POULTRY CRC LTD

FINAL REPORT

Sub-Project No: 3.2.2

PROJECT LEADER: Juliet R Roberts

Sub-Project Title:
Eggshell quality and the risks of food borne pathogens

DATE OF COMPLETION: September 15, 2013

ISBN 1 921010 94 0

Eggshell quality and the risks of food borne pathogens
Sub-Project No. 3.2.2

Researcher Contact Details

Juliet R Roberts
Animal Science
School of Environmental & Rural Science
University of New England
Armidale, NSW 2351

Phone: (02) 6773 1995
Fax: (02) 6773 3922
Email: jrobert2@une.edu.au

Kapil K Chousalkar
School of Animal & Veterinary Sciences
University of Adelaide
Roseworthy Campus
Roseworthy, SA 5371

(08) 8313 1502
(08) 8303 7956
Email: kapil.chousalkar@adelaide.edu.au

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

Poultry CRC Ltd Contact Details

PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: admin@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

Published in August 2014

Executive Summary

Eggs are periodically implicated in cases of food borne illness. This project examined the relationship between egg quality and food safety. Shell quality generally declines with increasing flock age and this was illustrated in the findings of Chapter 3 for traditional measures of egg shell quality as well as for egg shell ultrastructure. However, translucency did not vary consistently with increasing flock age and cuticle cover was not significantly different among flock age groups. Microbiological studies of eggs from the same flocks (Chapter 4) found that there were no significant differences among flock ages for total *Enterobacteriaceae* on the shell surface or in shell pores. *Salmonella* was found on 4.51% of egg shells and the most common serovar was *Salmonella* Infantis. Chapter 5 reports on a study on the incidence of antibodies for *Mycoplasma synoviae* (MS) in the egg yolk of eggs collected from commercial farms. There was a correlation between MS serological status and the egg quality variables of translucency, shell breaking strength, shell reflectivity and shell deformation but no correlation for egg weight, shell weight, percentage shell or shell thickness. The potential for egg washing to affect the cuticle and thereby the ability of bacteria to penetrate the eggshell was investigated in Chapter 6, using *Salmonella* Infantis. A higher incidence of bacterial penetration was associated with a higher incidence of shell ultrastructural features associated with poorer quality shells such as late fusion, type B bodies, type A bodies, poor cap quality, alignment, depression, erosion and cubics. However, although egg washing reduced the quality of cuticle cover, it did not significantly influence the incidence of bacterial penetration of the egg shells. This aspect of the project was investigated in more detail in the studies presented in Chapter 7 which used a range of phage types of *Salmonella* Typhimurium. All phage types of *S.* Typhimurium were able to penetrate eggshells and survive in egg albumen at 20°C. Polymerase chain reaction results showed that the incidence of penetration was higher for washed than for unwashed eggs. Again, a correlation was found between bacterial penetration and the incidence of unfavourable shell ultrastructural features. Egg washing was also shown to cause damage to the cuticle. The results of a small study conducted, using *Salmonella* Agona (Chapter 8), found that *S.* Agona could penetrate the eggshell but that there were no consistent differences between washed and unwashed eggs. A small study investigated the incidence of microcracks before and after commercial egg processing (Chapter 9). There was no difference in the incidence of translucency before and after processing. However, the incidence of microcracks following processing was significantly lower than prior to processing, suggesting that the commercial crack detection equipment was identifying a significant proportion of the eggs with microcracks. Chapter 10 reports on a study which compared eggs from one cage production system and one free range production system, at different ages of the flocks. As was reported in Chapter 3, there were age effects on shell quality measurements. Translucency score and shell reflectivity (shell colour lightness) were higher for the free range flock whereas egg weight, shell weight, percentage shell and shell thickness were higher for the cage flock. Shell ultrastructure was generally better for the cage flock. Although the overall bacterial load on the eggshells was low in this study, it was generally lower for the cage flock than for the free range flock. Concerns about a possible relationship between the incidence of shell translucency and the ease with which bacteria are able to penetrate eggshells led to a more detailed study of the underlying causes of shell translucency. Chapter 11 reports on the use of computer tomography to better study the shell structure, particularly the shell pores, of eggs with different translucency scores. These studies indicated that a higher score for translucency was correlated with a higher incidence of pores that branched towards the outside of the eggshell.

Contents

Chapter 1: Introduction

The eggs produced in Australia are of very high quality. However, it is widely recognised that bacteria such as *Salmonella* are a potential threat to the egg industry so the industry needs to be vigilant in monitoring the presence of these bacteria on or in eggs. Although there is the potential for vertical transmission of bacteria (as has been shown to occur for *Salmonella* Enteritidis and *Mycoplasma synoviae* - MS), it is generally accepted that horizontal transmission is the most likely source of contamination of shell eggs with most bacteria. MS was not detected in eggs from commercial laying hens during a previous Poultry CRC project (CRC Strategic Project 09-01-UNE). However, that project detected *Mycoplasma* in turkey eggs from a flock which was known to be positive for *Mycoplasma*. As MS has been associated with eggshell defects (Feberwee *et al*., 2009 a,b), it is essential to establish whether or not this organism can be detected from egg contents. It is difficult for bacteria to move across an intact good quality egg shell. However, earlier reports indicate that small defects in the egg shell may provide the means for the predominant bacterial species on the egg shell to penetrate the egg shell and move into the egg contents (De Reu *et al*., 2006).

The internal properties of eggs favour survival and growth of contaminating organisms which are Gram-negative (*E. coli* and *Salmonella* are Gram negative bacteria). Gram negative bacteria have a relatively simple nutritional requirement and have the ability to develop at low temperatures (Board and Tranter, 1995). To the egg industry, a good quality egg means the provision of an egg acceptable to the consumer. Egg shell quality can be influenced by many factors including age, strain of hen, temperature, disease (Roberts, 2004). Integrity of eggs and egg shells is very important for both egg producers and consumers. Translucent eggs and microcracks were observed during egg quality analysis of eggs collected from various farms across Australia during a previous AECL Project (AECL UNE-86). The role of the phenomenon known as “translucency”, and the presence of microcracks, in reducing egg shell strength required further definition. Also, the factors which are responsible for the formation of translucent eggs are not clear. A Dutch strain of *M. synoviae* has been found to be one of the factors responsible for formation of translucent eggs (Feberwee *et al*., 2009 a,b). However, there was little information about the effects of Australian strains of *M. synoviae* on the oviduct and the potential formation of translucent egg shells or egg shells with apical abnormalities.

The extent to which the cuticle of the egg shell covers the surface of the egg is important as the cuticle represents the first line of defence for the egg. It was not clear to what extent shell defects such as translucency, microcracks and poor cuticular covering increase the likelihood that bacteria on the outside of the shell will move into the egg contents. Preliminary studies conducted during CRC Strategic Project 09-01-UNE showed that egg shell translucency can potentiate the entry of microorganisms such as *Staphylococcus aureus, E. coli* and *Salmonella* Infantis into eggs at room temperature. It was observed that egg processing facilities, whether they use visual candling or electronic inspection and crack detection, allow through eggs with some small cracks (it would not be economically realistic to do otherwise) and the risk that such eggs pose to product safety is uncertain. According to Bain *et al*. (2006), microcracks are difficult to detect through the electronic crack detection system and eggs in retail outlets are likely to have micro cracks as a result of handling during collection, grading or packing. One of the strategies to minimize egg shell contamination is washing of eggs. However, the effects of washing on the egg shell cuticle, especially for eggs that contain microcracks, needs to be explored. The project documented the incidence of translucency and microcracks in egg shells. It also evaluated the extent to which these features and an incomplete cuticle increase the likelihood of bacteria penetrating the egg shell. This project was conducted in collaboration with that of Dr Margaret Sexton and colleagues (Sub-Project 3.2.1).

Objectives

The objectives of this Sub-Project, stated in the original application, were to:

- Elucidate the correlation between egg shell translucency, shell strength and product safety in laying hens.

- Determine the relationship between egg shell translucency and the occurrence of microcracks in the eggs of commercial laying hens.

- Determine the effect of egg shell translucency, the presence of microcracks, the quality of the shell cuticle and egg shell thickness on the ability of bacteria such as *Salmonella* and *E. coli* to penetrate the egg shell

- Determine the correlation between strain of hen, stage of lay and bacterial load on the eggshell surface and in the egg contents

- Sample flocks for the presence of food-borne pathogens, where problems are identified in eggs from these flocks

- Determine the role of egg washing in bacterial penetration of eggs.

Chapter 2: Methodology

This section outlines methodology that is common to more than one of the experimental chapters.

Traditional measurements of egg quality

Eggs were scored for translucency (0 lowest – 5 highest) using an egg candler and analysed for traditional egg shell quality measurements: shell colour by reflectivity, egg weight, egg shell breaking strength by quasi-static compression, shell deformation to breaking point and shell weight (egg quality equipment, Technical Services and Supplies, U.K.). Shell thickness was measured using a custom-made gauge based on a Mitutoyo Dial Comparator gauge Model 2109-10. Percentage shell was calculated from shell weight and egg weight. Egg internal quality was measured as albumen height, Haugh Units and yolk colour (TSS equipment).

Evaluation of eggshell cuticle cover using cuticle blue dye

Shell colour was measured by the L*a*b* colour system using a Konica Minolta spectrophotometer Model CM-2600d. "L*" has a maximum of 100 (white) and a minimum of 0 (black). For "a*", green is towards the negative end of the scale and red towards the positive end. For "b*", blue is towards the negative end and yellow towards the positive end of the scale. Shell reflectivity (%) was also measured with a shell reflectivity meter (Technical Services and Supply, U.K.). Eggs were then soaked in MST cuticle blue dye for 1 minute, rinsed in distilled water to remove excess stain and allowed to dry in air. Shell colour (L*a*b*) and shell reflectivity were measured on the stained eggs. A single score was calculated after the method of Leleu *et al.* (2011) as: $\Delta E^*_{ab} = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$.

