as the storage temperature x storage day interaction was significant (P = 0.013; see Table 4.2.). When eggs were stored in the refrigerator egg weight was similar on all storage days. Egg weight was lower on day 21 compared to days 7 and 14 when stored in the cool room (P < 0.05). On day 28 eggs stored at room temperature had lower egg weight compared to day 7 (P < 0.05).

On day 21, egg weight was higher when eggs were stored in the refrigerator than the cool room (P < 0.05) and on day 28 eggs stored in the refrigerator had higher weight than those stored at room temperature.

Table 4.2. The effect of storage time and storage temperature on the mean (± SEM) weight (g) of eggs collected on the same day from laying hens at 21, 30, 50 and 63 weeks of age and stored at room temperature, in a cool room or refrigerator for 28 days.

Storage day	Storage temperature			SEM
	Fridge	Cool Room	Room	
Day of lay	60.1	60.1_{AB}	60.1_{AB}	0.93
7	60.1	61.0_{A}	60.7_{A}	
14	61.6	60.4_{A}	59.0_{AB}	0.96
21	60.3^{a}	$57.5_{B}{}^{b}$	$59.1_{AB}{}^{ab}$	
28	61.1^{a}	$59.8_{AB}{}^{ab}$	$57.4_{B}{}^{b}$	
SE		0.96		

[a-b] Within a row values with without common superscripts are significantly different ($P < 0.05$).
[A-B] Within a column values with without common subscripts are significantly different ($P < 0.05$).

Haugh units (HU)

The effect of hen age on HU measurements (see Table 4.3.) was significant but it was influenced by storage temperature and storage time as the three way interaction, hen age x storage temperature x storage day was significant ($P < 0.001$). On the day of lay, the HU measurements were similar at 21 and 30 weeks of age but higher at 30 weeks of age than the HU values at 50 and 63 weeks ($P < 0.05$).

Storage in the refrigerator. On all storage days except for day 21, eggs collected for the 21 and 30 week age flocks had higher HU then those collected from the 50 and 63 week age flocks ($P < 0.05$). On day 21 of storage the HU was significantly lower for the 63 week old flock compared to other ages flocks ($P < 0.05$).

When stored in the refrigerator, there was a reduction in HU measures over the storage period but the pattern of change differed depending on the flock age. At 21 weeks of age, there was no significant decrease in HU measure in the first 14 days of storage, but then was lower at 21 and 28 days of age ($P < 0.05$). At 30, 50 and 63 weeks of age there was significant decrease in HU within the first 7 days of storage ($P < 0.05$). At 30 weeks of age there was no further reduction until day 21 and day 28 ($P < 0.05$). At 50 and 63 weeks of age, the HU measures were similar over 14 to 28 days of storage.

Storage in the cool room. The HU measure was always higher for eggs collected from the 21 week old flock than those collected from older aged flocks, except on day 7 and 28 when compared to eggs from the 30 week-old flock ($P < 0.05$). The HU measure was higher for the 30 week-old flock than the older flocks, except where is was similar to the 63 week-old flock on day 14 of storage (all, $P < 0.05$).The HU measures were similar for the 50 and 63 week old flocks at all storage times except day 14 when it was higher for eggs collected from the 63 week-old flock ($P < 0.05$).

For all flocks there was a significant drop in HU measure in the first 7 days of storage ($P < 0.05$). After day 7 the change in HU measures was different depending on flock age. At 21 and 30 weeks of age the HU measurements were similar over days 7 to 21 and then

significantly lower on day 28 ($P < 0.05$). For the 50 week-old flock the HU measurements was lower on day 14 than day 7 and then again lower on day 21 ($P < 0.05$), but similar on day 21 as day 28. For the 63 year-old flock, the HU measurement was similar on day 7 and 14 but higher on these days than day 21 and 28 ($P < 0.05$.).

Storage at room temperature. During storage, the 21 week old flock consistently had higher HU measures than the 50 and 63 week old flocks ($P < 0.05$). An except for day 14 of storage the 21 week old hens had higher HU than the hens aged 30 weeks ($P < 0.05$). On all storage days the HU measures were similar for the 50 and 62 week old flocks.

For all flocks there was a significant drop in HU measure in the first 7 days of storage ($P < 0.05$). After day 7 the change in HU measures was different depending on flock age. At 21 weeks of age the HU measurements were similar on days 14 and 21 but lower compared to day 7 ($P < 0.05$) and higher when compared to day 28 ($P < 0.05$). For the 30 week-old flock the HU measurement was lower on day 14 than day 7 and then again lower on day 21 and 28 ($P < 0.05$), but similar on day 28 as day 21. For the 63 year-old flock, the HU measurement was progressively lower on all storage days ($P < 0.05$).

Table 4.3. The effect of storage temperature and storage time on the mean (± SEM) HU measures of eggs collected on the same day from laying hens at 21, 30, 50 and 63 weeks of age and stored at room temperature, in a cool room or refrigerator for 28 days.

Sampling day	Flock age (weeks)				SEM
	21	30	50	63	
			Refrigerator storage		
0	96.8_{A}^{a}	102.7_{A}^{a}	90.8_{A}^{b}	88.6_{A}^{b}	
7	93.9_{A}^{a}	88.0_{B}^{a}	78.4_{B}^{b}	78.5_{B}^{b}	
14	90.4_{A}^{a}	89.7_{B}^{a}	74.5_{BC}^{b}	71.0_{C}^{b}	2.34
21	79.7_{B}^{a}	80.2_{C}^{a}	73.8_{BC}^{a}	66.5_{C}^{b}	
28	82.9_{B}^{a}	80.9_{C}^{a}	71.7_{C}^{b}	67.3_{C}^{b}	
SEM			2.32		
			Cool room storage		
0	96.8_{A}^{ab}	102.7_{A}^{a}	90.8_{A}^{bc}	88.6_{A}^{c}	
7	81.9_{B}^{a}	75.6_{B}^{ab}	65.1_{B}^{c}	70.7_{B}^{bc}	
14	84.3_{B}^{a}	73.1_{B}^{b}	58.1_{C}^{c}	68.1_{B}^{b}	2.34
21	79.7_{B}^{a}	73.0_{B}^{b}	50.4_{D}^{c}	51.1_{C}^{c}	
28	70.8_{C}^{a}	66.1_{C}^{a}	46.6_{D}^{b}	49.3_{C}^{b}	
SEM			2.32		
			Room storage		
0	96.8_{A}^{a}	102.7_{A}^{a}	90.8_{A}^{a}	88.6_{A}^{a}	
7	77.5_{B}^{a}	69.0_{B}^{b}	56.6_{B}^{c}	60.8_{B}^{c}	
14	68.2_{C}^{a}	62.4_{C}^{ab}	58.2_{B}^{bc}	52.1_{C}^{c}	2.34
21	64.0_{C}^{a}	45.6_{D}^{b}	34.4_{C}^{c}	34.8_{D}^{c}	
28	53.1_{D}^{a}	39.8_{D}^{b}	32.3_{C}^{c}	27.2_{E}^{c}	
SEM			2.32		

$^{a-b}$ Within a row values with different superscripts are significantly different ($P < 0.05$)
$_{A-D}$Within a column values with different subscripts are significantly different ($P < 0.05$)

The effect of hen age and moult on HU measurements is given in Table 4.4.

Table 4.4. The effect of storage temperature and storage time on the mean (± SEM) HU measures of eggs collected on the same day from laying hens at 83 and 90 weeks of age, having undergone a moulted at 67 and 70 weeks of age, respectively. The eggs were stored either at room temperature, in a cool room or refrigerator for 28 days.

Sampling day	Flock age (weeks)					
	82	90	82	90	82	90
	Refrigerator storage		Cool room storage		Room storage	
0	89.7	82.5	89.7	82.5	89.7	82.5
7	87.6	76.5	66.0	70.8	52.4	52.4
14	74.9	69.4	63.7	62.8	48.1	36.5
21	76.0	67.4	51.7	52.1	32.3	38.2
28	73.3	66.8	54.9	54.0	32.1	30.4

Albumen Index (AI)

Hen age, storage temperature and storage time had significant effects on AI but there were also significant interaction between hen age and storage temperature ($P = 0.042$) and between storage temperature and storage day ($P = 0.002$).

The effects of age and storage temperature on the AI are shown in Table 4.5. On the day of lay, the AI was higher for eggs collected from to 30 week-old flock than those collected from the 21 week-old flock which in turn was higher than the AI for the 50 and 63 week-old flocks, which were similar to one another (all, $P < 0.05$). At all storage temperatures the AI was similar for eggs collected from the 20 and 30 week-old flocks and higher than eggs collected from the 50 and 63 week-old flocks ($P < 0.05$), when again they were similar.

At all flock ages there was a progressively decrease in AI as the storage temperature increased (all, $P < 0.05$).

Table 4.5: The effect of flock age and storage time on the mean (± SEM) AI of eggs collected on the same day from laying hens at 21, 30, 50 and 63 weeks of age and stored at room temperature, in a cool room or refrigerator for 28 days.

Storage Temperature	Flock age (weeks)				SEM
	21	30	50	63	
Day of Lay	$0.121_A{}^b$	$0.156_A{}^a$	$0.108_A{}^c$	$0.102_A{}^c$	
Refrigerator	0.09_B2^a	$0.088_B{}^a$	$0.067_B{}^b$	$0.063_B{}^b$	0.004
Cool room	$0.069_C{}^a$	$0.055_C{}^a$	$0.041_C{}^b$	$0.041_C{}^b$	
Room	$0.044_D{}^a$	$0.032_D{}^{ab}$	$0.0.22_D{}^b$	$0.0.25_D{}^b$	
SEM		0.004			

[a-b] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different subcripts are significantly different (P < 0.05)

The effects of storage temperature and storage time on AI are given in Table 4.6. The AI was always higher when eggs that were stored in the refrigerator rather than the cool room (P < 0.05), and in turn were always higher in the cool room than when eggs were stored at room temperature (P < 0.05).

At all storage temperatures there was a significant decrease in AI over the first 7 days of storage (P < 0.05). When stored in the refrigerator the AI was similar on days 7 and 14 but higher than it was on day 21 and 28 (P< 0.05), when it was similar again. When eggs were stored in the cool room the AI was similar on days 7 to 21 but higher on these days than it was on day 28 (P < 0.05). When eggs were stored at room temperature the Al was similar on days 14, 21 and 28 but on these day lower than it was on day 7 (P < 0.05).

Table 4.6. The effect of storage temperature and storage time on the mean (± SEM) AI of eggs collected on the same day from laying hens at 21, 30, 50 and 63 weeks of age and stored at room temperature, in a cool room or refrigerator for 28 days.

Storage day	Storage temperature			SEM
	Refrigerator	Cool room	Room	
Day of lay	0.121_A	0.121_A	0.121_A	
7	$0.088_B{}^a$	$0.061_B{}^b$	$0.051_B{}^c$	0.06
14	$0.082_B{}^a$	$0.052_B{}^b$	$0.027_C{}^c$	
21	$0.069_C{}^a$	$0.054_B{}^b$	$0.027_C{}^c$	
28	$0.69_C{}^a$	$0.039_C{}^b$	$0.018_C{}^c$	
SEM		**0.06**		

[a-b] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different subscripts are significantly different (P < 0.05)

The effect of hen age and moult on AI is given in Table 4.7.