Evaluation of eggshell cuticle cover using scanning electron microscopy

Eggs were stained with cuticle blue dye and egg internal contents were removed by making a small hole at the blunt end of the egg using a Dremel High Speed rotary tool, 300 series. The inner shell walls were then rinsed with tap water to remove the adherent albumen, taking care not to wash off any cuticle stain. Small pieces of shell (1 cm^2) were cut out, mounted on aluminium stubs (0.9 cm diameter) using conductive silver paint (1005 aqueous conductive silver liquid-SEM adhesive, ProSciTech, Australia) and photographed under a dissecting microscope with attached camera. The same pieces of shell were then gold sputter coated in a Jeol MP-19020NCTR Neocoater and viewed and photographed under a Jeol JCM-5000 Neoscope benchtop scanning electron microscope

Scoring of ultrastructural features of the mammillary layer

Eggs were scored for the extent of translucency, photographed individually on a candling box, and processed for viewing of the ultrastructure of the mammillary layer. Small pieces of shell (1 cm^2) were cut from the equator of the egg shell, soaked overnight in distilled water and the shell membranes removed. The samples were then dried thoroughly and placed in a Biorad PT7 150 Plasma Asher for 4 hours to remove any remaining shell membrane (Reid, 1983). Samples were then mounted on aluminium stubs using conductive silver paint (1005 aqueous conductive silver liquid-SEM adhesive, ProSciTech, Australia), gold sputter coated in a Jeol MP-19020NCTR Neocoater and viewed under a Jeol JCM-5000 Neoscope desktop scanning electron microscope. Each sample was scored for ultrastructural features as

described by Solomon (1991). Mammillary cap size was scored as 1 (similar), 2 (variable), 3 (highly variable). Mammillary caps were scored according to their quality which was assessed as both the size of the cap in relation to its cone and the degree of membrane attachment, from best (1) to worst (5). Alignment, changed membrane (membrane not removed by plasma ashing), cubic cone formations, confluence, cuffing, early fusion, late fusion, depression, erosion, hole, type A bodies, type B bodies, aragonite and cubics were each ranked for incidence from 1 (none) to 4 (extensive).

Enumeration of *Enterobacteriaceae* from eggs

Visually clean eggs were processed for isolation of bacteria from the egg shell surface, egg shell crush and internal contents. Eggs were candled to ensure they were intact eggs without cracks.

Egg shell surface wash

Six pooled eggs were placed in 60 mL of sterile phosphate buffered saline (PBS, pH 7.4) in Whirl-Pak bags and rinsed by shaking for 2 mins. Before rinsing, PBS was warmed to 42°C to facilitate bacterial recovery. After a rinse sample was obtained, each egg was removed and transferred to a different sterile bag. Rinsates and intact eggs were then stored at 4°C overnight if required. A 100 µL of the PBS rinsate was plated onto each of nutrient agar, MacConkey and Violet Red Bile Glucose Agar (VRBGA) (Oxoid, Australia) to enumerate total bacterial count and *Enterobacteriaceae* counts, respectively. From the same rinsate, 1 mL was transferred to 4 mL of buffered peptone water (Oxoid, Australia) for *Salmonella* isolation. Plates and buffered peptone water were incubated at 37°C overnight. After incubation, colonies on the plates were counted. *Salmonella* were not enumerated separately from total bacterial count and *Enterobacteriaceae* counts.

Shell crush methodology

For the isolation of bacteria from egg shell pores, egg shells were processed as described by Musgrove *et al*. (2005a,b). Briefly, after egg shell surface processing, each egg shell surface was dipped into 70% alcohol for 1 min to kill any bacteria on the outside of the shell and was allowed to air dry in a biosafety cabinet. The eggs were cracked open into a sterile container. The inside of the egg shells was then washed with sterile phosphate buffered saline to remove the adhering egg albumen because of the antimicrobial activity of albumen. Shell and shell membranes of six pooled egg samples were transferred into a sterile bag (Nasco Whirl Pak Bags, USA) and crushed in order to expose all the shell. To each bag, 60 mL of buffered peptone water was added. The bag was incubated at 37°C overnight for further processing. The shell crush was processed for bacterial enumeration in the same way used for shell wash.

Egg internal contents

The egg internal contents collected in the sterile containers were thoroughly mixed and 1 mL of egg internal content was inoculated with 4 mL of either buffered peptone water (BPW) mixed thoroughly and incubated at 37°C. A 0.1 mL sample of the incubated BPW enrichment was inoculated into each of the MacConkey and VRBGA plates. The plates were incubated at 37°C overnight. After incubation, all the plates were examined for the growth of bacterial colonies.

Identification of bacteria

Isolation and identification of *Salmonella* spp.

The isolation of *Salmonella* was carried as described earlier by Cox *et al*. (2002). To isolate *Salmonella* spp., the inoculated buffered peptone water (from shell surface, shell crush and egg internal contents) was incubated at 37°C overnight and 100 µL of this sample was

inoculated into Rappaport Vasidalis (RV) broth (Oxoid, Australia) which was then incubated at 42 °C for 24 h. A loopful of the same sample was streaked on Xylose lysine deoxycholate agar (XLD; Oxoid Australia) and Bismuth Sulphite agar (BSA; Oxoid, Australia) plates. Individual bacterial colonies were selected and subjected to Gram staining. Presumptive *Salmonella* colonies from BSA or XLD agar were selected and used to stab inoculate Triple Sugar iron agar slopes (TSI; Oxoid, Australia). After incubation at 37°C, the inoculated TSI slopes were examined at intervals of 24 h up to 72 h, for typical *Salmonella* reactions. The presumptive *Salmonella* colonies were also tested by slide agglutination reaction using Poly O and Poly H antigens (BD, Australia) Slopes of isolates were sent to the Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia for *Salmonella* confirmation.

Characterisation of *Enterobacteriaceae*

The colonies of *Enterobacteriaceae* were selected and characterised using API© strips (Biomerieux, Australia). Strips were inoculated, handled as per the manufacturer's instructions and reactions were recorded using API webplus software (Biomerieux, Australia).

Egg penetration of washed and unwashed eggs

Egg washing

The egg washing process used in these studies involved the stages of pre-washing, washing with the aid of a surfactant/cleaner, sanitizing and drying. A laboratory based washer which could hold 15 eggs in three rows of five rotating rollers was used for the physical mechanics of the egg washing. Washing was performed using a hydroxide and hypochlorite based solution at the concentration of 0.45% (v/v) which equates to a pH of ~12 and ~200 ppm hypochlorite in the working solution at 40°C. Washing was followed by a compatible sanitizer (at a concentration of 0.16% (v/v)) which equated to ~200 ppm hypochlorite in the working solution at 32°C. Eggs were washed and sanitized for 46 and 22 seconds respectively. The pressure of the spray was 3 psi without brushes. Eggs were left on the bench for 15 minutes to dry and used for further experiments.

Inoculum preparation

Salmonella isolates (of different serovars and phage types), stored at -80°C in 80% glycerol were plated on xylose lysine deoxycholate (XLD) agar (Oxoid, Australia) and incubated overnight at 37°C. Colonies were selected from XLD agar and resuspended in phosphate buffered saline (PBS) to match the turbidity equivalent with a 0.5 McFarland standard (BioMerieux, Australia). Enumeration of viable bacteria was performed by serial dilution and spread plating on XLD agar, and incubated overnight at 37°C. Following enumeration, 200 mL inocula containing 10^3 and 10^5 colony forming units (CFU) per mL were prepared for each serovar. Agar filled eggs and whole eggs were immersed for 90 sec in one of three dilutions: PBS (control), ~10^3, and ~10^5 CFU/ mL *Salmonella* inocula.

Agar method for assessment of the eggshell penetration

The effects of washing, translucency, and eggshell quality on the bacterial penetration of the eggshell were assessed by the 'agar egg' method as described by De Reu *et al*. (2006). Fresh eggs were obtained from the cage front of layers. All eggs were candled, scored for translucency, and divided into two translucency groups based on candling score; where 1 = low translucency, and 2 = high translucency. For scoring translucency, a quantitative approach was used where a 1 cm^2 area of eggshell was marked and numbers of lighter coloured spots on the eggshell (as viewed over a light source) were counted. Eggshells with fewer than 10 spots/cm^2 were considered of low translucency. Eggs from each group were then allocated to washed and unwashed groups and subsequently allocated to inoculated and control groups. Each egg was dipped into 70% ethanol for 30 sec for sterilization of the egg shell surface and aseptically air dried for 10-15 min.

The internal contents of each egg were removed using an 18 g needle (BD, Australia) at the blunt end of the egg. Eggs were also washed internally with sterile PBS (pH 7.2) to remove residual albumen. Eggs were then filled with XLD agar and sealed after the agar solidified. Agar-filled eggs from each treatment group (washed and unwashed) were immersed for 90 sec in approximately 10^5 CFU/mL solution of *Salmonella*. Eggs from the control groups (washed and unwashed) were immersed in sterile PBS for 90 sec. After inoculation, agar-filled eggs were incubated at 20˚C for 21 days. After incubation, the eggs were aseptically opened and the penetration of *Salmonella* spp. was assessed by the blackening of the interior egg shell.

Whole egg penetration experiments

The effects of egg washing on *Salmonella* survival on the eggshell surface and penetration across the eggshell, as well as the survival of *Salmonella* in the internal contents of the egg, were investigated using a 'whole egg penetration' approach. Eggs were divided into two groups: washed (n=30) and unwashed (n=60). Washed eggs were divided into one control (PBS) and two treatment groups (10^3 and 10^5 CFU/mL) with 10 eggs each. All the washed eggs were incubated at 20˚C after exposure to *Salmonella* or the sham PBS treatment. Unwashed eggs were divided into two groups of eggs. Group 1 was further divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. Eggs from group 1 were incubated at 20°C after exposure to *Salmonella* or the sham PBS treatment. Group 2 was also divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. These unwashed eggs were incubated at 37˚C. The reason that only unwashed eggs were incubated at 37˚C is that washed eggs are not used for hatching purposes in Australia. Each egg was dipped into 70% ethanol for 30 sec to sterilize the outer shell and allowed to air dry in a biosafety cabinet for 10-15 min. Eggs were then immersed for 90 sec in 10^3 CFU/mL or 10^5 CFU/mL of *Salmonella*. After inoculation, eggs were incubated at 20˚C or 37˚C for 21 days.

Chapter 3: Egg quality and age of laying hens: implications for product safety

Abstract

The hen's egg is a highly nutritious food source which is generally safe from microbiological contamination owing to a number of innate antimicrobiological properties. However, defects in the egg shell have the potential to increase the probability of bacterial entry into the egg. This study investigated two aspects of shell quality which have the potential to influence the food safety of table eggs: eggshell translucency and the integrity of the cuticle covering the outside of the egg, in relation to the age of a flock. Eggs were collected from commercial caged layer flocks in early, mid, late and very late lay. Eggs were candled and scored for translucency. Cuticle cover was estimated using MST Cuticle Stain and a Konica Minolta hand-held spectrophotometer. Traditional measures of egg quality were determined using specialised equipment (TSS, U.K.) Shell ultrastructural features were scored following plasma ashing of shell samples and viewing under a benchtop scanning electron microscope. Translucency score was significantly higher in late lay than for all other age groups. Shell quality declined with increasing flock age. However, the extent of cuticle cover on the egg shell was not significantly different among flock age groups. The incidence of shell ultrastructural features associated with good quality shells was lower for older flocks and incidence of ultrastructural features associated with poorer quality shells was higher for older flocks. Translucency score had a low correlation with the ultrastructural features of the mammillary layer.

Introduction

Eggs produced in Australia are considered medium to low risk for food borne illness. However, the egg industry in Australia is periodically implicated in cases of food poisoning (OzFoodNet Working Group, 2009). It is difficult for bacteria to move across an intact good quality egg shell. However, small defects in the egg shell may provide means for bacteria on the egg shell to penetrate the shell and move into the egg contents (Messens *et al.,* 2005b; De Reu *et al*., 2006). The importance of the cuticle as a barrier against water loss and bacterial ingress into the egg has been discussed by a number of authors (Sparks and Board, 1984) and it has also been suggested that egg shell translucency may facilitate bacterial penetration (Chousalkar *et al*., 2010). In the present study, unwashed eggs collected directly from the cage front were scored for translucency and tested for egg quality measurements. Egg shells were stained for estimation of cuticle deposition and the ultrastructural characteristics of the mammillary layer of the eggshell were scored and related to the translucency scores.

Materials and methods

Eggs were collected from commercial flocks in different stages of lay: early (<25-40 wks – 10 flocks), mid (41-55 wks – 10 flocks), late (56-65 wks – 6 flocks) and very late (>65 wks – 8 flocks).

Ninety eggs were used for measurements of egg quality which were conducted in the Egg Quality Laboratory at the University of New England, Australia. Thirty eggs were analysed for traditional measures of egg quality as described in Chapter 2.

Thirty eggs were stained with MST cuticle blue stain and the cuticle colour measured as described in Chapter 2. The difference between the reading before and after staining was measured for 24 out of the total of 34 flocks (6 early, 7 mid, 4 late and 7 very late lay

flocks). A single score was calculated after the method of Leleu *et al.* (2011) as: $\Delta E^*_{ab} = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$.

The remaining thirty eggs were scored for the extent of translucency, photographed individually on a candling box, and processed for viewing of the ultrastructure of the mammillary layer as described in Chapter 2.

To verify the suitability of MST cuticle blue stain as an indicator of the presence and extent of cuticle, 90 eggs (30 from each of 3 flocks aged 33, 50 and 67 weeks) were processed as described in Chapter 2.

Results

Egg quality

All egg shell quality measurements varied significantly among age categories (Table 1). Translucency score was significantly higher in late lay than for all other age groups. Shell reflectivity increased (shells became lighter in colour) from early to late lay, although late and very late lay were not significantly different from each other. Egg weight increased from early to mid lay, remained relatively constant into late lay and then increased again from late to very late lay. Shell breaking strength decreased with flock age although late and very late lay were not significantly different from each other. Shell deformation to breaking point decreased from early to mid lay but then remained relatively constant. Shell weight increased from early to mid lay before decreasing in late lay and then increasing in very late lay to levels not different from mid lay. Percentage shell was higher at early and mid lay than for late and very late lay. Shell thickness increased from early to mid lay, decreased in late lay to values significantly different lower than all other age groups and then increased in very late lay to values not significantly different from early and mid lay.

Egg internal quality, as measured by albumen height and Haugh Unit, decreased consistently with increasing flock age (Table 1), with each age group being statistically significantly different from the others. However, there were no significant differences among age categories for yolk colour score.

Shell Cuticle Cover

The spectrophotometric measurements of shells with stained cuticle indicated that the value for "L*" increased from early to mid lay, remained constant to late lay before increasing again in very late lay (Table 2). The value for "a*" was not significantly different among age categories and the coefficient of variation was very high for all age categories. The value for "b*" was similar for early, mid and late lay and then decreased for the very late lay group. The difference in "L*" values before and after staining was significantly higher for early and very late lay than for mid and late lay. The difference in "a*" before and after staining was highest in mid and late lay and lowest in very late lay, with early lay intermediate. The difference in "b*" before and after staining was highest in mid and late lay and lowest in early lay, with very late lay intermediate. There was no significant difference among age categories for the single score value which was calculated after the method of Leleu *et al.* (2011).

Egg shell ultrastructure

For the eggs studied for shell mammillary layer ultrastructure, there were no statistically significant differences among age categories for translucency score (measured 7 days after the egg was laid by the hen), as shown in Table 3. However, there were statistically significant effects of age category on some of the ultrastructural scores. Cap size variability was lower in early lay than for all other age categories. The incidence of confluence of mammillary caps was higher in early lay than for all other ages. Cap quality score was lower (i.e. cap quality was higher) for early and mid lay than for late lay although the score for very late lay was similar to that for early and mid lay. The incidence of early fusion was higher for early and mid lay than for late lay, with very late lay intermediate between the two. Late

fusion had a lower incidence in early lay than for all other age categories. The incidence of Type B bodies was lower in early and mid lay than in late and very late lay. The incidence of aragonite was lowest in early and mid lay, highest in late lay, with very late lay intermediate. The incidence of cubic cones was lower in mid lay than for all other ages. The incidence of cuffing and changed membrane was higher in early lay than for all other age categories. The incidence of alignment of mammillary cones, Type A bodies, cubics, depression and erosion were not significantly different among age categories. Holes were not recorded for any age category.

There were significant positive correlations (higher incidence for higher translucency score) between translucency score and the incidence of mammillary layer variations for alignment, Type A bodies, Type B bodies, aragonite and cubic cones. There were significant negative correlations for cap quality (higher translucency, lower cap quality) and changed membrane (higher translucency score, lower incidence). However, there was no significant correlation with translucency score for the incidence of confluence, early fusion, late fusion, cubics, cuffing, depression, erosion and cap size variability.

Verification of cuticle stain

There was a high correlation between the presence of cuticle blue stain on the egg shells and the amount of cuticle present, as viewed under the scanning electron microscope. Eggs with good quality intact cuticle stained well; eggs with patchy cuticle acquired patchy stain whereas, in the absence of the cuticle, the eggs did not stain at all (Figure 1). These findings confirmed the suitability of MST cuticle blue dye as an indicator of the amount of cuticle present on the surface of the egg shell.

Table 1: Traditional measures of egg shell quality

Measurement	Early Lay	Mid Lay	Late Lay	Very Late Lay	P Value
Shell Quality					
Translucency score	^{b}2.52 ± 0.05	^{b}2.59 ± 0.06	^{a}3.07 ± 0.08	^{b}2.53 ± 0.08	<0.0001
Shell reflectivity (%)	^{c}28.0 ± 0.2	^{b}30.5 ± 0.3	^{a}31.6 ± 0.4	^{a}32.1 ± 0.3	<0.0001
Egg Wt (g)	^{c}57.6 ± 0.3	^{b}62.3 ± 0.3	^{b}62.6 ± 0.4	^{a}65.0 ± 0.4	<0.0001
Breaking strength (N)	^{a}44.1 ± 0.5	^{b}42.1 ± 0.6	^{c}37.2 ± 0.7	^{c}36.2 ± 0.6	<0.0001
Deformation (μm)	^{a}330.0 ± 3.4	^{b}293.3 ± 4.0	^{b}281.2 ± 5.6	^{b}285.2 ± 6.5	<0.0001
Shell weight (g)	^{c}5.44 ± 0.03	^{a}5.94 ± 0.03	^{b}5.74 ± 0.06	^{a}5.88 ± 0.04	<0.0001
Percentage Shell (%)	^{a}9.46 ± 0.05	^{a}9.54 ± 0.05	^{b}9.15 ± 0.08	^{b}9.08 ± 0.06	<0.0001
Shell thickness (μ)m	^{b}384.5 ± 1.5	^{a}391.8 ± 2.0	^{c}378.1 ± 3.0	^{ab}382.5 ± 3.4	0.0012
Internal Quality					
Albumen height (mm)	^{a}8.94 ± 0.07	^{b}8.22 ± 0.07	^{c}7.82 ± 0.10	^{d}7.14 ± 0.11	<0.0001
Haugh Units	^{a}94.1 ± 0.3	^{b}89.6 ± 0.4	^{c}86.9 ± 0.6	^{d}81.8 ± 0.7	<0.0001
Yolk colour score	10.49 ± 0.06	10.60 ± 0.05	10.43 ± 0.13	10.46 ± 0.07	NS

Values are Mean ± SEM. Values across a row with different superscripts are significantly different. NS is not significant (P>0.05).

Table 2: Spectrophotometric measurements of stained cuticle

Measurement	Early Lay	Mid Lay	Late Lay	Very Late Lay	P Value
L*	^{c}54.42 ± 0.29	^{b}55.85 ± 0.29	^{b}55.59 ± 0.39	^{a}57.24 ± 0.46	<0.0001
a*	1.861 ± 0.295	0.876 ± 0.371	1.007 ± 0.446	1.400 ± 0.375	NS
b*	^{a}32.46 ± 0.15	^{a}32.42 ± 0.16	^{a}32.88 ± 0.24	^{b}31.66 ± 0.21	0.0002
ΔL*	^{a}5.247 ± 0.509	^{b}3.323 ± 0.305	^{b}3.428 ± 0.313	^{a}4.887 ± 0.597	0.0051
Δa*	^{ab}14.02 ± 0.33	^{a}15.04 ± 0.39	^{a}14.94 ± 0.47	^{b}13.31 ± 0.44	0.0060
Δb*	^{c}2.806 ± 0.313	^{ab}4.027 ± 0.240	^{a}4.541 ± 0.313	^{bc}3.470 ± 0.402	0.0022
Single Score (Leleu *et al*. 2011)	16.10 ± 0.58	16.53 ± 0.44	16.55 ± 0.51	15.73 ± 0.72	NS

Values are Mean ± SEM. Values across a row with different superscripts are significantly different. NS is not significant (P>0.05).