Table 4.7. The effect of storage temperature and storage time on the mean (± SEM) AI of eggs collected on the same day from laying hens at 83 and 90 weeks of age, having undergone a moulted at 67 and 70 weeks of age, respectively. The eggs were stored either at room temperature, in a cool room or refrigerator for 28 days.

Sampling day	Flock age (weeks)					
	82	90	82	90	82	90
	Refrigerator storage		Cool room storage		Room storage	
0	0.098	0.082	0.098	0.082	0.098	0.082
7	0.078	0.065	0.047	0.053	0.031	0.032
14	0.063	0.063	0.045	0.043	0.023	0.016
21	0.071	0.070	0.031	0.032	0.016	0.017
28	0.063	0.063	0.034	0.031	0.014	0.013

Yolk Index (YI)

The effect of hen age on YI (see, Table 4.8), was significant but it was influenced by storage temperature and storage time as the three way interaction, hen age x storage temperature x storage day was significant ($P < 0.001$). On the day of lay, the YI was similar at 21 and 30 weeks of age but higher than the values at 50 and 63 weeks of age ($P < 0.05$), when the YI was again similar.

Storage in the refrigerator. When eggs were stored in the refrigerator, age had no effect on YI at day 7 and 28 days of storage. On day 14 the YI was higher for eggs collected from the 21 week-old flock compared to eggs from the 30 week of age ($P < 0.05$) which in turn was higher YI than for eggs collected from the 50 and 63 week-old flocks ($P < 0.05$), which were similar. After 21 days of storage, the eggs collected from the 21 week-old flock had lower YI compared to other aged flocks ($P < 0.05$) and the YI for the 30 week-old flock was higher than the YI for the 50 and 63 week-old flocks ($P < 0.05$), which were similar.

The YI changes over the storage time varied extensively for the different aged flocks with no clear pattern obvious.

Storage in the cool room. When eggs were stored in the cool room, age had no effect on YI at day 14 of storage. On all other storage days the YI was higher for eggs from the 30 week-old flock than the 50 and 63 week-old flocks and higher than the 21 week-old flock on days 7 and 21 but not day 28 (all, $P < 0.05$). On all storage days except day 7 the YI for the 50 and 63 week-old flocks were similar but on day 7 it was higher for the 63 week-old flock ($P < 0.05$).

At 21 week of age the YI was significantly lower on all storage days compared to the day of lay ($P < 0.05$). For the 30 week-old flock the YI was lower on days 14 and 21 compared to other days ($P < 0.05$). For the 50 week-old flock the YI was higher on the day of lay and day 14 compared to other days ($P < 0.05$). In the 63 week-old flock the YI was lower on days 21 and 28 than the other days including the day of lay ($P < 0.05$).

Storage at room temperature. When eggs were stored at room temperature, flock age had no effect on the YI during the first 7 days of storage. For the 21 week-old flock the YI on days 14 and 21 and 28 were lower than on day 7 ($P < 0.05$) but values on days 21 and 28 were similar. For the 30 week-old flock the YI on days 14 and 21 and 28 were lower than on day 7 ($P < 0.05$) but values on days 14 and 21 were similar, however, both were higher than the value recorded on day 28 ($P < 0.05$). For the 50 week-old flock, the YI on day 7 was higher than values on days 14, 21 and 28 ($P < 0.05$), which were all similar. For eggs collected from the 63 week-old flock the YI was higher on day 7 than day 14 which in turn was higher than day 21 and day 28 ($P < 0.05$), when they were similar.

When eggs were stored at room temperature the was significant drop in YI from day of lay until day 7 of storage except for the 50 week-old flock ($P < 0.05$). For the 21 week-old flock the YI was higher on day 7 than on other days, similar on days 14 and 21 but higher on day 14 compared to day 28 ($P < 0. 05$). The changes during storage were similar for the 30 week-old flock as the younger 21 week-old flock except for day 14 and 21 values which were again similar but both different to the day 28 YI ($P < 0.05$). For the 50 week-old flock the YI was higher on day 7 than other storage days ($P < 0.05$). For the 63 week-old flock the YI on day 7 was higher than other days as was day 14 YI compared to later storage days ($P < 0.05$).

Table 4.8. The effect of storage temperature and storage time on the mean (± SEM) YI of eggs collected on the same day from laying hens at 21, 30, 50 and 63 weeks of age and stored at room temperature, in a cool room or refrigerator for 28 days.

Sampling day	Flock age (weeks)				SEM
	21	30	50	63	
	Refrigerator storage				
0	0.496_{B}^{a}	0.496_{B}^{a}	0.432_{B}^{b}	0.450_{C}^{b}	
7	0.494_{B}	0.508_{B}	0.492_{A}	0.508_{A}	0.010
14	0.531_{A}^{a}	0.494_{B}^{b}	0.445_{B}^{c}	0.464_{BC}^{c}	
21	0.432_{C}^{c}	0.549_{A}^{a}	0.485_{A}^{b}	0.487_{AB}^{b}	
28	0.459_{C}	0.484_{B}	0.455_{B}	0.455_{C}	
SEM	0.010				
	Cool room storage				
0	0.496_{A}^{a}	0.496_{A}^{a}	0.432_{A}^{b}	0.450_{A}^{b}	
7	0.448_{B}^{b}	0.478_{A}^{a}	0.380_{B}^{c}	0.449_{A}^{b}	0.010
14	0.439_{B}	0.424_{B}	0.433_{A}	0.431_{A}	
21	0.430_{B}^{b}	0.467_{A}^{a}	0.393_{B}^{c}	0.391_{B}^{c}	
28	0.426_{B}^{a}	0.437_{B}^{a}	0.385_{B}^{b}	0.395_{B}^{b}	
SEM	0.010				
	Room storage				
0	0.496_{A}^{a}	0.496_{A}^{a}	0.432_{A}^{b}	0.450_{A}^{b}	
7	0.407_{B}	0.432_{B}	0.422_{A}	0.415_{B}	
14	0.372_{C}^{a}	0.362_{C}^{ab}	0.336_{B}^{b}	0.339_{C}^{b}	0.010
21	0.347_{CD}^{a}	0.347_{C}^{a}	0.301_{B}^{b}	0.290_{D}^{b}	
28	0.334_{D}^{a}	0.292_{D}^{b}	0.273_{B}^{b}	0.275_{D}^{b}	
SEM	0.010				

[a-b] Within a row values with different superscripts are significantly different ($P < 0.05$)
[A-D] Within a column values with different subscripts are significantly different ($P < 0.05$)

The effect of hen age and moult on YI is given in Table 4.9.

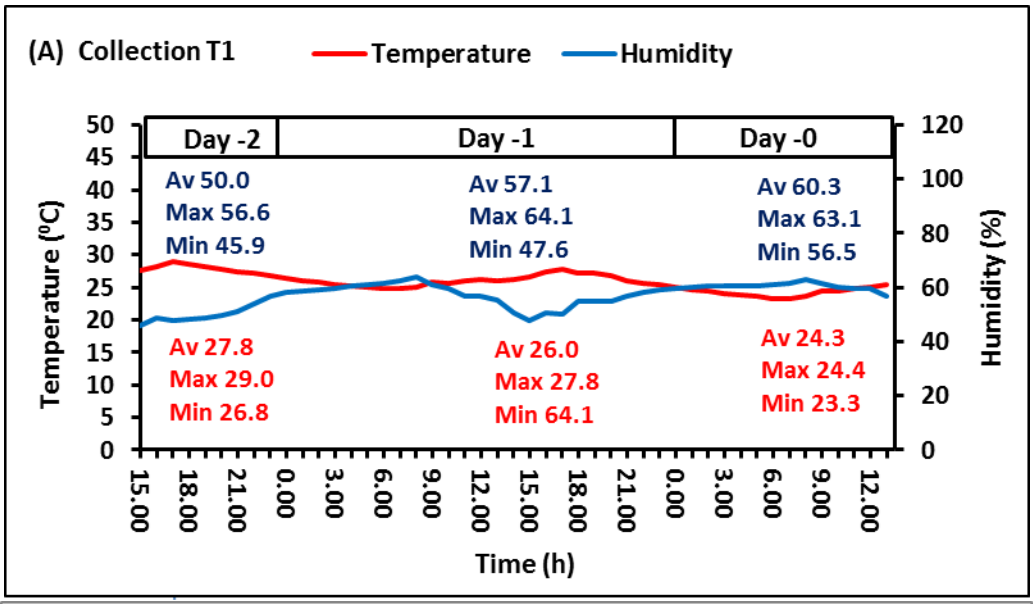
Table 4.9. The effect of storage temperature and storage time on the mean (± SEM) YI of eggs collected on the same day from laying hens at 83 and 90 weeks of age, having undergone a moulted at 67 and 70 weeks of age, respectively. The eggs were stored either at room temperature, in a cool room or refrigerator for 28 days.

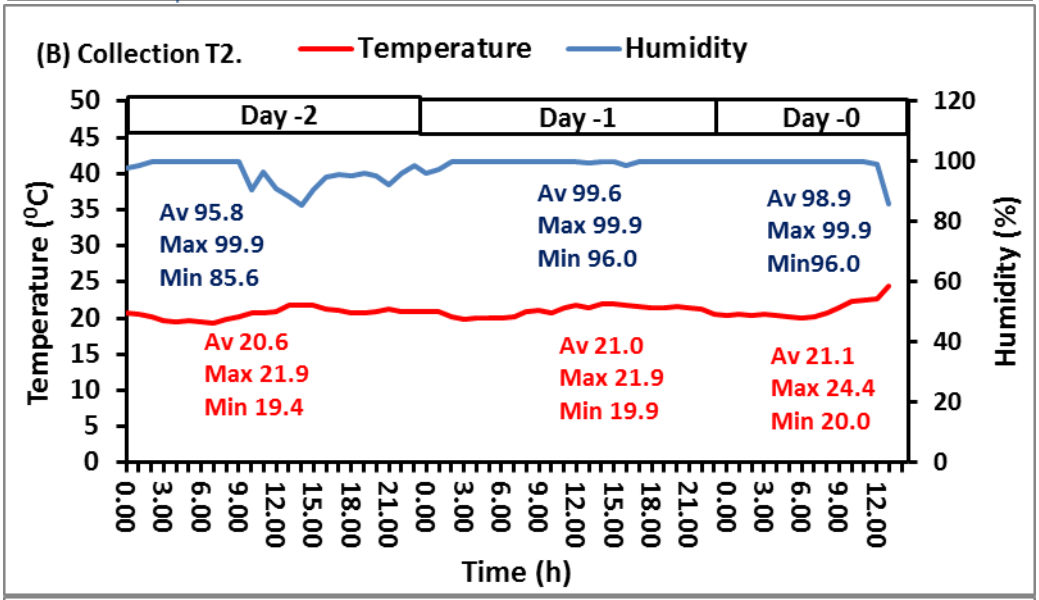
Sampling day	Flock age (weeks)					
	82	90	82	90	82	90
	Refrigerator storage		Cool room storage		Room storage	
0	0.459	0.461	0.459	0.461	0.459	0.461
7	0.465	0.472	0.478	0.483	0.415	0.431
14	0.433	0.473	0.449	0.462	0.341	0.323
21	0.402	0.413	0.508	0.486	0.303	0.306
28	0.413	0.394	0.463	0.454	0.279	0.263