L*, a*, b* measured on flocks 1-34

ΔL*, Δa*, Δb* and single score measured on flocks 11-34

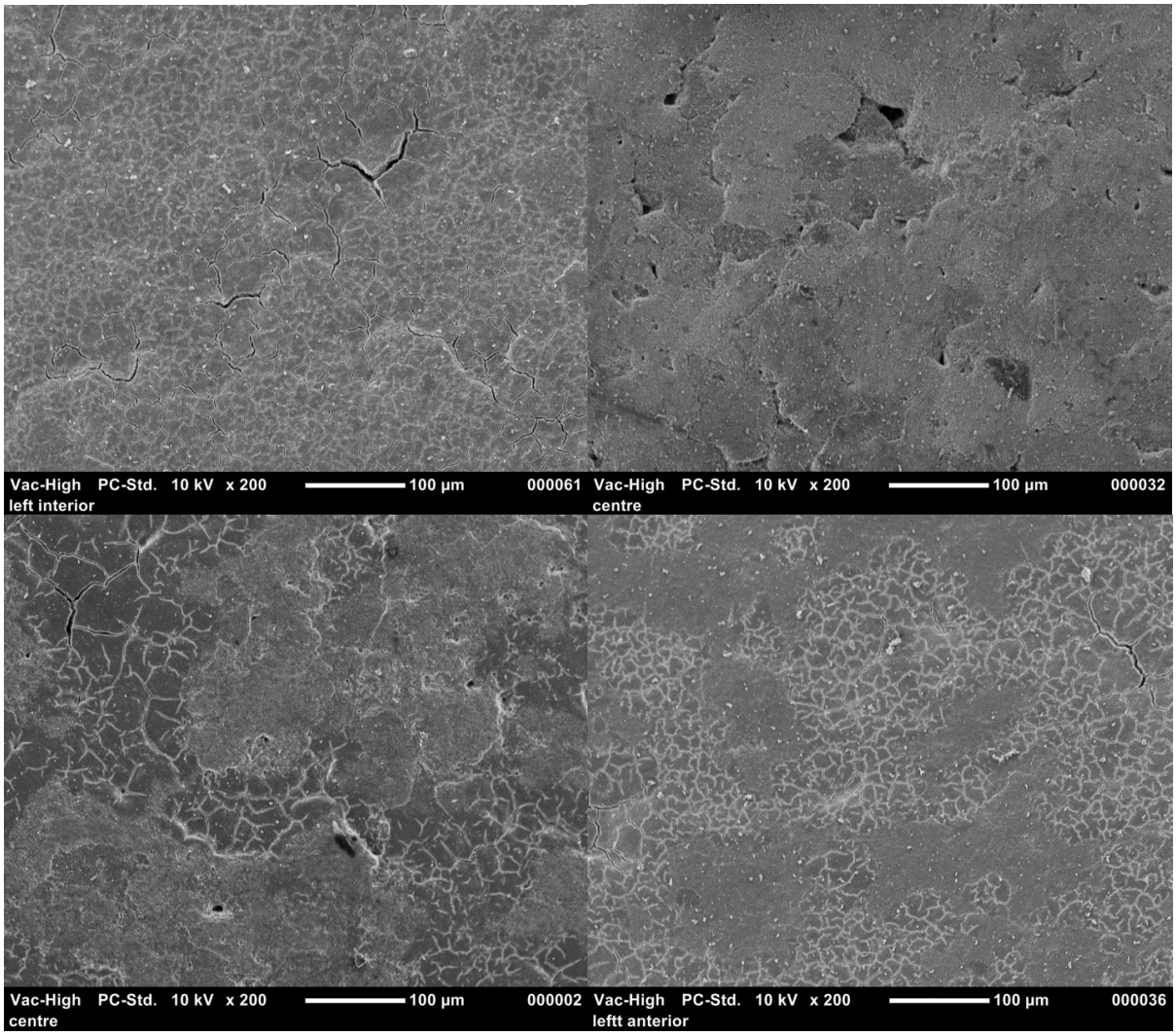
Table 3: Mammillary ultrastructure scores

Measurement	Early Lay	Mid Lay	Late Lay	Very Late Lay	P Value
Translucency score	2.62 ± 0.06	2.54 ± 0.07	2.71 ± 0.09	2.52 ± 0.09	NS
Cap size variability	[b]2.25 ± 0.03	[a]2.44 ± 0.04	[a]2.43 ± 0.04	[a]2.46 ± 0.04	<0.0001
Confluence	[a]2.88 ± 0.05	[b]2.63 ± 0.06	[b]2.65 ± 0.06	[b]2.65 ± 0.06	0.0029
Cap quality	[b]2.93 ± 0.04	[b]2.89 ± 0.05	[a]3.16 ± 0.06	[b]3.01 ± 0.05	0.0014
Early fusion	[a]2.27 ± 0.04	[a]2.17 ± 0.06	[b]2.03 ± 0.05	[ab]2.17 ± 0.04	0.0041
Late fusion	[b]3.61 ± 0.03	[a]3.71 ± 0.04	[a]3.80 ± 0.04	[a]3.76 ± 0.03	0.0009
Alignment	2.47 ± 0.04	2.46 ± 0.06	2.41 ± 0.05	2.58 ± 0.05	NS (0.0552)
Type A bodies	1.37 ± 0.04	1.39 ± 0.04	1.43 ± 0.05	1.44 ± 0.05	NS
Type B bodies	[b]1.96 ± 0.06	[b]2.11 ± 0.07	[a]2.51 ± 0.08	[a]2.43 ± 0.07	<0.0001
Aragonite	[c]1.22 ± 0.04	[c]1.27 ± 0.05	[a]1.68 ± 0.09	[b]1.46 ± 0.07	<0.0001
Cubics	1.21 ± 0.03	1.20 ± 0.04	1.16 ± 0.03	1.26 ± 0.04	NS
Cubic cones	[a]1.70 ± 0.04	[b]1.52 ± 0.04	[a]1.68 ± 0.05	[a]1.71 ± 0.05	0.0046
Cuffing	[a]1.74 ± 0.04	[b]1.52 ± 0.04	[b]1.51 ± 0.06	[b]1.51 ± 0.05	<0.0001
Changed membrane	[a]2.17 ± 0.08	[b]1.85 ± 0.08	[b]1.88 ± 0.10	[b]1.84 ± 0.09	0.0069
Depression	1.04 ± 0.04	1.34 ± 0.13	1.04 ± 0.02	1.03 ± 0.01	NS
Erosion	1.02 ± 0.01	1.03 ± 0.01	1.02 ± 0.01	1.03 ± 0.02	NS

Values are Mean ± SEM. Values across a row with different superscripts are significantly different. NS is not significant (P>0.05).

Figure 1

Shell surface showing clockwise from top left: good cuticle cover, little or no cuticle, patchy cuticle cover, patchy cuticle cover. Scale bars represent 100 µm



Discussion

The translucency score was higher for the late lay age group than for all other groups. The significance of this finding is not clear and there are no published data against which to compare these results. Results on egg quality obtained in the current study are consistent with previous reports of the changes that occur in egg quality as flocks get older both in Australia (Roberts and Ball, 2003; Roberts, 2004; Roberts and Ball, 2006) and in other countries (Washburn, 1982; Travel *et al.,* 2011). The increased shell reflectivity (indicative of lighter shell colour) has been reported previously by a number of authors (Roberts and Ball, 2006; Tumova and Ledvinka, 2009; Zita *et al.,* 2009). Increased egg weight with increasing hen age may be viewed as positive or negative depending on the market involved. Increased egg weight with increasing hen age has been reported previously by many authors (Guesdon and Faure, 2004; Roberts and Ball, 2006; Tumova and Ledvinka, 2009). The decrease in shell breaking strength as hens age, found in this study, has also been reported previously (Rodriguez-Navarro *et al.,* 2002; Roberts and Ball, 2006). Shell deformation to breaking point is an indicator of the degree of elasticity of the eggshell. A higher shell deformation was found in the younger flocks in the current study as has been described by Roberts and Ball (2006) although the results of Zita *et al.,* (2009) for shell deformation varied with the breed of laying hen. Although egg weight increased with increasing hen age, as has been described previously (Nys, 1986; Silversides and Budgell, 2004; Zita *et al.,* 2009), shell weight did not always increase in proportion. This resulted in percentage shell decreasing with increased hen age as has been described previously (Roberts and Ball, 2006; Silversides and Scott, 2001). Shell thickness fluctuated among the age groups, being highest in mid lay. Most studies report a decrease in shell thickness with increasing hen age (Roland *et al.,* 1975; Roland, 1979). The higher shell weight and shell thickness for the very late lay group, as compared with the late lay group, correlate with a larger egg size (65 g as compared with 62.6 g for the late lay group). However, the percentage shell was not significantly different between the late lay and very late lay groups. In addition, shell breaking strength was similar for late lay and very late lay groups, suggesting that the increases in shell weight and shell thickness in very late lay were commensurate with the increase in egg size. The steady reduction in albumen quality with increased flock age has also been reported by a number of researchers (Williams, 1992; Silversides, 1994). The heritability of many egg quality parameters is high (Dunn, 2011; Dunn *et al*., 2011) which means that genetic selection by major breeding companies can assist with improvements in quality, in addition to management improvements.

The use of MST cuticle blue stain for determining the extent of cuticle cover in avian egg shells has been verified by correlating the extent of staining with the appearance of the shell cuticle under the scanning electron microscope. Therefore, MST cuticle blue stain is a reliable indicator of the presence of cuticle on the egg shell surface. However, quantification of the degree of staining using the Konica Spectrophotometer proved more complex than had been anticipated. The use of shell colour, as measured by the spectrophotometer, is confounded by the underlying colour of the egg shell. The L* values show the same pattern as shell reflectivity which is that the shells became paler in colour with increased flock age. The results from the b* spectrum suggest that the shells from the eggs of the very late lay flocks were less yellow than for the other three flocks which may correlate with the paler brown coloured shells of this group. Even when the extent of shell colour is measured by a range of techniques, including the calculation of a single value to integrate the L*a*b* values, there appears to be no clear correlation between average cuticle cover and flock age. However, the higher mean values for Δa* in mid and late lay suggest the cuticle is thickest during these stages of the laying life of a hen. Previous studies report that the cuticle becomes thinner with increasing flock age (Sparks and Board, 1984). The significance of the extent of cuticle cover is still uncertain. The most important role of the cuticle in relation to food safety may be the presence of sufficient cuticle to block the outside of most of the pores.

The translucency scores for the eggs used for ultrastructural examination of the mammillary layer were not significantly different among flock age groups. Translucency score was measured at an average of 3 days following lay for the egg quality eggs and an average of 7 days following lay for the eggs used for ultrastructure studies which may explain the difference. Shell translucency increases with time following oviposition. In general, the incidence of mammillary layer variations which appear to be associated with better shell quality (confluence, early fusion, cuffing) decreased with increasing flock age and mammillary cap quality decreased. Conversely, the incidence of mammillary layer variations which appear to be associated with reduced shell quality (cap size variability, late fusion, Type A bodies, Type B bodies, aragonite) increased with increasing flock age. Our data accord with previous studies (Parsons, 1982; Nascimento and Solomon, 1991; Solomon, 1991, 1992a,b; Nascimento *et al.,* 1992; Roberts and Brackpool, 1994; Solomon, 2009). It is not clear why the incidence of "changed membrane" was higher for the early lay flocks. Changed membrane is membrane remaining following the plasma ashing procedure. It is possible that, in the younger flocks, the attachment between the outer shell membrane and the mammillary cones is greater, making it more difficult to remove the membrane. However, this suggestion needs to be confirmed.

The correlations between egg shell translucency scores and the scores obtained from examination of the mammillary layer of the egg shells suggest that the ultrastructure scoring system only partially explains the phenomenon of egg shell translucency. It is still not completely clear what structural features of the eggs cause translucency. Shell translucency appears to be associated with the ultrastructure of the egg shell and is associated with the presence of moisture in the spaces of the mammillary layer and perhaps also the pores. Studies are underway to examine egg shells using a Phoenix v|tome|x dual X-ray system in an attempt to further explain the phenomenon of translucency.

Eggs from the same flocks utilised for the egg quality studies were evaluated for total bacterial counts and Enterobacteriacae on the outside of the shells, in shell crush (to incorporate any bacteria present in the shell pores) and egg internal contents, as described in Musgrove *et al.* (2005a) and results are presented in other chapters of this report. There were no effects of flock age on the bacterial counts on the shell surface and in shell crush and no bacteria were isolated from the egg contents. However, some *Salmonella* were isolated from the shell. In Australia and most European countries, there is some debate about the benefits of washing eggs. Previous research suggests that washing removes faecal material and reduces microbial load on the egg shell surface and thus reduces the likelihood of horizontal transmission occurring as well as reducing the potential for cross contamination during food handling/preparation (Musgrove *et al.,* 2004a). However, research has also shown that wet washing can damage the cuticle layer (which prevents the entry of bacteria across the egg shell) thereby leaving pores exposed and potentiating bacterial penetration (Sparks and Burgess, 1993). Egg washing is widely used in many countries including Australia (Hutchison *et al*., 2004). It has been demonstrated that the extent of cuticle deposition can influence the egg shell penetration of *Salmonella* Enteritidis at 20˚C (De Reu *et al*., 2006). However, it is not clear how the measurements of cuticle cover undertaken in the present study correlate with the ease with which bacteria can pass through the egg shell. It is possible that the presence of cuticle in the egg shell pores is the most significant barrier to bacterial entry and that a thick cuticle on the outside of other parts of the egg is of lesser significance.

The fact that cuticle cover, as measured by MST cuticle blue staining, was not significantly different among age categories, coupled with the relatively constant translucency scores from mid to very late lay, and the absence of bacteria inside the eggs tested, suggests that translucency is not a major risk factor for food safety of table eggs. However, this remains to be tested in future experiments using whole eggs and agar filled eggs exposed to specific serovars of *Salmonella*. In addition, experiments are on-going to better characterise the basis of egg shell translucency. Similarly, the extent to which variation in cuticle cover represents a risk factor for food safety is still not fully understood.

This topic is also under further investigation using both washed and unwashed eggs with varying degrees of cuticle cover in whole egg and agar filled egg experiments.

Chapter 4: Enumeration and identification of *Enterobacteriaceae* from eggs (Mr. V. Gole, PhD thesis)

Abstract

In the present study, eggs from commercial caged layer flocks at different stages of lay in Australia were collected. Enterobacteriaceae populations from egg shell surface and egg shell pore were enumerated. *Enterobacteriaceae* populations isolated from egg shell surface and egg shell pores were characterized using API® strips. The egg shell surface, egg shell pore and egg internal content samples were also processed for the isolation of *Salmonella* and these isolates were tested for the presence or absence of several virulence genes (*prgH, sopB, spiC, orfL, invA, sifA, sitC, misL*). Results indicated that there was no significant difference in total *Enterobacteriaceae* count on the eggs of the flock from early, mid or late lay flocks. *Enterobacteriaceae* isolates were of 11 different genera which included: *Escherichia, Salmonella, Enterobacter, Serratia, Yersinia, Klebsiella, Pantoea, Kluyvera, Leclercia, Cedecea and Citrobacter*. Out of all identified *Enterobacteriaceae* isolates, the *Escherichia* genus were reported most frequently (60.78%). Results also indicated that 4.51% of eggshells were positive for *Salmonella*. Out of 14 *Salmonella* strains isolated in this study, serotyping confirmed that 12 isolates were *Salmonella* Infantis and 2 strains were *Salmonella* ser 4, 12d. Polymerase chain reaction (PCR) results indicated that all *Salmonella* Infantis isolates harboured *prgH, sopB, spiC, orfL, invA, sifA, sitC, misL* genes which suggest that *Salmonella* Infantis strains isolated from egg shell surface may have the capacity to invade and survive in macrophages.

Introduction

The egg contents are an ideal growth medium for microorganisms which are hazardous to humans. It has been observed that the microflora of the eggshell is dominated by Gram-positive bacteria, whereas Gram-negative bacteria are best equipped to overcome the antimicrobial defenses of the egg content (De Reu *et al.,* 2006). The egg industry in Australia is periodically implicated in cases of *Salmonella* food poisoning (Chousalkar *et al*., 2010). Cage laying production systems are the major source of whole shell eggs in Australia. It is possible that egg shell translucency increases the incidence of bacterial penetration (Chousalkar *et al.,* 2010). Cox *et al*., (2002) reported that *Salmonella* Infantis was the predominant *Salmonella* serovar in the Australian egg industry. For the shelf life of an egg and from a food safety perspective, it is important to lower the level of bacterial contamination on eggs. Studies on microbial contamination of egg shells have been performed earlier (Musgrove *et al.,* 2004b; Musgrove *et al.,* 2005a). Abnormalities in egg shells (thin shells, increased shell pore numbers) can potentiate the entry of food borne pathogens into the eggs (De Reu *et al.,* 2008). With an increase in number of bacteria present on the eggshell surface, the chances of eggshell penetration and contamination of internal contents by bacteria increases (Smith *et al.,* 2000). It has also been reported that bacterial contamination of air cells, shells, and egg contents are more common in eggs from older hens compared to younger hens (Jones *et al.*, 2004). At oviposition, 90% of eggs are germ free (Board, 1966). The eggshell can be contaminated by any surface with which egg comes in contact (Board and Tranter, 1995). Faeces, water, caging material, nesting material, insects, hands, broken eggs, dust on egg belt, blood, soil are the most common sources of eggshell contamination (Board and Tranter, 1995; Ricke *et al.,* 2001; Davies and Breslin, 2003). Overall, food quality and sanitary processing conditions can be judged by Coliforms, *Enterobacteriaceae* and *E. coli* populations (Kornacki and Johnson, 2001; Ricke

et al., 2001). Food borne illness costs Australia an estimated $1.2 billion per year (Hall *et al.,* 2005). The annual report of the OzFoodnet network (2009) reported 9,533 cases of *Salmonella* infection in Australia. It is important to study the prevalence of *Salmonella* on the eggshell surface because contaminated eggshells may play an important role in cross contamination in the kitchen. Also, food stuffs in restaurants can be cross contaminated with *Salmonella* due to improper food handling of food (Slinko *et al.,* 2009).
Complex pathogenesis is a characteristic of *Salmonella* infections. The virulence capacity of *Salmonella* is encoded by multiple genes which are clustered together on *Salmonella* Pathogenicity Islands (SPI) (Hensel, 2004). Using the Type III secretion system, various pathogens deliver effector proteins into the cytosol of host cells (Marcus *et al.,* 2000). SPI 1 and SPI 2 encode distinct type III secretion systems. By delivering effector proteins, SPI 1 helps the *Salmonella* to penetrate the intestinal epithelium and also it induces apoptosis in macrophages (Lostroh and Lee, 2001; Courtney *et al.,* 2006). SPI 2 is important in systemic disease as it contains genes which are essential for survival and replication of *Salmonella* within host macrophages and epithelial cells (Shea *et al*., 1996). SPI 3 encodes the high affinity Mg^{2+} uptake system (Marcus *et al.,* 2000) which is important for survival of *Salmonella* in macrophages, whereas SPI 4 is involved in secretion of toxins (Gassama-Sow *et al*., 2006). Hence, it is essential to investigate the presence or absence of virulence genes located on different SPIs. Currently, in Australia, there is limited information on the presence of virulence genes in *Salmonella* strains isolated from eggs. Keeping this perspective in mind, in the present study, visibly clean eggs collected from commercial egg farms from hens at various stages of lay were tested for the presence of *Salmonella* spp. The *Enterobacteriaceae* populations on the egg shell surface and in the egg shell pores were monitored. *Salmonella* isolates from eggs were tested for the presence of a wide range of virulence genes.

Materials and Methods

Collection of eggs

Visually clean eggs (n=1860, 60 eggs from 31 flocks), collected from the cage fronts of commercial layer farms, were processed for isolation of *Salmonella* spp from the egg shell surface, egg shell crush and internal contents. The farms included in this study had either HyLine or Isa Brown laying hens at different stages of lay. Eggs were candled to ensure they were intact eggs without cracks. All the 31 flocks were divided into three different categories based on the age of flock. Early lay (22-40 weeks), Mid lay (41-55 weeks) and Late lay (> 55 weeks) categories, including 10, 8 and 13 flock respectively.

Egg shell surface wash

Egg shell surface wash was prepared by placing six pooled eggs in 60 mL of sterile PBS in Whirl-Pak bags and processing as described in Chapter 2.

Shell crush methodology

For the isolation of bacteria from egg shell pores, egg shells were processed as described in Chapter 2. Following crushing of the shells, 60 mL of PBS was added to each bag. A 100 ul of PBS was plated on violet red bile glucose agar and also, 1 mL of PBS was transferred to 4 mL of buffered peptone water. Plates and buffered peptone water was incubated at 37°C overnight. After incubation, colonies on the plates were counted.

Egg internal contents

The egg internal contents were collected and processed as described in Chapter 2

Isolation and identification of Salmonella spp.

The isolation of *Salmonella* was carried as described in Chapter 2.

Characterisation of *Enterobacteriaceae*

Enterobacteriacae were isolated and characterised using API© strips (Biomerieux, Australia). as described in Chapter 2.

DNA extraction and Polymerase chain reaction (PCR) for *Salmonella* Infantis typing

Salmonella Infantis isolated from egg shell wash were grown in 5 mL Brain heart infusion broth (BHI, Oxoid, Australia) overnight at 37°C with shaking. The cells were pelleted using a centrifuge at 1,500 g for 10 minutes. DNA was extracted and purified using Wizard® Plus Minipreps DNA purification system (Promega, Australia) as per manufacturers' instructions. The extracted DNA suspended in nuclease free water was stored at -20°C until further use. *Salmonella* Infantis isolates were tested for eight different virulence genes (Table 1). For PCR, each reaction mixture contained 1 X reaction buffer (Fisher Scientific, Australia), 1.8 mM $MgCl_2$, 200 µM dNTPs, 1 uM of each primer, 1 U *Taq* polymerase, and 50 pg DNA template made up to 20 µL with nuclease free water. Samples were amplified using a Bio-Rad Thermal Cycler with an initial denaturation step at 95°C for 5 min followed by 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing temperature (as per primer-Table 1) for 30 sec and extension at 72°C for 1 min 30 sec, with a final extension step at 72°C for 5 minutes, followed by a holding temperature of 10°C.

The details of primers used in the PCR reactions, annealing temperature and size of amplified product are described in Table 1. PCR products were separated by 2% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer. Gel red was used to visualize bands under ultra-violet light. Size of the PCR products was determined by 100 bp DNA ladder (Qiagen, Australia).

Statistical analysis

Statistical analysis was performed following log transformation of the bacterial counts. One way analysis of variance (ANOVA) and Kruskal Wallis test were used with Graph Pad Prism 5 software to compare bacterial counts of early, mid and late lay.