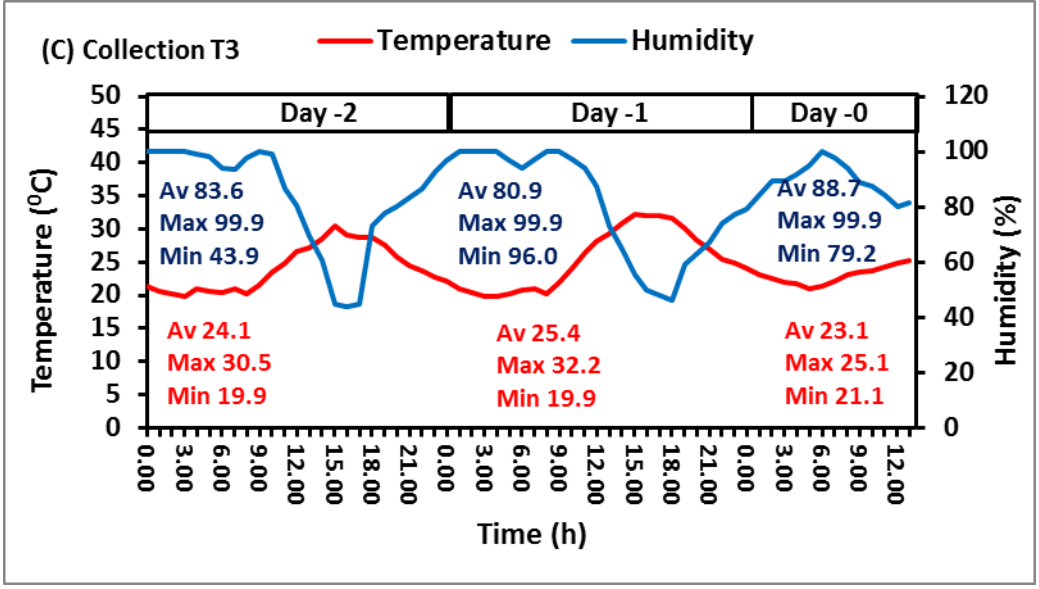
Experiment 5. The effect of layer house ambient temperature on egg quality during storage under three different temperatures

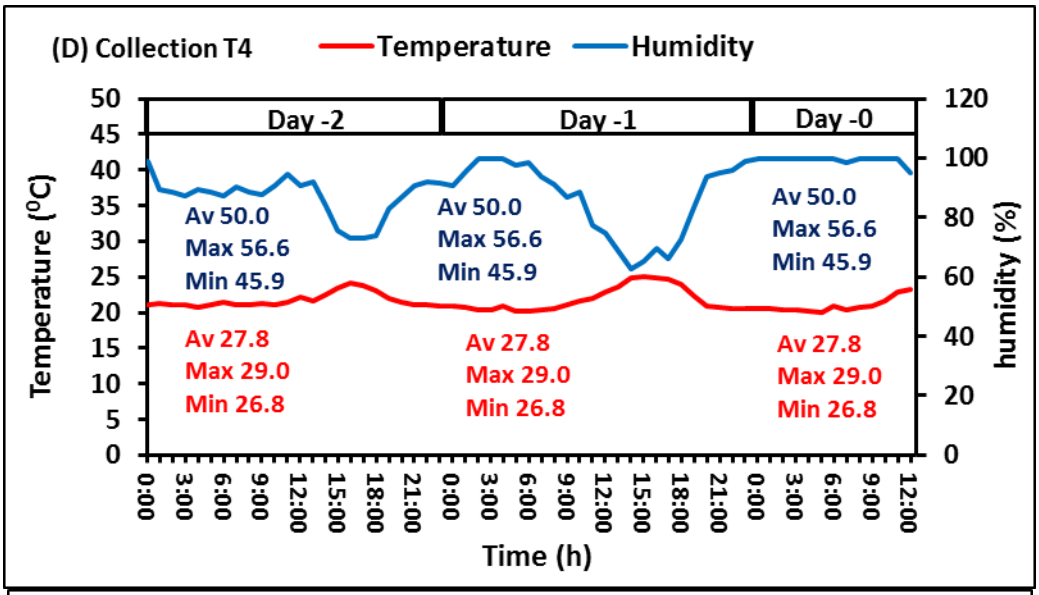
Shed temperatures

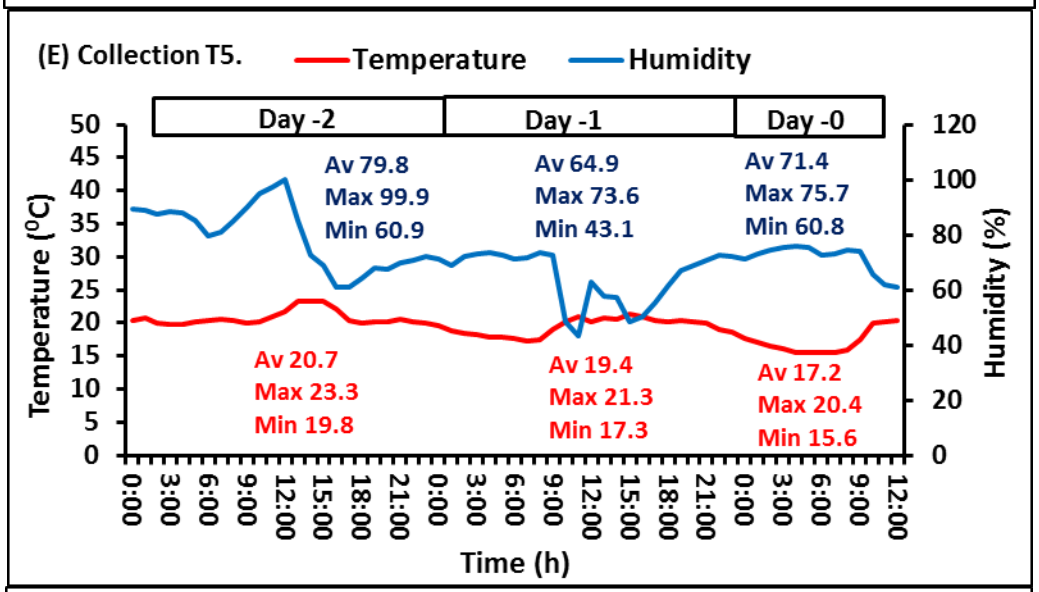
The shed temperature and humidity during the two days before egg collections and up to 12:00 on the day of egg collection are shown in Figure 5.1 A-G. Because the collections were made over a period of 25 weeks the data were analysed as two separate periods to take account of the potential interaction between hen age and shed temperature on egg quality. The first three collections were made in summer when the flock age was 30 weeks for collection T1, 33 weeks for T2 and 37 weeks for T3. The range in hen age was 7 weeks for these three collections. The last four egg collections were made in autumn (T4) and three (T5-T7) in winter. The flock age at T4 was 40 weeks, T5 was 47 weeks, T6 was 56 weeks and T7 was 57 weeks. The data analysis suggested that the autumn (T4) collection should be treated separately to T5-T7 collections and so the range in hen age for collections T5-T7 was 10 weeks.

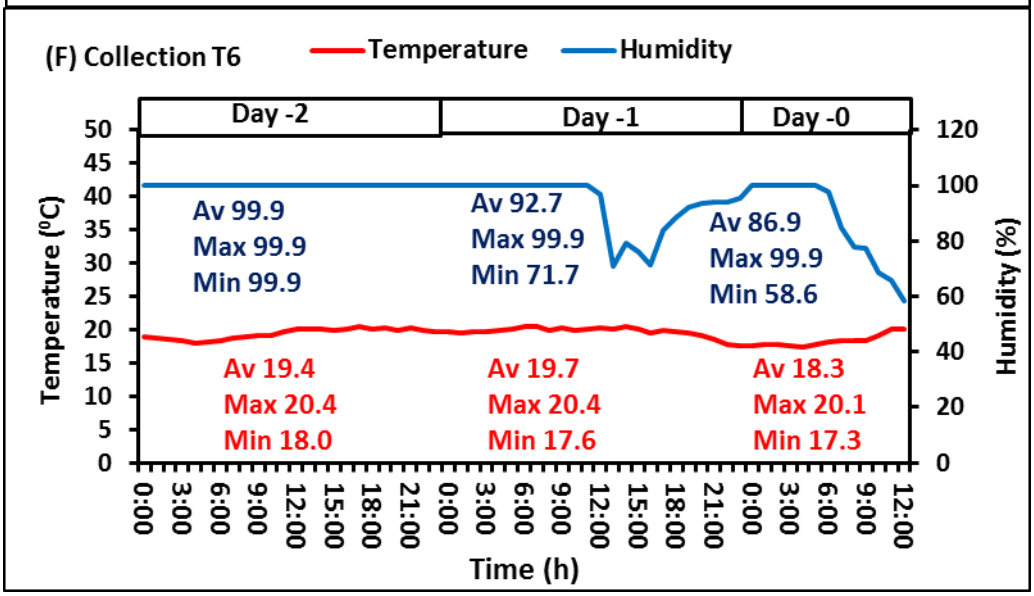

(A) Collection T1
Temperature
Humidity
Day -2
Day -1
Day -0
Av 50.0
Max 56.6
Min 45.9
Av 57.1
Max 64.1
Min 47.6
Av 60.3
Max 63.1
Min 56.5
Av 27.8
Max 29.0
Min 26.8
Av 26.0
Max 27.8
Min 64.1
Av 24.3
Max 24.4
Min 23.3
Temperature (°C)
Humidity (%)
Time (h)


(B) Collection T2.
Temperature
Humidity
Day -2
Day -1
Day -0
Av 95.8
Max 99.9
Min 85.6
Av 99.6
Max 99.9
Min 96.0
Av 98.9
Max 99.9
Min96.0
Av 20.6
Max 21.9
Min 19.4
Av 21.0
Max 21.9
Min 19.9
Av 21.1
Max 24.4
Min 20.0
Temperature (°C)
Humidity (%)
Time (h)


(C) Collection T3
Temperature
Humidity
Day -2
Day -1
Day -0
Av 83.6
Max 99.9
Min 43.9
Av 80.9
Max 99.9
Min 96.0
Av 88.7
Max 99.9
Min 79.2
Av 24.1
Max 30.5
Min 19.9
Av 25.4
Max 32.2
Min 19.9
Av 23.1
Max 25.1
Min 21.1
Temperature (°C)
Humidity (%)
Time (h)


(D) Collection T4
Temperature
Humidity
Day -2
Day -1
Day -0
Av 50.0
Max 56.6
Min 45.9
Av 50.0
Max 56.6
Min 45.9
Av 50.0
Max 56.6
Min 45.9
Av 27.8
Max 29.0
Min 26.8
Av 27.8
Max 29.0
Min 26.8
Av 27.8
Max 29.0
Min 26.8
Temperature (°C)
humidity (%)
Time (h)


(E) Collection T5.
Temperature
Humidity
Day -2
Day -1
Day -0
Av 79.8
Max 99.9
Min 60.9
Av 64.9
Max 73.6
Min 43.1
Av 71.4
Max 75.7
Min 60.8
Av 20.7
Max 23.3
Min 19.8
Av 19.4
Max 21.3
Min 17.3
Av 17.2
Max 20.4
Min 15.6
Temperature (°C)
Humidity (%)
Time (h)


(F) Collection T6
Temperature
Humidity
Day -2
Day -1
Day -0
Av 99.9
Max 99.9
Min 99.9
Av 92.7
Max 99.9
Min 71.7
Av 86.9
Max 99.9
Min 58.6
Av 19.4
Max 20.4
Min 18.0
Av 19.7
Max 20.4
Min 17.6
Av 18.3
Max 20.1
Min 17.3
Temperature (°C)
Humidity (%)
Time (h)