Table 1: Details of the genes and primers used for *Salmonella* typing

Gene	Function	Forward Primer (F) (5' - 3') and Reverse Primer (R) (5' - 3')	Annealing temperature	Product Size	Reference
prgH	Invasion of macrophages	F-GCCCGAGCAGCCTGAGAAGTTAGAAA R-TGAAATGAGCGCCCCTTGAGCCAGTC	55^{o}C	755 bp	Hughes *et al.* (2008)
sopB	Invasion of macrophages	F-GAAGACTACCAGGCGCACTT R-TTGTGGATGTCCACGGTGAG	55^{o}C	804 bp	Designed
InvA	Invasion of macrophages	F-CTGGCGGTGGGTTTTGTTGTCTTCTCTATT R-AGTTTCTCCCCCTCTTCATGCGTTACCC	60^{o}C	1062 bp	Hughes *et al.* (2008)
sitC	Invasion of macrophages/ iron acquisition	F- CAGTATATGCTCAACGCGATGTGGGTCTCC R-CGGGGCGAAAATAAAGGCTGTGATGAAC	64^{o}C	740 bp	Hughes *et al.* (2008)
spiC	Survival in macrophages	F-CCTGGATAATGACTATTGAT R-AGTTTATGGTGATTGCGTAT	56^{o}C	300 bp	Hughes *et al.* (2008)
sifA	Survival in macrophages	F-TTTGCCGAACGCGCCCCCACACG R-GTTGCCTTTTCTTGCGCTTTCCACCCATCT	62^{o}C	448 bp	Hughes *et al.* (2008)
misL	Survival in macrophages	F-GTCGGCGAATGCCGCGAATA R-GCGCTGTTAACGCTAATAGT	58^{o}C	540 bp	Hughes *et al.* (2008)
orf L	Survival in macrophages/ colonisation	F-GGAGTATCGATAAAGATGTT R-GCGCGTAACGTCAGAATCAA	56^{o}C	331 bp	Hughes *et al.* (2008)

Results

Total *Enterobacteriaceae* count on egg shell and in egg shell pore

The results of the enumeration of bacteria from egg shell surface and shell pores are shown in Table 2. There was no significant effect of flock age on total *Enterobacteriaceae* counts on either the shell surface or in the shell pores.

Results indicated that the average *Enterobacteriaceae* count on the eggshell was 1.46 log CFU/eggshell and in eggshell pore the average count was 0.34 log CFU/ eggshell. On the egg shell, the total *Enterobacteriaceae* count ranged between 0.43 and 3.75 log CFU/eggshell whereas, in egg shell pores, the count varied between 0 and 2.1 log CFU/eggshell. Fourteen samples were found positive for *Salmonella.* All of these samples were from the eggshell surface. Serotyping confirmed that twelve samples were *Salmonella* Infantis whereas two were *Salmonella* sub 1, ser 4, 12:d isolates. All of the egg shell pore samples were negative for *Salmonella.*

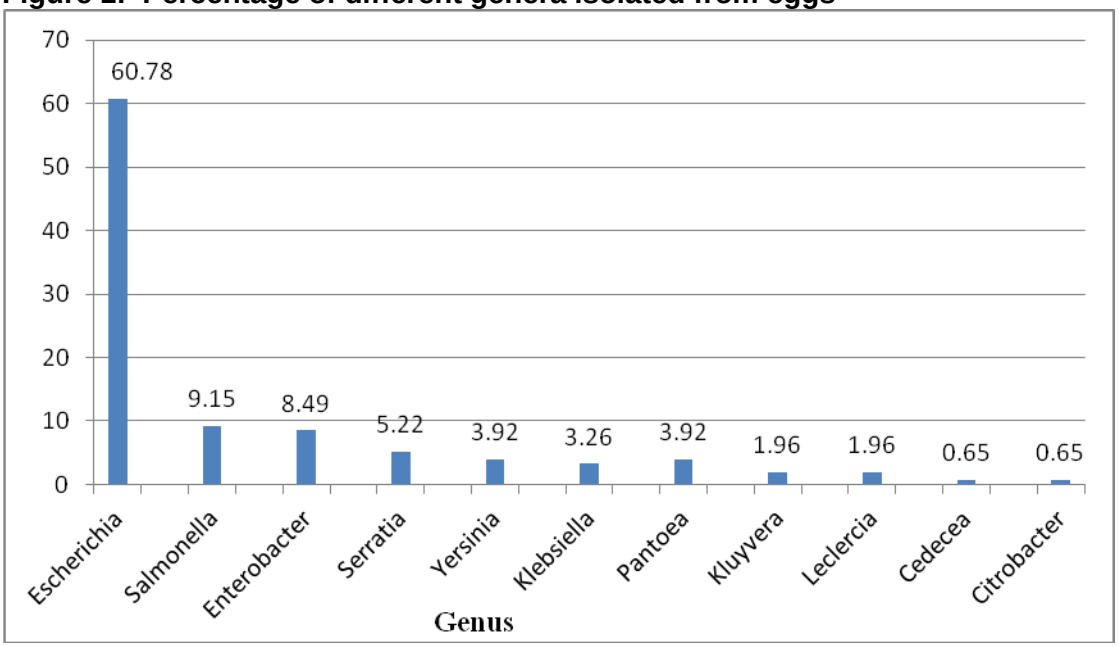
Table 2: Total *Enterobacteriaceae* count (log cfu/eggshell) at different stages of lay

Bacterial count	Early lay	Mid lay	Late lay
Total *Enterobacteriaceae* count (shell surface)	1.78±0.33	1.46±0.22	1.22±0.15
Total *Enterobacteriaceae* count (shell pore)	0.32±0.17	0.44±0.28	0.29±0.10

Identification of *Enterobacteriaceae* isolated from eggs

API Rapid 20E was used to identify the various members of *Enterobacteriaceae* at genus and species level. Identified isolates belonged to 11 different genera which included: *Escherichia, Salmonella, Enterobacter, Serratia, Yersinia, Klebsiella, Pantoea, Kluyvera, Leclercia, Cedecea and Citrobacter* (Table 3). *Salmonella* isolates are indicated in bold font. Out of all isolates identified, isolates from *Escherichia* genus were reported most frequently (60.78%), followed by *Salmonella* (9.15%), *Enterobacter* (8.49%) and *Serratia* (5.22%) (Figure 1). All other genera were identified less frequently (< 5%). Isolates reported at least once were: *Yersinia enterocolitica, Klebsiella pneumonia, Escherichia coli, Escherchia fergusonii, Serratia plymuthica, Serratia* spp*, Serratia odorifera, Salmonella* Infantis, *Enterobacter carcinogenous, Enterobacter spp, Kluyvera spp, Leclercia adecarboxylata, Pantoea spp.* 3, *Pantoea spp.* 2, *Pantoea spp, Cedecea spp*, *Citrobacter freundii.* Also, three *Escherichia coli* isolate were identified from eggshell pores.

Figure 1: Percentage of different genera isolated from eggs



Salmonella typing using virulence genes

PCR results indicated that all of the *Salmonella* Infantis isolates tested positive for *prgH, sopB, spiC, orfL*, *invA, sifA, sitC* and *misL* genes.

Table 3: Characterization of *Enterobacteriaceae* isolated from eggs

Flock Number	Age (weeks)	*Enterobacteriaceae* Reported (Number isolated)
1	22	*Yersinia enterocolitica* (2), *Klebsiella pneumoniae* (1), *E. coli* 1 (1)
2	24	*Serratia plymuthica* (1), *E. coli*1 (1)
3	24	*E. coli* 1 (8), *Klebsiella pneumoniae* (1)
4	26	*Serratia plymuthica* (1)
5	27	*E. coli* 1 (1), *Klebsiella pneumoniae* (1),
6	27	*E. coli* 1 (10)
7	30	*E. coli* 1 (8), *E. coli* 2 (2), ***Salmonella* Infantis (2)**
8	30	*E. coli* 1 (1), *E. coli* 2 (1)
9	31	*Enterobacter* (2), *Kluyvera* spp (1), *Serratia* spp (1), *E. coli* 1 (2), *Leclercia adecarboxylata* (1)
10	34	*E. coli* (3), *Enterobacter carcinogenous* (1), *Serratia odorifera* (1)
11	41	*E. coli 1* (5), *E. coli* 2 (2), *E. fergusonii* (1), *Klebsiella pneumonia* (1), ***Salmonella* Infantis (1)**
12	45	*E. coli 1* (2), *Pantoea* spp. 3 (1), *Pantoea* spp. 2 (1)
13	45	*E. coli* 1 (1)
14	46	*E. coli* 1 (8), *E. fergusonii* (1), ***Salmonella* Infantis (1)**
15	50	-
16	52	*Kluyvera* (1), *Klebsiella pneumoniae* (1), *E. coli* 1 (2), ***Salmonella* Infantis (2)**
17	53	*Serratia plymuthica* (1), *Pantoea* spp (1)
18	55	*E. coli* 1 (1)
19	56	*E. coli* 1 (5), *Pantoea* spp. (1), *E. coli 1* (2)
20	58	*E. coli* 1 (5), *Escherichia* genus (1), *Serratia odorifera* (1), *Enterobacter cloacae* (2), ***Salmonella* Infantis (2)**
21	59	*Yersinia entercolitica* (1), *Serratia plymuthica* (1), *Kluyvera* spp. (1)
22	62	*Cedecea* spp. (1), *E. coli 1* (1), *Citrobacter freundii* (1), *Enterobacter* (2)
23	63	*E. coli* 1 (1)
24	64	*Leclercia adecarboxylata* (1), *E. coli* 2 (1), ***Salmonella* spp.** (1), *Enterobacter cloacae* (1)
25	66	*Enterobacter* (1), *E. fergusonii* (1), *E. coli* 1 (3), *E. coli* 2 (1), ***Salmonella* Infantis (1)**
26	66	*Yersinia entercolitica* (2), *E. coli* 1 (1)
27	66	*E. coli* 1 (1)
28	68	*Enterobacter cloacae* (1), *E. coli* 1 (5), *E. coli* 1 (1)
29	72	*Pantoea* spp. 3 (1), *Pantoea* spp. 1 (1), *Yersinia entercolitica* (1), *Enterobacter cloacae* (1)
30	74	*Leclercia adecarboxylata* (1), ***Salmonella* Infantis (3), *Salmonella* serovar 4, 12d (1)**
31	80	*E. coli* 1 (3), *Enterobacter cloacae* (1), *Enterobacter aerogenes* (1), *Serratia odorifera* (1)

Discussion

In Australia, there has been a continuous increase in egg production for the last decade. The egg industry produced 392 million dozen eggs in 2011 and per capita consumption of the eggs has increased to 213.5 eggs (AECL annual report, 2011-12). In recent years, food borne illness has emerged as a serious problem throughout the world, including Australia, where approximately 28000 cases were reported in 2009 (OzFoodNet Working Group, 2010). *Salmonella* (9533 cases) was one of the most important pathogens responsible for these food borne infections (OzFoodNet Working Group, 2010). Many of these outbreaks were related to eggs or egg products. Hence, the Australian egg industry is under continuous pressure to improve its approach towards public health aspects of eggs. At the time of oviposition, as compared to the ambient temperature, the egg temperature is warmer. Variation in temperature after laying can create a negative pressure in the egg that can suck the contaminants present on the eggshell surface into the shell pores, leading to the contamination of the egg internal contents. A high bacterial load present on the eggshell surface could increase the chance of eggshell penetration and contamination of internal contents (Smith *et al.,* 2000). In the present study, shell rinse and crush methods were used to recover *Enterobacteriaceae* from commercial shell eggs as described earlier by Musgrove *et al.* (2005a,b). A relatively low average *Enterobacteriaceae* count on the eggshell (1.46 log CFU/eggshell) was reported, which is in agreement with De Reu *et al.* (2009) who reported 1.51 log CFU/eggshell count in eggs from furnished cages. Musgrove *et al*. (2005b) reported average *Enterobacteriaceae* count of 1.32 log CFU/ml from eggs which were collected at the accumulator. The difference in the count might be due to the variation in sampling as, in the present study, eggs were directly collected from the farm whereas, in Musgrove's study, eggs were collected from the accumulator at a commercial facility. Similarly, Jones *et al.* (2007) reported a higher *Enterobacteriaceae* count (2.00 log CFU/ml) from egg shell wash. However, the study of Jones *et al.* (2007) was conducted on restricted shell eggs which did not meet the quality standard for retail. The presence of microorganisms on the eggshell surface, eggshell pores and the shell membrane is a determining factor for the recovery of microorganisms by either surface rinsing/swabbing or by shell blending (Moats, 1980, 1981). As bacteria can move from eggshell surface into eggshell pores and further into egg internal contents, it is important to study the bacterial count in eggshell pores. In this study, the average *Enterobacteriaceae* count in eggshell pores was 0.32 log CFU/ eggshell. In the present study, we did not find any significant difference in *Enterobacteriaceae* count (on eggshell and in shell pore) across early, mid and late lay. There is a dearth of literature regarding *Enterobacteriaceae* counts in eggshell pores or effect of flock age on *Enterobacteriaceae* count on eggs, which precludes comparison of our findings with those of other workers. However, there is a number of studies which have investigated the effect of flock age on bacterial contamination of eggshells. De Reu *et al.* (2006) and Protais *et al.* (2003) reported that there was no significant difference in eggshell contamination between beginning and end of the laying period in furnished cages or aviaries. Huneau-Salaun *et al.* (2010) found that eggshell contamination increased significantly with increasing age of flock but Mallet *et al.* (2006) reported that contamination decreased with age. However, both of these authors attributed the variation in their results to seasonal or environmental effects rather than flock age.

Cleaning of poultry sheds is generally limited to the areas which are easily accessible (Huneau-Salaun *et al.,* 2010). The area on which an egg is laid has an impact on eggshell contamination (Harry, 1963) and accumulation of dust on egg belt or cage in the shed may result in increased bacterial contamination of eggshells (Huneau-Salaun *et al.,* 2010). Hence, cleaning of dust from sheds and general hygiene are of vital importance. Our findings regarding the presence of various genera of *Enterobacteriaceae* on eggshells are in agreement with Musgrove *et al.* (2004a) who also reported *Escherichia, Salmonella, Enterobacter, Serratia, Yersinia, Klebsiella, Pantoea, Kluyvera and Citrobacter* on eggshell surface. However, *Cedecea* spp. and *Leclercia adecarboxylata* were not reported in their

study. Out of these, *Leclercia adecarboxylata* is infrequently isolated from eggshells. This microorganism was rarely reported in humans (Hess *et al.,* 2008). There are very few reports of *Cedecea* isolation from eggshells and the clinical significance of *Cedecea* is not fully understood (Abate *et al.,* 2011). In the present study, out of all *Enterobacteriaceae* genera reported, *Escherichia* was reported most frequently. This finding is in agreement with earlier reports by Musgrove *et al.* (2004a) and Stępień-Pyśniak (2010).

In the present study, 4.51% eggshells were found positive for *Salmonella*. Similar findings were reported by Stępień-Pyśniak (2010) who reported 3.2% prevalence of *Salmonella* on egg shells. Cox *et al.* (1973) reported that less than 10% of the eggshells were contaminated in hens individually artificially infected with *Salmonella enterica* serovars Senftenberg, Thompson and Typhimurium, whereas Jones *et al*. (1995) reported that 7.8% of the egg shells were contaminated. In the present study, *Salmonella* spp were not detected in any of the egg internal contents. This finding is in agreement with Daughtry *et al.* (2005) who undertook a microbiological survey of commercial eggs in Australia to determine the prevalence of *Salmonella* contamination. During Daughtry's study, *Salmonella* spp. was not isolated from the internal contents of any of the 20,000 eggs sampled.

All of the *Salmonella* Infantis isolates from the present study possessed *prgH, sopB, spiC, orfL, invA, sifA, sitC, misL* virulence genes. These genes play an important role in invasion of macrophages and are also essential for survival of *Salmonella* within macrophages. Out of these genes, *prgH, sopB, invA and sitC* are located on *Salmonella* pathogenicity island (SPI) 1 whereas *spiC and sifA* are located on SPI-2. *misL* and *orfL* genes are located on SPI-3 and SPI 4 respectively (Skyberg *et al.,* 2006; Gassama-*Sow et al.*, 2006). SPI 1, SPI 2 and SPI 3 play an important role in invasion of macrophages and survival of *Salmonella* within macrophages (Hughes *et al*., 2008) whereas SPI 4 is involved in secretion of toxins (Gassama-Sow *et al.,* 2006) and survival of *Salmonella* in macrophages. *Salmonella* Typhimurium, SPI 1 and SPI 2 are important in causing systemic and gastrointestinal tract infection in young chicks (Jones *et al.,* 2007). *Salmonella* Infantis isolates in the present study possessed genes located on SPI 1 (*prgH, sopB, invA, sitC*) and SPI 2 (*spiC, sifA*) which suggest that these isolates may have the capacity to cause systemic and gastrointestinal infection in day old chicks. However, further animal trials are essential to confirm these findings. PCR results also indicated that all the *Salmonella* Infantis isolates possessed *orfL* and *misL* genes which are involved in survival of *Salmonella* in macrophages. However it is essential to note that possession of a single or a few virulence genes does not endow a strain with pathogenic status unless that strain has acquired the appropriate virulence gene combination to cause disease in a specific host species (Gilmore and Ferretti, 2003). *Salmonella* Infantis strains isolated from eggshells may have capacity to invade and survive in macrophages. However, further studies such as a macrophage invasion assay are essential to confirm these findings.

Salmonella Infantis was found to be a prevalent serovar in the poultry industry in a number of countries. In Finland, in 1975, a large epidemic of *Salmonella* Infantis in broiler chicken caused human Salmonellosis (Raevuori *et al.,* 1978). Poppe *et al.* (1991) reported *Salmonella* Infantis as the second most important serovar isolated from layer flocks in Canada. *Salmonella* Infantis was also reported to be the second most important serovar after *Salmonella* Enteritidis in the German layer industry (Hinz *et al.,* 1996). In Australia, *Salmonella* Infantis has been widely isolated from 1987-1992, more frequently from pigs and with high frequency in chickens (Murray, 1994). *Salmonella* Infantis has been isolated from the ovaries of commercial layers at the time of slaughter which raised public health concerns supported by the hypothesis that transovarian infection is the main cause of transmission of this organism to the eggs (Barnhart *et al.,* 1991).

Even though, in the present study, the prevalence of *Salmonella* on eggshells was low, proper handlings of eggs in the kitchen is essential as improper handling may cause cross contamination of other food materials leading to food poisoning outbreaks (Slinko *et al*. 2009). It is essential to adopt safe food handling practises in the food service sector, so that cross contamination can be avoided which will, potentially, reduce the risks of *Salmonella* food poisoning cases.

Another way to reduce egg related food poisoning cases is egg washing. Egg washing is used to reduce eggshell contamination in many countries such as the United States, Australia and Japan (Hutchison *et al.,* 2004). Egg washing can reduce the microbial load on the eggshell surface (Messens *et al.,* 2011). Musgrove *et al.* (2004a) compared the different *Enterobacteriaceae* present on the eggshell surface of washed and unwashed eggs. They found that most of the isolates from the unwashed control eggs belonged to *E. coli* and *Enterobacter spp.* However, in washed eggs, they were able to recover very few isolates and concluded that the egg washing removed many of the *Enterobacteriaceae* species from the eggshell surface. On the other hand, research has also shown that wet washing can damage the cuticle layer (which prevents the entry of bacteria across the egg shell), thereby leaving pores exposed and potentiating bacterial penetration (Sparks and Burgess, 1993). It has been demonstrated that the extent of cuticle deposition can influence the egg shell penetration of *Salmonella* Enteritidis (De Reu *et al.,* 2006). However, it is not clear whether the extent of cuticle deposition can also influence egg shell penetration by *Salmonella* Infantis at various temperatures.

Chapter 5: Prevalence of antibodies to *Mycoplasma synoviae* in laying hens and possible effects on egg shell quality (Mr. V. Gole, PhD thesis)

Abstract

Mycoplasma synoviae can cause respiratory disease, synovitis, or result in a silent infection in chickens and turkeys. The importance of *M. synoviae* is well established in broilers but only a few studies have been conducted in layers. In the present study, the prevalence of *M. synoviae* in commercial layer flocks was determined using ELISA. For this study, 19 commercial layer flocks have been selected randomly from New South Wales and Queensland region of Australia from producers who were willing to participate in the survey. Thirty eggs per flock were sampled and used for extraction of antibodies from the egg yolk. Similarly, thirty eggs per flock were collected and used to determine various egg shell quality parameters. Subsequently, a possible correlation between the serological status of eggs for *M. synoviae* and egg shell quality was studied. In the flocks under study, seroprevalence of *M. synoviae* was found to be high at 69 % (95 % confidence interval (CI) = 47 to 91). Statistical analysis showed a possible correlation between serological status for *M. synoviae* and egg quality parameters such as translucency, shell breaking strength, shell reflectivity and shell deformation. On the other hand, there was no significant correlation between serological status for *M. synoviae* and other egg quality parameters such egg weight, shell weight, % egg shell or shell thickness.