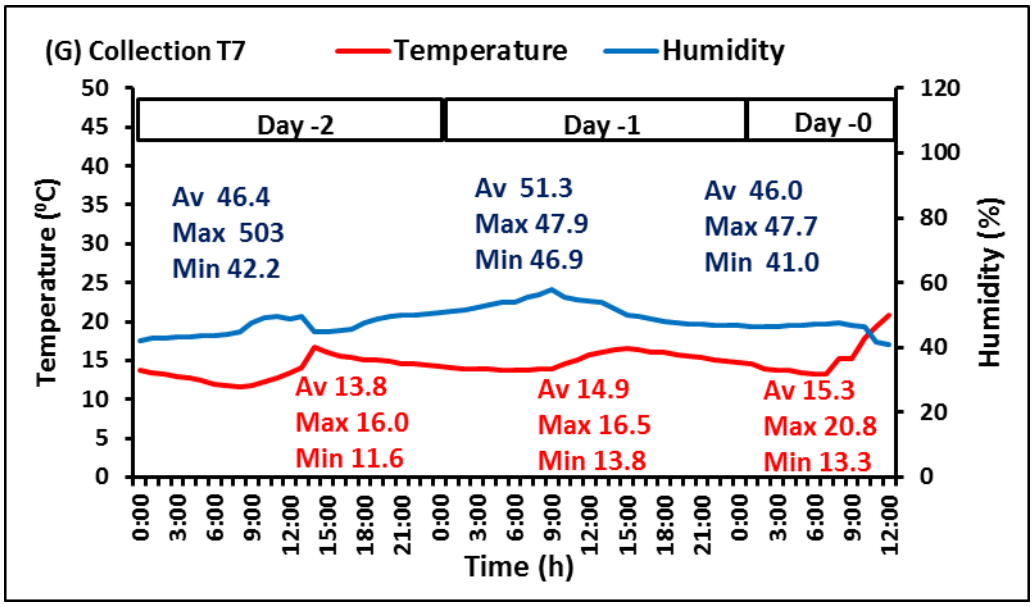


Figure 5.1. A-G: The layer shed ambient temperature and relative humidity patterns during the two days (day -2 and day -1) before egg collection and up to 12:00 on the day of collection (day 0). The average daily temperature and humidity and the range in both for the each collection period are embedded into the graphs.

For all the egg collections the average temperature, maximum and minimum temperatures for the 26 hours before 6:00 h on the day of individual collections are shown in the Table 5.1. This time span was selected based on the time sequences of egg formation. The time period would cover the period of albumen deposition and later stages of egg formation for eggs collected on the individual days.

For the three summer collections, T1 and T3 had similar average temperatures. The T2 collection was made during a summer wet weather period and accounts for the low average temperature and limited temperature range.

For the autumn and winter collections, (T4, T5, T6 and T7), the average temperatures varied between 21.6ºC and 14.8ºC, remained fairly stable with the range being limited to only about 3-5ºC. The following comparisons were made for these collections. The collection at T4 (week 40 of age) was compared with collection T5 (47 weeks) as the average temperature for these two collections were the similar. A further comparison was made between collection T5, T6 and T7 where the flock age varied between 47 and 57 weeks. From experiment 4, this is a range where hen age was found to have limited, if any, effects on egg quality during storage.

Table 5.1. The average, the maximum and the minimum temperatures during the 26 h before 06:00 h on the day of individual egg collections.

Season	Collection day	Flock age (weeks)	Temperature		
			Average (^{0}C)	Maximum (^{0}C)	Minimum (^{0}C)
Summer	T1	30	25.5	27.9	23.3
	T2	33	20.9	22.0	20.0
	T3	37	25.3	32.0	21.0
Winter	T4	40	21.6	25.0	20.0
	T5	47	18.7	21.3	15.6
	T6	56	19.1	20.4	17.3
	T7	57	14.8	16.5	13.3

Summer collections (T1, T2 and T3)

Egg Weight

Collection day had an effect on egg weight ($P < 0.001$) with it higher at theT3 collection (63.7 ± 0.3 g) than T2 collection (62.6 ± 0.3 g) which in turn was greater than for the T1 collection (61.7 ± 0.3 g) (all, $P < 0.05$).

Storage temperature had significant effect on egg weight ($P < 0.001$). The egg weight when stored at room temperature was lower (61.8 ± 0.3 g) than when stored in the refrigerator (63.4 ± 0.3 g), the cool room (62.8 ± 0.3 g) or the weight on the day of lay (62.8 ± 0.3 g) ($P < 0.05$).

Storage day had a significant effect on egg weight ($P < 0.05$). Egg weight was lower after 7 days of storage (63.0 ± 0.3 g) compared to the day of lay (64.7 ± 0.5 g) ($P < 0.05$). Egg weight was similar on days 7 and day 14 (62.2 ± 0.3 g) but lower on day 21 (61.7 ± 0.3 g) no day 28 (61.7 ± 0.3 g) than day 7 ($P < 0.05$).

Haugh unit (HU)

The HU measures for the collections made in summer (T1, T2 and T3) are given in Table 5.2. There was a significant three interaction between collection day, storage time and storage temperature ($P < 0.001$). For all storage temperatures there was a significant reduction in HU measure over the first 7 days of storage ($P < 0.05$). The HU on the day of collection was higher for the T1 collection compared to the other collection days ($P < 0.05$).

Storage in the refrigerator

For the T1 collection, the HU measure was similar on day 7, 14 and 28. The value for day 21 was lower than the measures on day 7 and 14 ($P < 0.05$) but not day 28. For the T2 collection, the HU measure was similar on day 7 and 14 with these values being higher than those recorded on days 21 and 28 ($P < 0.05$). For the T3 collection, The HU measures were similar on days 7, 21 and 28 with the differences between day 7 and 14 being the only significant difference ($P < 0.05$).

On day 14 the HU measure was lower for the T3 collection than the T1 and T2 collections ($P < 0.05$). On day 28, the HU measure was lower for the T2 collection than the T1 and T3 collections ($P < 0.05$). On other storage days there were no differences between the collections.

Storage in the cool room. For all summer collections the change in HU measure during storage followed similar patterns. The HU measure were similar on days 14 and 21 and these were lower than those recorded on day 7 ($P < 0.05$). The HU measure on day 28 was lower than on all other storage days ($P < 0.05$). The only difference between collection days was on day 7 where the HU measure was lower for collection T2 than collection T1 and T3 ($P < 0.05$).

Storage at room temperature. For the T1 collection, the HU measure was similar on days 7 and 14 which were higher than on day 21 which in turn was higher than on day 28 (all, $P < 0.05$). For the T2 collection, the HU measure was similar on day 7 and 14 with these values being higher than those recorded on days 21 and 28 ($P < 0.05$). For the T3 collection, the HU measure was higher on day 7 than day 14 which in turn was higher than the measures on day 21 and 28 ($P < 0.05$), with the values on these later two days similar.

The HU measure on day 7 was similar for all collection days. On the other storage days the HU measures were higher for collection T1 than collection T3 ($P < 0.05$). It was only on day 21 that the HU was higher for collection T1 than collection T2 ($P < 0.05$).

Table 5.2. The effect of storage temperature and storage time on the mean (± SEM) HU measures of eggs collected from laying hens and housed in individual cages and exposed to different shed ambient temperatures during summer and then stored at room temperature, in a cool room or refrigerator for 28 days.

*Shed Temperatures	Collection day			
	T1	**T2**	**T3**	
Av	25.5	20.9	25.3	SEM
Max	27.9	22.0	32.0	
Min	23.3	20.0	21.0	
Sampling day	Flock age (weeks)			
	30	33	37	
Stored in the refrigerator				
0	103.4_{A}^{a}	97.7_{A}^{b}	95.5_{A}^{b}	1.69
7	87.9_{B}	87.2_{B}	85.6_{B}	
14	87.4_{B}^{a}	87.4_{B}^{a}	79.9_{C}^{b}	
21	81.6_{C}	79.6_{C}	84.2_{BC}	1.95
28	84.3_{BC}^{a}	76.2_{C}^{b}	83.3_{BC}^{a}	
SEM	1.95			
Stored in the cool				
0	103.4_{A}^{a}	97.7_{A}^{b}	95.5_{A}^{b}	1.69
7	76.9_{B}^{a}	71.0_{B}^{b}	74.5_{B}^{a}	
14	68.6_{C}	64.4_{C}	68.1_{C}	
21	63.4_{C}	63.1_{C}	62.9_{C}	1.95
28	55.1_{D}	55.8_{D}	54.0_{D}	
SEM	1.95			
Stored in the room				
0	103.4_{A}^{a}	97.7_{A}^{b}	95.5_{A}^{b}	1.69
7	62.8_{B}	60.4_{B}	60.6_{B}	
14	57.6_{B}^{a}	58.3_{B}^{a}	48.5_{C}^{b}	
21	51.1_{C}^{a}	40.6_{C}^{b}	35.6_{D}^{b}	1.95
28	39.3_{D}^{a}	43.4_{C}^{a}	32.7_{D}^{b}	
SEM	1.95			

$^{a-b}$ Within a row values with different superscripts are significantly different ($P < 0.05$)
$_{A-D}$Within a column values with different subscripts are significantly different ($P < 0.05$)
* The average, maximum and minimum shed temperature over the 26 h before 06:00 h on the day of egg collection

Albumen Index (AI)

The effect of collection day on AI was marginally non-significant (P = 0.08). Collection T3 (0.066 ± 0.002) tended to have lower AI than collection T2 (0.071 ± 0.022) and T1 (0.075 ± 0.022).

Storage temperature had a significant effect on AI (see Table 5.3.), but this changed over time as the interaction between storage day and storage temperature was significant (P = 0.009).

For all storage temperatures there was a significant decrease in AI after 7 days of storage (P < 0.05). When stored in the refrigerator the AI was similar on all storage days except for the difference between day 21 and 28 (P < 0.05). When stored in the cool room the AI was higher on day 7 compared to day 14 and day 28 (P < 0.05), but similar on days 14 and 21. When stored at room temperature the AI was higher on day 7 compared to day 21 and day 28 (P < 0.05), but similar to the AI on day 14.

On all storage days the AI was higher when stored in the refrigerator than the cool room (P < 0.05) and higher when stored in the cool room than at room temperature (all, P < 0.05).

Table 5.3. The effect of storage temperature and storage time on the mean (± SEM) AI of eggs collected from laying hens housed in individual cages and exposed to different shed ambient temperatures during summer and then stored at room temperature, in a cool room or refrigerator for 28 days.

Storage day	Storage temperature			SEM
	Refrigerator	Cool room	Room	
Day of lay	0.127_{A}	0.127_{A}	0.127_{A}	
7	$0.093_{BC}{}^{a}$	$0.065_{B}{}^{b}$	$0.041_{B}{}^{c}$	0.06
14	$0.090_{BC}{}^{a}$	$0.052_{C}{}^{b}$	$0.032_{BC}{}^{c}$	
21	$0.085_{C}{}^{a}$	$0.045_{CD}{}^{b}$	$0.024_{C}{}^{c}$	
28	$0.099_{B}{}^{a}$	$0.036_{D}{}^{b}$	$0.019_{C}{}^{c}$	
SEM		0.06		

[a-b] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different subscripts are significantly different (P < 0.05)

Yolk Index (YI)

The YI for the collections made in summer (T1, T2 and T3) are given in Table 5.4. There was a significant three interaction between collection day, storage time and storage temperature (P < 0.001).

On the day of lay the YI was similar for all collection days.

Storage in the refrigerator. For the T1 collection, the YI was lower on day 21 compared to day 14 ($P < 0.05$) with there being no other differences. For the T2 collection, YI was similar on days 7, 14 and 21, but compared to these days it was lower on day of lay ($P < 0.05$). The YI was lower on day 28 than it was on days 7 and 14 ($P < 0.05$). For the T3 collection, the YI was similar on all storage days and the day of lay.

On all storage days except day 21 the YI was higher for eggs collected on day T1 than day T3. The YI was similar on days 7 and 14 for the T1 and T2 collections but differed on days 21 and 28 with the YI being lower for T1 on day 21 but higher on day 28 ($P < 0.05$). On days 7 and 21 the YI for T2 was higher than T3 ($P < 0.05$) but similar on days 14 and 28.

Storage in the cool room. For the T1 collection YI was lower on day 7 than the day of lay. The YI was similar on day 14 and 21 and lower on these days than day 7 and then lower on day 28 than previous days (all, $P < 0.05$). For collection T2, the YI was similar on the day of lay and day 7 of storage and higher at these times compared to day 14 and 21 ($P < 0.05$) where they were similar but higher than the YI on day 28 ($P < 0.05$). For collection T3, the YI was similar on days 14, 21 and 28 but lower on these days than day 7 ($P < 0.05$) which was in turn lower than on the day of lay ($P < 0.05$).

The YI for eggs collected on days T1 and T2 were similar on all day storage days and the day of lay. On days 7, 14 and 21 the YI for collection T1 was higher than for T3 ($P< 0.05$) but not on other days. It was only on day 21 that YI for collection T2 was higher than T3, with no differences on other days.

Storage in the room. For the T1 collection, the YI was similar on day of lay and day 7 of storage, but higher than on days 14 and 21 ($P < 0.05$) when they were similar but higher than on day 28 ($P < 0.05$). For the T2 collection, the YI was higher on the day of lay compared to days 7 and 14 ($P < 0.05$) where it was similar but higher on these days than on day 21 which in turn where it was higher than on day 28 (all, $P < 0.05$). For the T3 collection, there was a progressively lower YI from the day of lay until day 21 (all, $P < 0.05$) and then it was similar on days 21 and 28.

The YI for the T2 collection was higher than that for the T1 collection on days 7 and 14 ($P < 0.05$) but similar on days 21 and 28. After day 7 the YI for the T2 eggs were higher than the T3 eggs ($P < 0.05$). The YI of the T1 eggs were higher than T3 eggs on day 21 and 28 ($P < 0.05$).

Table 5.4. The effect of storage temperature and storage time on the mean (± SEM) YI of eggs collected from laying hens housed in individual cages and exposed to different shed ambient temperatures during summer and then stored at room temperature, in a cool room or refrigerator for 28 days.

*Shed temperatures	Collection day			
	T1	T2	T3	
Av	25.5	20.9	25.3	SEM
Max	27.9	22.0	32.0	
Min	23.3	20.0	21.0	
Sampling day	Flock age (weeks)			
	Flock age (weeks)			
	30	33	37	
	Stored in the refrigerator			
0	0.489_{AB}	0.470_{C}	0.473	0.69
7	0.495_{AB}^{a}	0.494_{AB}^{a}	0.468^{b}	
14	0.501_{A}^{a}	0.515_{A}^{ab}	0.486^{b}	
21	0.478_{B}^{b}	0.502_{AB}^{a}	0.464^{b}	0.79
28	0.505_{AB}^{a}	0.473_{BC}^{b}	0.471^{b}	
SEM	0.79			
	Stored in the cool room			
0	0.489_{A}	0.470_{A}	0.473_{A}	0.69
7	0.464_{B}^{a}	0.455_{A}^{ab}	0.438_{B}^{b}	
14	0.435_{C}^{a}	0.419_{B}^{ab}	0.404_{C}^{b}	
21	0.428_{C}^{a}	0.435_{B}^{a}	0.385_{C}^{b}	0.79
28	0.399_{D}	0.392_{C}	0.389_{C}	
SEM	0.79			
	Stored in the room			
0	0.489_{A}	0.470_{A}	0.473_{A}	0.69
7	0.384_{A}^{b}	0.409_{B}^{a}	0.409_{B}^{a}	
14	0.349_{B}^{b}	0.396_{B}^{a}	0.352_{C}^{b}	
21	0.327_{B}^{a}	0.334_{C}^{a}	0.284_{D}^{b}	0.79
28	0.294_{C}^{a}	0.299_{D}^{a}	0.265_{D}^{b}	
SEM	0.79			

$^{a-b}$ Within a row values with different superscripts are significantly different ($P < 0.05$)
$^{A-D}$ Within a column values with different superscripts are significantly different ($P < 0.05$)
* The average, maximum and minimum shed temperature over the 26 h before 06:00 h on the day of egg collection

Winter and autumn collections (T4, T5, T6 and T7).

The comparisons between autumn T4 collection and winter T5 collection.

Egg Weight

Storage time had no effect on egg weight (P = 0.088). Collection day had a significant effect on egg weigh but it depended on the storage temperature (see, Table 5.5) as the collection day x storage temperature interaction was significant (P < 0.001). Egg weight was not different on the day of lay but eggs from collection day T5 had higher weight when stored in the cool room or room (P < 0.05).

Eggs collected on day T4 had lower weight when stored in the cool room and room compared to the refrigerator or day of lay (P < 0.05). Eggs collected on day T5 had lower weight when stored in the room compared to other temperatures and the day of lay (P < 0.05).

Table 5.5. The effect of collection day (T4 and T5) and storage temperature on the mean (± SEM) weight of eggs (g) collected from laying hens of different ages and housed in individual cages and exposed to similar ambient shed temperatures.

Collection day	Hen age (weeks)	Storage temperature				SEM
		Day of lay	Refrigerator	Cool room	Room	
T4	40	66.1^{a}	64.9^{a}	62.5$_{B}^{b}$	61.3$_{B}^{b}$	1.36
T5	47	65.1^{ab}	64.6^{a}	65.5$_{A}^{a}$	63.5$_{A}^{b}$	
SEM		2.36		1.36		

$^{a-b}$ Within a row values with different superscripts are significantly different (P < 0.05)
$_{A-D}$Within a column values with different superscripts are significantly different (P < 0.05)

Haugh Unit (HU)

Collection day had a significant effect on HU but this was influenced by storage temperature as the collection day x storage temperature interaction was significant (P < 0.001). On the day of lay and when stored in the cool room, the T4 eggs had lower HU then eggs collected on day T5 (P < 0.05). For both T4 and T5 eggs there was a significantly progressive decrease in HU as the storage temperature increased (all, P < 0.05).

Table 5.6. The effect of collection day (T4 and T5) and storage temperature on the mean (± SEM) HU of eggs collected from laying hens of different ages and housed in individual cages and exposed to similar ambient shed temperatures.

Collection day	Hen age (weeks)	Storage temperature				SEM
		Day of lay	Refrigerator	Cool room	Room	
T4	40	91.1_{B}^{a}	77.3^{b}	63.1_{B}^{c}	49.7^{d}	1.36
T5	47	96.3_{A}^{a}	77.8^{b}	72.2_{A}^{c}	50.4^{d}	
SEM		2.36		1.36		

[a-b] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different superscripts are significantly different (P < 0.05)

Storage day had an effect on HU but this depended on storage temperature (see, Table 5.7) as the interaction between the two was significant (P < 0.001). On all storage days eggs stored in the refrigerator had higher HU measures compared to those stored in the cool room, and in turn eggs stored in the cool room had higher HU than those stored at room temperature (all, P < 0.05). At all storage temperatures there was a significant decrease in HU after 7 days of storage (P < 0.05). When stored in the refrigerator or cool room the HU measure was similar on days 7 and 14 and higher than on these days compared to days 21 and 28 (P < 0.05), when they were again similar. When stored in the room there was a significant decrease in HU as storage time increased (P < 0.05).

Table 5.7. The effect of storage day and storage time on the mean (± SEM) HU of eggs collected from laying hens housed in individual cages and exposed to different shed ambient temperatures during winter.

Storage day	Storage Temperature			SEM
	Refrigerator	Cool Room	Room	
Day of lay	93.7_{A}	93.7_{A}	93.7_{A}	1.49
7	82.0_{B}^{a}	73.8_{B}^{b}	62.3_{B}^{c}	
14	77.7_{B}^{a}	68.7_{B}^{b}	52.7_{C}^{c}	
21	75.0_{C}^{a}	68.1_{C}^{b}	47.1_{D}^{c}	1.72
28	75.5_{C}^{a}	61.5_{C}^{b}	38.4_{E}^{c}	
SEM		1.72		

[a-b] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different superscripts are significantly different (P < 0.05)

Albumen Index (AI)

The effect of collection day on AI (see Table 5.7.) was significant but it was influenced by storage temperature and storage time as the three way interaction, collection day x storage temperature x storage day was significant ($P = 0.004$). On the day of lay, the AI was similar for the T4 and T5 collections.

Storage in the refrigerator. The AI was higher for the T5 collection on day 7 of storage ($P < 0.05$) but on day 21 and day 28 it was eggs from collection T4 that had higher AI ($P < 0.05$). For both collection days there was a significant decrease after 7 days of storage and a further decrease after 14 days ($P < 0.05$). The AI on day 14 of storage was similar to day 21 for both collection days but different to day 28 of storage ($P < 0.05$) while values on day 21 and 28 were similar.

Storage in the cool room. The AI index was higher for the T5 collection on days 21 and 28 of storage ($P < 0.05$). For the T4 collection the AI was similar on all storage days and significantly lower than on the day of lay ($P < 0.05$). For collection T5, the AI was similar on storage days 14, 21 and 28 and lower on these days than day 7 ($P < 0.05$), which was in turn lower than the AI on the day of lay ($P < 0.05$).

Storage in the room. The AI was similar for the T4 and T5 collections on all storage days. For both collections the AI was lower on day 7 than the day of lay ($P < 0.05$). For the T4 collection it was again lower on both days 14 and 21 ($P < 0.05$) but similar on days 21 and 28. For the T5 collection the day 14 AI was similar to day 21 and lower than day 7 ($P < 0.05$), while the AI on day 21 and 28 were similar.

Table 5.7. The effect of collection day and storage time on the mean (± SEM) AI of eggs collected from laying hens of different ages and housed in individual cages and exposed to similar ambient shed temperatures.

		Storage day				
Collection day	Day of Lay	7	14	21	28	SEM
		Stored in the refrigerator				
T4	0.110^{a}	$0.074_{B}{}^{b}$	0.076^{c}	$0.074_{A}{}^{cd}$	$0.071_{A}{}^{d}$	0.004
T5	0.118^{a}	$0.097_{A}{}^{b}$	0.069^{c}	$0.061_{B}{}^{cd}$	$0.054_{B}{}^{d}$	
		Stored in the cool room				
T4	0.110^{a}	0.063^{b}	0.052^{b}	$0.043_{B}{}^{b}$	$0.034_{B}{}^{b}$	0.004
T5	0.118^{a}	0.072^{b}	0.061^{c}	$0.060_{A}{}^{c}$	$0.054_{A}{}^{c}$	
		Stored in the room				
T4	0.110^{a}	0.046^{b}	0.035^{c}	0.021^{d}	0.018^{d}	0.004
T5	0.118^{a}	0.047^{b}	0.033^{c}	0.024^{cd}	0.017^{d}	
SEM	0.003		0.004			

$^{a-b}$ Within a row values with different superscripts are significantly different ($P < 0.05$)
$_{A-D}$ Within a column values with different superscripts are significantly different ($P < 0.05$)

Yolk Index (YI)

The YI was higher for the T5 collection (0.44 ± 0.01) compared to the T4 collection (0.39 ± 0.01) ($P = 0.009$). Storage day had no effect ($P = 0.451$). Storage temperature had a significant effect ($P < 0.001$). Storage at room temperature (0.34 ± 0.01) significantly decreased YI ($P < 0.05$) compared to storage in the refrigerator (0.44 ± 0.02) or the cool room (0.43 ± 0.01) and also compared to the day of lay (0.43 ± 0.01).

Comparisons between the winter collections T5, T6 and T7.

Egg weight

Collection day had no effect on egg weight ($P = 0.312$). Storage temperature had a significant effect but this was dependent on the storage day as the interaction between storage temperature and storage day was significant ($P = 0.054$). It was only when eggs were stored at room temperature that the egg weight changed during the storage period. Egg weight was lower after 21 and 28 days compared to the day of lay or day 7 of storage ($P < 0.05$).

On day 21 eggs stored at room temperature had lower weight than those stored in the refrigerator and then on day 28 the weight of eggs stored at room temperature had lower weight than those stored at other temperatures (P < 0.0).

Table 5.8. The effect of collection day (T5, T6 and T7) and storage time on the mean (± SEM) weight of eggs (g) collected from laying hens housed in individual cages and exposed to different shed ambient temperatures during winter.

Storage day	Storage Temperature			SEM
	Refrigerator	Cool Room	Room	
Day of lay	65.7	65.7	65.7_{A}	0.59
7	64.1	65.5	65.8_{A}	
14	65.9	64.4	64.5_{AB}	
21	64.8^{a}	65.7^{ab}	$63.2_{B}{}^{b}$	0.68
28	65.5^{a}	65.7^{a}	$62.9_{B}{}^{b}$	
SEM		0.68		

[a-b] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different subscripts are significantly different (P < 0.05)

Haugh Unit (HU)

The collection day had no effect on the HU measures (P = 0.241) and the HU was not influenced by storage time (see, Table 5.9).

Table 5.9. The effect of collection day (T5, T6 and T7) and storage time on the mean (± SEM) HU measures of eggs collected from laying hens housed in individual cages and exposed to different shed ambient temperatures during winter.

Storage day	Collection day			SEM
	T5	T6	T7	
Day of lay	93.6	93.6	93.6	3.2
7	75.6	76.9	76.3	
14	67.3	68.9	70.6	
21	65.2	68.1	64.0	2.1
28	60.3	66.0	61.6	
SEM		2.1		

The effect of storage temperature on HU was significant (see, Table 2.10) but changed with storage time as the interaction between the two was significant (P < 0.001). At all storage temperatures there was significant drop in HU after 7 days of storage. When eggs were stored in the refrigerator the HU measures were the same on days 14, 21 and 28 but lower on these days than on day 7 (P < 0.05).The changes were similar when storage was in the cool room except that the measures on day 21 and 28 were similar. When storage was at room temperature there was a progressively significant decrease in HU as storage time increased (P < 0.05).

Table 5.10. The effect of storage time and storage temperature on the mean (± SEM) HU measures of eggs collected from laying hens housed in individual cages and exposed to different shed ambient temperatures during winter.

Storage day	Storage Temperature			SEM
	Refrigerator	Cool Room	Room	
Day of lay	93.6_{B}^{b}	93.6_{A}^{b}	93.6_{A}	0.59
7	82.6_{B}^{a}	80.1_{B}^{a}	66.1_{B}^{b}	
14	77.3_{C}^{a}	75.3_{C}^{a}	54.0_{C}^{b}	
21	74.1_{C}^{a}	74.4_{CD}^{a}	47.1_{D}^{b}	0.68
28	76.0_{C}^{a}	71.4_{D}^{b}	40.8_{E}^{c}	
SEM		0.68		

[a-b] Within a row values with different superscripts are significantly different (P < 0.05)
[A-D] Within a column values with different subscripts are significantly different (P < 0.05)

Albumen index (AI)

Collection day had no effect on AI (P = 0.303). The interaction between storage temperature and collection day was marginally non-significant (P = 0.060). The effects of storage temperature and collection day are given in Table 5.11.

Storage temperature had a significant effect on AI but was influenced by storage time as the interaction between these was significant (P = 0.018).The effect of storage temperature and storage time is given in Table 5.12.

Table 5.11. The effect of collection day (T5, T6 and T7) and storage temperature on the mean (± SEM) AI of eggs collected from laying hens housed in individual cages and exposed to different shed ambient temperatures during winter.

Collection Day	Storage Temperature			SEM
	Refrigerator	Cool Room	Room	
T5	0.074	0.061	0.030	0.004
T6	0.073	0.069	0.033	
T7	0.067	0.069	0.033	0.002
SEM		0.002		

There was significant drop in AI after 7 days of storage at all storage temperatures ($P < 0.05$). When stored in the refrigerator and the cool room there was a further drop in AI at 14 days ($P < 0.05$) where it remained similar to day 28. When stored in the room the AI at 14 days ($P < 0.05$) was lower than day 7 ($P < 0.05$), similar on days 14 and 21 but lower on day 28 than day 14 ($P < 0.05$).

The AI on days 14, 21 and 28 were similar when stored in the refrigerator and cool room but higher on day 7 when storage was in the refrigerator ($P < 0.05$). On all storage days the AI for eggs stored at room temperature was lower compared to storage at other temperatures ($P < 0.05$).

Table 5.12. The effect of storage time and storage temperature on the mean (± SEM) AI of eggs collected from laying hens housed in individual cages and exposed to different shed ambient temperatures during winter.

Storage day	Storage Temperature			SEM
	Refrigerator	Cool Room	Room	
Day of lay	0.114_{A}	0.114_{A}	0.114_{A}	0.002
7	0.087_{B}^{a}	0.077_{B}^{b}	0.050_{B}^{c}	
14	0.070_{C}^{a}	0.066_{C}^{a}	0.034_{C}^{b}	
21	0.062_{C}^{a}	0.065_{C}^{a}	0.026_{CD}^{b}	0.002
28	0.066_{C}^{a}	0.060_{C}^{a}	0.019_{D}^{b}	
SEM		0.002		

[a-b] Within a row values with different superscripts are significantly different ($P < 0.05$)
[A-D] Within a column values with different subscripts are significantly different ($P < 0.05$)

Yolk Index (YI)

The collection day had no effect YI (P = 0.780). For collection day T5 the YI was 0.439 ± 0.011, collection day T6 it was 0.430 ± 0.011 and collection day T7 it was 0.429 ± 0.011. Storage day had a significant effect (P = 0.011) on YI with it being 0.418 ± 0.021, 0.457 ± 0.014, 0.0422 ± 0.013, 0.403 ± 0.013 and 0.0461 ± 0.013, on day of lay and days 7, 14, 21 and 28 days of storage, respectively. The only significant difference was where the YI was lower on day 14 than days 7 and 28 (P < 0.05). Storage temperature had a significant effect on YI (P < 0.001), with it significantly lower (P < 0.05) when storage was at room temperature (0.370 ± 0.12) compared to storage in the refrigerator (0.440 ± 0.012), in the cool room (0.458 ± 0.011) or the YI at the time of lay (0.458 ± 0.010).

Discussion

Experiment 1. **A model for generating suitable samples for detecting proteomic changes in egg albumen protein during aging: The effects of storage temperature and time on egg albumen quality.**

The effects of temperature and storage time on conventional measures of egg quality are well established. The essential objective of the current study was to determine the pattern of change in egg quality and identify samples that would be suitable and relevant to establishing the proteomic changes in egg albumen as eggs age.

Storage in refrigerator at 4.20 ± 0.05ºC resulted in limited changes in conventional measures of egg quality until at least 29 days of storage. Storage in a cool room at 15.87 ± 0.07°C resulted in measurable and progressive changes in egg quality over the 29 storage period. The changes that occurred by 14 and 21 days of storage provided samples that would be suitable for proteomic analysis of egg aging. Storage in the room at 21.8 ± 0.06ºC resulted in major progressive changes in egg quality up to 21 days of storage. After this there was no further change in egg quality. The HU measure is the most common conventional measure of egg quality and relies on egg weight the albumen height. When storage was at room temperature, the egg albumen had deteriorated to a point where the albumen was extremely fluid (thinning) and the height was at its lowest point. Therefore, storage for 14 and 21 days at room temperature provided samples suitable for proteomic analysis of egg aging.

Experiment 2. Determination of candidate proteomic biomarkers for egg freshness

2D-PAGE methodology

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is based on a first dimension, where proteins are separated by their isoelectric point (isoelectric focusing, IEF) and a second dimension, where proteins are further separated by their electrophoretic mobility using SDS-PAGE (May et al., 2012). The 2D-PAGE methodology has been used to analyse for bio-markers by quantifying individual proteins by identifying specific protein spots on the 2D-gel image.

In this particular project, the 2D-PAGE procedure was used to examine the changes in proteins during the deterioration of egg albumen and identify a bio-marker of egg aging and indirectly egg age. While it is possible to start the development of the methodology based on published data, there will always be a need to refine this information to meet the specific needs of individual objectives. In the current project this has been complicated by the fact the egg albumen is dominated by a small number of proteins with ovalbumin making up over 50% of all protein. The predominance of a small number of proteins is an obstacle to obtaining good protein separation using 2D-PAGE analysis.

In Figures 2.10 to 2.15 the areas of the gel profile that are of interest are marked in red and yellow triangles. At this point there has only been a visual appraisal of the protein profiles by comparing the profiles to published proteomic analysis by Wang *et al.* (2011) and Omana *et al.* (2011), by using the location according to pH in the first dimension and MW in the second dimension of the 2D-PAGE analysis. The protein spots located in the red rectangle are proposed to potentially be ovoinhibitor and the spots identified in the yellow triangle are potentially clusterin proteins. The next step is to remove some of these protein spots for sequencing identification. These proteins spots are of interest as there appear to be changes in staining density as the eggs age and the albumen changes seen when egg were stored at room temperature rather than in a refrigerator. These proteins have consistently shown up in those gels providing the best clarity and separation.

Omana *et al.* (2011) stored eggs at room temperature for 40 days and performed 2-D PAGE analysis on samples after 10, 20, 30 and 40 days of storage. They identified 80 proteins and after 10 days of storage the researchers found that 7 protein spots increased in abundance and one decreased in abundance. The same number albumen thinning was observed of changes in protein abundance was identified at day 20 of storage, but after day 20 albumen thinning was observed and there were minimal changes in the abundance of proteins. During aging clusterin, ovoinhibitor, ovotransferrin and some ovalbumin family proteins were found to increase in abundance. Figure 6 is a reproduction of a 2D-PAGE gel from the work of Omana et al., (2011) with the clusterine proteins identified by the red markings.

Wang *et al.* (2011) investigated differences in the protein profiles of albumen for different egg varieties sort from supermarkets using 2D-PAGE analysis. They found 19 proteins that differed between some of the egg varieties. Clusterine was one of these and identified to have MW of around 32 kDa which is different to its theoretical MW of 51.9 kDa. Wang et al., (2011) proposed that the proteins identified in their work as fragments of clusterine and that clusterine had been degrading during the albumen processing. The proposal that the protein spots identified in Figures 2.10 and 2.15 of the current project are likely to be clusterine is feasible based on the proposal that clusterine degrades during storage into smaller fragments and that the MW of these fragments would probably decrease over time. Until the protein spots from the current work are identified it is only possible to speculate what changes are occurring during egg aging.

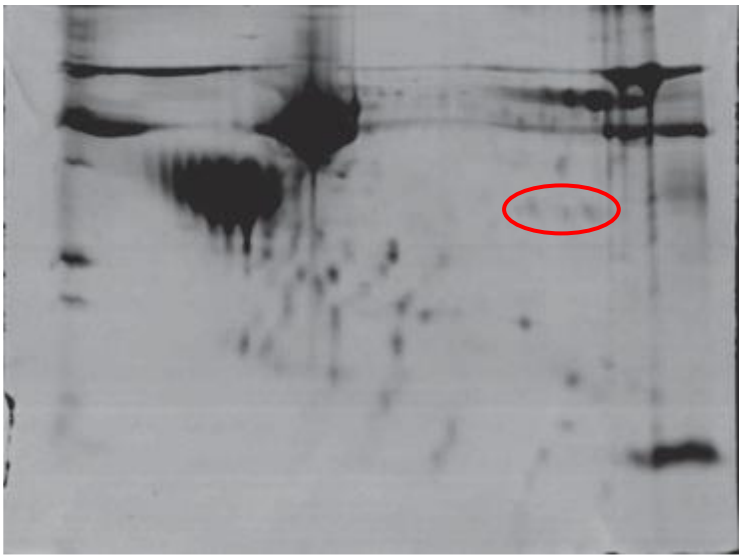


Figure 6. The The two dimesional protein gel profile refproduced from Omana et al., (2011) for albumen extracted form a freshly laid egg and loaded onto a 13cm IGF strip with a pH range of 4-7.

Protein separation using terminal amine isotopic labelling of albumen proteins (N-**TAILS**)

With the difficulties experienced using 2D-PAGE analysis it was decided to investigate using N-TAILS as a potential method of identifying albumen proteins that could be used as a bio-marker of egg aging. N-TAILS is a method in quantitative proteomics which identifies proteins based on the N-terminal fragments. The N-termini peptides and ε-amino groups of lysine ware labelled with water-soluble formaldehyde via reductive methylation (Hsu *et al.*, 2006). The dimethyl labelling using 'heavy' or 'light' formaldehyde treatment of the control and stored albumen samples results in a nominal mass difference of 6 Da between the peptide pairs allows negligible interference between the two isotopic clusters for quantification of peptides (Ji *et al*., 2005).

The N-TAILS approach to identifying protein changes in egg albumen was instigated late in the current project and so only an early analysis is possible. However, the results do provide encouragement that it could achieve our initial objective of identifying a suitable biomarker of albumen quality and indirectly egg aging.

At this point of the analysis there has been good consistency between all of the sample runs, and it appears that there has been excellent coverage of the egg white proteome. The analysis of the LC-MS profiles is continuing.

Experiment 3. **The effect of acute stress on egg quality during storage at different temperatures**

Experiment 3a. The effect of acute relocation stress on egg albumen quality

The relocation of hens had no significant effect on HU or AI while there were some limited effects on YI when storage was in the refrigerator or at room temperature but not the cool room.

The failure to observe a significant difference in the albumen corticosterone concentration immediately after relocation suggests that the hens were not unduly stressed. Earlier work where hens, previously housed individually in conventional layer cages were transferred to group cages and housed at 5, 4, 3 or 2 hens per cage or as a single hen per cage, there significant changes in egg albumen corticosterone concentrations (Downing and Bryden, 2005). The albumen corticosterone concentrations increased from around 1 to 2.6 ng/g, with the effect of group size after relocation just failing to be significant ($P = 0.06$). However, the albumen corticosterone concentrations were significantly higher on days 2 to 4 than later days after the relocation (Downing and Bryden, 2005).

Egg albumen is laid down over a 3-4 h period after oviposition and unless the stress results in elevated corticosterone of sufficient magnitude during this period, there is not likely to be any measurable change in albumen corticosterone concentration. The increase in albumen corticosterone concentration during storage has to be due to movement of corticosterone from the yolk into the albumen. The higher concentrations in eggs collected after relocation of hens suggests that the yolk content in these eggs was higher and in turn the higher yolk accumulation does suggest that hens had been stressed but not to an extent that there were changes in the albumen corticosterone concentration.

Experiment 3b. The effect of transport and relocation acute stress on egg quality

On the day following the transport and relocation, 32.5% of the eggs laid had some form of shell abnormality with the majority being of the target morphology. These target eggs had a lower HU at the time of lay compared to eggs having normal shell morphology but this was not the case for eggs that were laid with misshapen or soft shells. It needs to be made clear that there were only small numbers of eggs with these shell features. While the target eggs had lower HU at the time of lay there was no difference between these eggs and normally shelled eggs at any storage temperature.

Shell morphology had an effect on AI but this depended on storage temperature. On the day of lay, the AI was lower for eggs having the target shell morphology but this difference did was not evident at any storage temperature. For normal shelled eggs the AI was lower as the storage temperature increased but for target eggs the AI was not different when stored in the refrigerator compared to the day of lay but the AI was lower when store in the cool or at room temperature. Shell morphology had no effect on YI.

Stress can delay oviposition in hens (Mills *et al*., 1991; Reynard and Savory, 1999), and these delays often result in eggs with abnormal shells. The type of abnormality seems to depend on the length of the delay in oviposition with Hughes *et al.* (1986) suggesting that the minimum delay necessary to cause an abnormality is around 1.35 h. The abnormalities seen include alterations to the shell, absence of a shell, modification of the normal egg shape (misshapen, slab-sided and equatorial bulges) and various levels of calcium deposited on the outer cuticle (Watt and Solomon, 1985). The frequency of eggs with extra calcium on the outer surface is greatest at peak production and this was found to diminish with age (Mills *et al*., 1991; Yue and Duncan, 2003).

Relocating hens from pens to cages and housing them in groups of 4, 3 or 2 caused a delay in oviposition with some hens even retaining the egg overnight (Hughes *et al*., 1986). Over the first 6-8 days egg production was reduced and a higher percentage of the eggs laid were abnormal, especially during the first 6 days after the move (Hughes *et al*., 1986). Crating and wing banding of hens increased the number of abnormal eggs laid and

interestingly, the number of abnormal eggs laid by hens in a pen adjacent to the wing banded hens increased, even though they themselves were not handled (Hughes *et al.,* 1986).

There is evidence indicating that corticosterone can affect oviposition time and the incidence of abnormal eggs. In hens 66-74 weeks of age, plasma corticosterone concentration was higher, an increase of approximately 1.0 ng/ml, in hens laying soft-shelled or membranous eggs in the afternoon compared to hens laying normal hard shelled eggs (Klingensmith *et al*., 1984).

In the current study, when considering eggs with normal shell morphology there was a significant decrease in HU measures after 7 days of storage at any temperature but it was more severe when egg were stored at room temperature. On the day of lay the HU was similar for all treatment days and it was only after 3 weeks of storage was there significant effects of treatment day on HU measures. In general the HU measures were higher for eggs collected after the stressors were applied (day 3 and 4) compared to treatment day 1. The treatment day had no effect on AI, although there was an effect on YI. But only on the day of lay where it was higher for eggs collected the day after the stressors were applied. .

On the day of lay the egg albumen corticosterone concentration was lower for eggs collected on day S4 than day S1 while the difference between days S1 and S3 was not significant. However, while the pattern of change in corticosterone concentrations differed for the different storage temperatures. At the end of the storage period the albumen corticosterone concentrations where significantly higher in the eggs collected after the stressors were applied.

For egg abnormalities to occur, Reynard and Savory (1999) proposed that the stress needed to be imposed during the time of oviposition. This proposal was derived after subjecting hens to a 6 h period of relocation stress and looking at the effect of oviposition time on and egg quality. For those hens failing to lay during the period of stress, oviposition occurred soon after the stress ceased, if the delay from their normal oviposition time was less than 2.4 h. Generally if the delay was > 2.4 h at the time the stress was removed then oviposition time was much longer and these eggs showed the most extensive abnormalities. Reynard and Savory (1999) make a very feasible argument that once the delay is greater than the critical time, oviposition delay is prolonged because the increase in uterine contractions needed for explosion of the egg is disrupted. During stress, elevated catecholamines or corticosterone at a critical period may interfere with the normal uterine function delaying oviposition and the eventual laying of abnormal eggs.

In the current study, the higher albumen corticosterone concentrations seen in eggs collected after the stress were not higher on the day of lay. The much higher concentrations seen in eggs collected after the stress and stored for more than 3 weeks at any of the three temperatures suggested that the hens had experienced higher corticosterone concentrations and that this had resulted in greater corticosterone accumulation in yolk which later diffused into the albumen during storage.

Experiment 4. The effect of hen age and storage temperature and storage time on egg quality

There is extensive data in the literature reporting on the effects of hen age on egg quality and some extended the work to include the effects storage time and storage conditions on

egg quality. In studies looking at the effects of hen age on egg quality there are two protocols commonly used to evaluate these effects. In the first option, an individual flock of hens is followed through a full production cycle from the point of lay with egg samples collected at different ages to measure changes in egg quality. For the second option, eggs are collected at one time from different aged flocks and egg quality compared. Both experimental designs include some confounding problems. For the first approach, while the genetics remains the same there are potential interactions with changes in climatic conditions, nutrition and management. Even if the studies are performed in a controlled environment, changes in the nutrition are most likely changed over a full production cycle. The second protocol uses flocks of different ages where the environment, nutrition and management could differ and influence the egg quality. Some examples of these approaches are given below.

de Menezes *et al*. (2012) reared hens as one flock and eggs were collected at 35, 40, 45 and 50 weeks of age and stored at 8°C or 25°C. During the collection period the housing temperature varied between 19-32^{0}C and the humidity ranged between 75 and 95%, meaning that there is the potential influence of temperature on egg quality. Hosseinisyar *et al*. (2007) collected eggs from two commercial flocks at 28 and 68 weeks of age and stored them at room temperature or 6°C and measured egg quality. However, as the flocks were in commercial facilities, differences in environment and management are likely confounding issues. Akyurek and Okur (2009) obtained eggs from brown-nick layer hybrids that were housed on a commercial egg farm. Eggs were collected over two-age periods when the hens were 22 and 50 weeks old. The eggs were sampled fresh and after storage for 3, 7, 10 and 14 days at 4°C and 20°C. In this study, the confounding issues are again differences in climatic condition, changes in dietary ingredients and other management changes. Similar issues are likely with the study of Chung and Lee (2014) where eggs were collected from two age flocks (40 and 60 weeks) from a commercial layer operation and egg quality determined at lay and after 7, 14, 21 and 28 days storage at room temperature or in a refrigerator. Roberts *et al.* (2013) collected eggs from commercial flocks in different stages of lay: early (< 25–40 weeks – 10 flocks), mid (40–55 weeks – 10 flocks), late (55–65 weeks – 6 flocks) and very late (>65 weeks – 8 flocks). Again, there are potential confounding issues associated with differences in nutrition, management and environment but the researchers did use a number of flocks in each age category and so this would help account for some of the differences on individual farms.

In the current study, hens were housed in a single shed where hens of different aged hens confined to individual pens in the facility. The hens were all Isa Brown strain and had been transferred to the layer housed at 15-16 weeks of age from the same breeder and rearing farm. The birds experienced the same environmental conditions and were fed the same mash diet prepared on the farm. Under these conditions differences due to strain, environment, nutrition and management are controlled and would not interfere with measuring age effects on egg quality.

When determining the effect of production systems on egg quality limiting the collections to Isa brown flocks between 50 and 63 weeks age would eliminate the confounding problem of hen age on the results.

Experiment 5. The effect of layer house ambient temperature on egg quality during storage under three different temperatures

In looking at the results from the summer collections (T1, T2 and T3 collections), the HU measure was higher for the T1 collection than the T2 and T3 collections, with the HU similar for T2 and T3 collections on the day of lay. The temperature pattern experienced by the hens leading up to the T1 and T3 collections were similar and the pattern for both these collections were different to the pattern experienced by the hens leading up to the T2 collection. So, while the hens prior to the T1 and T3 collections had similar temperature patterns there was a significant difference in HU and for these hens there was an age difference of 7 weeks. So, the difference in HU is most likely due to differences in age. Prior to the T2 and T3 collections the hens experienced distinctly different temperature patterns but had similar HU measures at the time of lay. The age difference between hens at the T2 and T3 collections was 4 weeks. Here it would appear that neither the flock age nor the shed temperature had any effect on the HU measures at the time of lay.

There was a significant drop in HU after 7 days at all storage temperatures although it was more pronounced as the storage temperature increased. When eggs from the summer collections were stored in the refrigerator there was no consistent pattern to the changes for the different egg collections. However, over the 28 days of storage the rate of change in HU was similar for all three summer collections. After day 7 of storage the day of collection (T1-T3) had no effect on the HU measures. When stored at room temperature the eggs collected at T3 (37 weeks) had a lower HU measures compared to the earlier collection days.

Collection day had no effect on the AI with the main influence being storage temperature with the AI being lower as the storage temperature increased. Collection day had no effect on YI when eggs were stored in the refrigerator. When stored in the cool room or the room eggs collected on day T3 had lower YI than those eggs collected on day T1. Prior to these two collections hens were exposed to similar temperature patterns and so the differences in YI are more likely due to the differences in the hen ages. When stored in the cool room or the room eggs collected on day T2 had similar YI than those eggs collected at day T1. Prior to these two collections hens were exposed to different temperature patterns and so there being limited differences in YI it is unlikely that the ambient temperature had any effect on YI.

When looking at the winter colletions (T5, T6 and T7), the collection day had no effects on egg HU, AI or YI measures at the time of lay or at any storage day. The only effects seen were the typical interaction between storage day and storage temperature, with storage at room temperature resulting in lower egg quality measures during storage. The average temperatures for the winner collections varied between 19 and 15ºC and the temperatures ranged between 21.3 and 13ºC. In this range shed temperature had no effect on egg quality.

The effect of shed temperature on hen physiology and egg quality depends on the absolute temperature, the duration of the exposure, humidity and strain susceptibility. In studies examining the effects on temperature on egg quality an array of experimental designs are used. Typical designs are to expose hens to a fixed temperature pattern over a period or use a cyclic temperature pattern that attempts to mimic heat wave conditions seen as part of the normal environmental conditions in commercial practice. However, many studies expose hens to the conditions for extended periods and in some ways are not typical of commercial conditions.

Most poultry houses in Australia, while not being completely environmentally controlled do have some form of temperature mitigation strategy. Serve heats stress conditions can be experienced in the summer but more commonly hens need to deal with moderately high temperatures during the day but then lower temperatures during the night. Experimental designs that use cyclic periods of high temperature are more representative of conditions hens are likely to experience in commercial sheds. However, these designs often use long durations of high cyclic temperature and would be representative of extreme heat wave conditions (> 32ºC) rather than extended periods of moderately high temperature (25-30ºC). This latter more moderate high temperature is the range in temperature that can often be achieved using temperature mitigation strategies such as tunnel ventilation, roof insulation and wetting, circulation fans and misting.

Roberts and Ball (1998) using small numbers of hens from different strains and exposing them to cyclic periods of high temperature (33ºC for 06:00 h and 20ºC for 18:00 h) over 2 weeks found the HU measures were lower during the period of high temperature compared to the next 2 weeks when hens were held at 20ºC. However, when compared to the eggs collected before the cyclic heats stress there were no differences in HU. In commercial practice it is possible to have extended periods of heat stress but egg production would not be sustainable in areas where this was a regular rather than infrequent occurrence. Faria *et al*. (2001) exposed hens over a two week experimental period to a constantly high ambient temperature (30-32 ºC), cyclic high temperature (32ºC for 6-8 h and 23-25 for 16-18 h) or constant temperature (24.8 -27.0ºC) and determined that in eggs collected over the last 2 days of the treatments period there was no effect of temperature on HU measures. Durmus and Kamanli (2015) maintained hens at three temperatures, 12 ± 2ºC, 20 ± 2ºC and 32 ± 2ºC over 27-29 weeks of age with quality determined on eggs collected over the last 2 days of this period. The researchers found no effect of temperature on HU measures. Based on these and the current observations, shed temperature has no effect on HU measures when the daily shed temperature averages less than 26ºC. However, if the goal is to measures differences in egg quality between production systems, then collecting egg during the winter would guarantee that shed temperature had no influence on the quality measures.

Implications

1. Storage of eggs in a cool room with a mean (± SEM) temperature of 15.87 ± 0.07 ^{0}C, or in a room temperature with a mean (± SEM) temperature of 21.8 ± 0.06 ^{0}C, storage after 14 and 21 days resulted in significant deterioration in egg albumen quality and provided samples suitable for use in the proteomic analysis of egg aging.

2. Hen age is known to effect egg quality but in the current work the main influence occurred in the early stages of the production stages. Hens aged between 50 and 63 weeks had similar measures of egg quality and the changes during storage were similar and so independent of hen age at this time in the production cycle. The effect of age was independent of any effect on diet, environment and strain as all egg collections were made on the same day from a barn production facility where the different aged Isa Brown flocks were housed in the one layer shed and maintained under the same management conditions.

3. It is clear from the published literature that prolonged periods of heat stress (> 32ºC) cause poorer egg and shell quality but in commercial practice it is not common for hens to have to experience such extreme condition for protracted periods. In the current work when hens were faced with summer shed temperatures where the daily average prior to lay was less than 26ºC, the shed temperature had no effect on egg quality at the time of lay or during storage. Winter temperatures where the daily average temperature was between 15-20ºC, the shed temperature had no effect on egg quality.

4. Using short duration transport and pen relocation to induce acute stress resulted in approximately one third of hens laying eggs with some form of shell abnormality with the main condition being the 'target' shell morphology. The 'target' eggs had lower HU measures at the time of lay but, after this, storage had similar effect as seen for eggs laid with normal shell morphology. Those eggs that had normal shell morphology and laid the day after the acute stress was applied or the day before, had similar egg quality at lay and during storage. During storage eggs laid after the acute stress had higher albumen corticosterone concentrations and the source of this corticosterone would be the egg yolk.

5. In this particular project the 2D-PAGE procedure was used to examine the changes in proteins during the deterioration of egg albumen and identify a bio-marker of egg aging and indirectly egg age. Extensive effort was required to modify the different stages of the 2D-PAGE procedure and was complicated by the fact the ovalbumin is such a dominating protein in the albumen. At completion of the project protein profiles were developed that located potential bio-markers of albumen deterioration but at this time their identification needs to be confirmed.

6. The N-TAILS approach to identifying protein changes in egg albumen was instigated late in the current project and so the analysis is in its earlier stages but does provide encouragement that it could achieve our initial objective of identifying a suitable biomarker of albumen quality and indirectly egg aging. At this point of the analysis there has been good consistency between all of the sample runs, and it appears that there has been excellent coverage of the egg white proteome. The analysis of the LC-MS profiles is continuing.

Recommendations

1. Extensive effort was committed to developing 2D-PAGE conditions that would allow separation of egg albumen proteins and the identification of a potential biomarker of aging. By the end of the project there were potential proteins proposed to change during egg albumen deterioration with the next step being to isolate and identify these.

2. In the later stages of the project the use of N-terminal amine isotopic labelling of albumen proteins provided good evidence that it is a procedure that can be used to identify bio-markers of egg aging. While sample preparation is time consuming the instrumentation available allows quick throughput and raid scanning using mass spectrometery.

3. An early objective of the project was to evaluate egg quality from different production systems. In retrospect, such an evaluation would have little relevance if some of the other factors such as hen age, ambient temperature and acute stress, could not be accounted for. The project has identified limits to these factors and if applied would allow for the evaluation of production system effects on egg quality. If eggs are collected from Isa Brown hens aged between 50 and 63 weeks of age, the average daily shed temperature is less than 20^0C (winter) and only eggs with normal shell morphology are used then the effects of hen age, shed temperature and acute stress will not be a consideration in any assessment of production system effects on egg quality.

Acknowledgements

Mr Don Nicholson's technical contribution to the project is greatly appreciated.

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	Proteomic measures of albumen degradation as indicators of egg freshness
Poultry CRC Sub-Project No.:	3.2.4
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Sub-Project Overview	Provide an understanding of the proteome changes in egg albumen associated with a deterioration in egg freshness. Develop an understanding of how stress influences egg quality. Define a set of limits to hen age, ambient temperature and stress which could then be used to evaluate the quality of eggs from different production systems.
Background	Changes in the albumen especially the viscosity of the thick albumen is used as measure of freshness. The measurement of protein changes is likely to provide an accurate determination of freshness. A core part of the current project was to use 2D-PAGE analysis to separate and isolate albumen proteins and identify a protein bio-marker that could be used to develop an assay to measure egg age.
Research	The initial 2D-PAGE was based on published procedures but needed extensive refined to better meet the needs of the current project. Because of the difficulties associated with the 2D-PAGE analysis, an alternative procedure was used. This was Terminal Amine Isotopic Labelling of Substrates (N-TAILS). In two experiments the effect of acute stress on egg quality was investigated. The effects of hen age on egg quality was evaluated by collecting eggs from different aged flock housed in barn facility, where the strain, rearing, shed environment, nutrition and management were the same. The effect of ambient temperature on egg quality has been investigated over summer and winter.
Sub-Project Progress	Using the 2D-PAGE procedures, the most obvious changes occurred when eggs were stored at room temperature and were obvious after just 7 days storage. After refinement of the 2D-PAGE procedures, protein spots were isolated from stored albumen samples and by comparing their location based on pH and MW with published data it appeared that the abundance of ovoinhibitor and clusterin proteins had changed during storage. The next step is to remove these protein spots and have them identified. The N-TAILS approach was instigated late in the current project and so the analysis is in its earlier stages but does provide encouragement that it be used to identify a suitable biomarker of egg aging. Hens aged between 50 and 63 weeks had similar measures of egg quality and the changes during storage were similar and so independent of hen age. When the daily average temperature was between 15-20ºC, temperature had no effect on egg quality. Those eggs having normal shell morphology and laid the day after acute

	stress had similar egg quality at lay and during storage as eggs lain the day before the stress was applied.
Implications	The N-TAILS approach to identifying bio-markers of albumen deterioration and aging is likely to be a better strategy that using the 2D-PAGE analysis because the predominance of ovalbumin in egg albumen makes separation difficult using the 2D-PAGE procedure. The project has identified limits to hen age, shed temperature and acute stress that if applied in an experimental design would allow for a valid assessment of production system effects on egg quality.
Publications	