Introduction

Mycoplasma species are well-known pathogens of domestic poultry, causing significant economic losses (Lierz *et al*., 2007). *Mycoplasma synoviae* is one of the most pathogenic species of this genus and can cause respiratory disease, synovitis or sometimes result in a silent infection in poultry. The importance of *M. synoviae* is well established in broilers but only a few studies have been conducted in layers (Hagan *et al*., 2004). *M*. *synoviae* is known to be transmitted vertically through eggs (Jordan, 1975). The prevalence of egg *M. synoviae* antibodies in egg yolk is reported to be an appropriate way of assessing the flock prevalence of *M. synoviae* infection in laying hens (Hagan *et al*., 2004) and is correlated with serum antibodies (Mohammed *et al*., 1986 a,b). Vertical transmission of a respiratory isolate of *M. synoviae* was confirmed in broiler breeders (Macowan *et al*., 1984). Earlier, the Dutch strain of *M. synoviae* was found to be one of the factors causing egg shell translucency (Feberwee *et al.*, 2009a,b). However, there is little information available regarding the effects of Australian strains of *M. synoviae* on egg shell quality. In the present study, the prevalence of *M. synoviae* in commercial layer flocks was studied using ELISA. Finally, correlations between egg shell quality parameters and the seropravalence of *M. synoviae* in eggs from the same flock were investigated.

Materials and Methods

Eggs were collected randomly from 19 different commercial layer flocks located in New South Wales and Queensland, Australia. Of these 19 flocks, three flocks were vaccinated (Bioproperties, Vaxsafe® MS). *M. synoviae* was expected to spread quickly within a flock so, from each flock, 30 eggs were collected in order to extract antibodies from the egg yolk which were further used in an ELISA. Thirty eggs from each flock were analyzed for egg quality parameters: translucency score (0 = no translucency, 1 = mild translucency, 2 =

moderate translucency, 3 = severe translucency), shell reflectivity (%), egg weight (g), shell breaking strength (Newtons), shell deformation to breaking point (μm), shell weight (g), percentage shell (%) and shell thickness (μm). All the equipment used for egg quality analyses, except egg shell thickness, was supplied by Technical Services and Supply, UK. Egg shell thickness was measured by taking shell pieces from three equidistant points on the equator with shell membrane intact and measuring their thickness using a gauge constructed from a Mitutoyo Dial Comparator gauge Model 2109-10.

Antibody extraction from egg yolk and ELISA

The yolk extraction method was adapted from Mohammed *et al.* (1986b). For a saline extraction, 3 mL of egg yolk was collected from each of the 570 eggs (n=30 from 19 flocks) and mixed with 3 mL saline, vortexed and left for 48 h at 4°C. For the chloroform extraction, 0.5 mL saline extraction and 1 mL chloroform were vortexed to a thick paste. This was allowed to stand for 30 min at room temperature before being centrifuged at 850 g for 20 min. The upper aqueous layer was removed and used in the ELISA. These extracted yolk antibodies were stored at -20°C. Each extracted antibody sample was diluted 1:50 ratio in PBS and was then used in the ELISA. The BioChek Mycoplasma synoviae antibody kits (BioChek, catalog code CK 115) were used in this study, according to the manufacturer's instructions, in order to study the prevalence of *M. synoviae* in the sampled commercial layer flocks. Absorbance of controls and test samples was measured at 405 nm (Multiskan Ascent pathtech). Dilutions of chloroform-extract egg yolk antibody were prepared from the pools of known positive (*M. synoviae* vaccinated) and known negative eggs and tested for the following titres; 1:10, 1:50, 1:100, 1:500 and 1:1000. From the curve produced, the linear part was expanded. Reading the known positive and negative samples individually at the selected dilution produced a cut-off point for the test. The cut off values were determined using the model described by Greiner *et al.* (1995). The results were used to calculate the optimum sensitivity (Se) and specificity (Sp). Depending upon ELISA results, the flocks were divided into three groups, infected, uninfected and vaccinated. The flocks with 10% or more positive reactions were considered positive serologically, based on the criterion of Kleven and Bradbury (2008). Using the ELISA results, seroprevalence of *M. synoviae* was determined at 95% confidence interval and the ANOVA of the S-PLUS statistical software was used to compare egg shell quality parameters of infected, uninfected and vaccinated groups.

Results and Discussion

Egg yolk antibodies were used for studying the prevalence of *M*. *synoviae* amongst the layer flock. Using the chloroform-extracted egg yolks, a dilution factor of 1:50 was chosen as it was on the linear part of the standard curve produced. The Se and Sp for each threshold value were calculated as the proportion of positive results in the positive reference population and negative results in the negative reference population, respectively (Greiner *et al*., 1995). It was observed that the optimized cut-off point for egg yolk was 0.390 with 90% Se and Sp. Of the 19 flocks screened under this study, numbers of serologically positive (infected) and negative (uninfected) flocks were found to be 11 and 5, respectively, and the remaining 3 flocks were vaccinated. Thus, the prevalence *of M. synoviae* serologically positive flocks in commercial layers was high {11/16 (69 %), 95 % CI = 47 to 91}. Table 1 shows the individual flock-wise seroprevalence of *M. yynoviae.* This study has established a method for using egg yolk antibodies to test the flock prevalence of *M. synoviae* in hens. Laying hens are efficient producers of antibodies. Following immunization against a specific pathogen, higher levels of antibodies are usually found in egg yolk than serum of hens (Malik *et al*., 2006). Using egg yolk samples for routine screening is beneficial as it avoids the expense and stress of blood sampling. The present study was conducted in order to determine the seroprevalence of *M. synoviae* infection in the commercial layer flocks by ELISA and a high seroprevalence of *M. synoviae* in commercial layer stock was found. The

prevalence study of Hagan *et al*. (2004), which was also based on the detection of *M. synoviae* antibodies in eggs, reported a prevalence of 78.6% in commercial layer flocks in East England. In another study (Mohammed *et al*., 1986a), prevalence of *M. synoviae* was 87% in commercial layer flocks in Southern California. Our finding is in accordance with other researchers mentioned above. It has been found that multiple age flocks and low biosecurity standards amongst the layer farms are responsible for the high prevalence and persistence of *M. synoviae* infections (Stipkovits & Kempf, 1996; Kleven and Bradbury, 2008). *M. synoviae* infected commercial layer stocks therefore pose a significant epidemiological risk for other categories of poultry. Suzuki *et al.* (2009) reported a high rate of *M. synoviae* seroprevalence (up to 53%) in backyard flocks in Paraguay, measured by ELISA test. Kapetanov *et al.* (2010) reported that, in Serbia, the overall seroprevalence of *M. synoviae* in the flocks decreased from 47.49% in 2000 to 22.17% in 2009.

Table 2 shows correlations between *M. synoviae* serological status and different egg shell quality parameters including translucency, egg shell reflectivity, egg weight, shell breaking strength, shell deformation, egg shell weight, % egg shell and shell thickness. Statistical analysis showed that the vaccinated group (3.1 ± 0.1) had the highest translucency score as compared to the infected (2.4 ± 0.1) and uninfected (2.3 ± 0.1) groups whereas the infected group (31.5 ± 0.2 %) had significantly higher % shell reflectivity, followed by the vaccinated group (29.7 ± 0.5 %) and the uninfected group (28.0 ± 0.3 %), respectively. Shell breaking strength (38.9 ± 0.5) was found significantly lower in the infected group, as compared to the uninfected and vaccinated groups. In the uninfected group, shell deformation (343.5 ± 6.0 μm) was significantly higher as compared to the other two groups. However, there was no significant difference for other egg quality parameters such as egg weight, egg shell weight, % egg shell and shell thickness among these three groups.

For the economic viability of the egg industry, it is critical to produce eggs with good egg shell quality. Egg quality problems are responsible for economic losses within the Australian egg industry so it is important to study the factors which are responsible for decreased egg internal quality and egg shell quality. Many factors have been identified as having direct or indirect effects on egg shell quality including bird strain, bird age, nutrition, stress and disease (Roberts, 2004). The mechanism by which *M. synoviae* affects the normal eggshell calcification process and egg shell quality is not clear. Eggshell matrix proteins in the uterine fluid are essential for the regulation of the growth of calcite during eggshell calcification. *M. synoviae* may affect the composition and concentration of eggshell matrix proteins (Hinke *et al*., 2003). *M. synoviae* may also affect the ciliary motility in the oviduct, which could lead changes in the uterine fluid content affecting the deposition of calcium carbonate crystals (Dominquez-Vera *et al*., 2000). Feberwee *et al*. (2009a,b) reported that a Dutch strain of *M. synoviae* was associated with formation of egg apex abnormalities (EAA) and also reported a synergism between *M. synoviae* and infectious bronchitis virus. However, there is little information available regarding synergism between uterotropic strains of Australian infectious bronchitis virus and *M. synoviae*. In the present study, it was found that shell breaking strength was significantly lower in the infected group as compared to the uninfected and vaccinated groups. Also, the vaccinated group had the highest translucency score as compared to the infected and uninfected groups, whereas the infected group had lighter shells followed by the vaccinated and negative groups, respectively. On the other hand, there was no significant difference between serological status of *M. synoviae* and other egg shell quality parameters such as shell reflectivity, egg weight, egg shell weight, % egg shell and shell thickness. Findings of the study are in contrast to earlier findings by Lott *et al.* (1978) who found that *M. synoviae* infection had no effect on egg shell strength in an experimental study. The present study was a field investigation and not performed under experimental/controlled conditions, hence there could be other confounding factors affecting egg quality. Feberwee *et al*. (2009a) reported that vaccination with a live *M. synoviae* vaccine reduces the occurrence of *M. synoviae*-induced egg apex abnormality significantly. However, the current study reported a high translucency score in the vaccinated group. The high egg shell translucency in *M. synoviae* vaccinated

flocks needs further investigation as it would not be prudent to deduce direct effects of *M. synoviae* on egg shell translucency due to limited sample size.

Table 1: Individual flockwise seroprevalence of *M. synoviae* with 95% exact binomial confidence interval model

Flock age (weeks)	*M. synoviae* serologically positive samples/sample size	Flockwise (%) seroprevalence (95% CI)
22	20/30	66.7 (47.2-82.8)
24	0/30	0.00 (0.0-11.6)
26	1/30	3.3 (0.1-17.2)
27	10/30	33.3 (17.3-52.8)
31	1/30	3.3 (0.1-17.2)
41	0/30	0.0 (0-11.6)
45	11/30	36.7 (19.9-56.1)
53	12/30	40.0 (22.7-59.4)
55	4/30	13.3 (3.8-30.7)
58	29/30	96.7 (82.8-99.9)
59	8/30	26.7 (12.3-45.9)
62	15/30	50.0 (31.3-68.7)
64	11/30	36.7 (19.9-56.1)
66	0/30	0.0 (0.0-11.6)
66	3/30	10.0 (2.1-26.5)
72	12/19	63.2 (38.4-83.7)
30[a]	15/30	50.0 (31.3-68.7)
52[a]	14/30	46.7 (28.3-65.7)
74[a]	20/30	66.7 (47.2-82.7)

[a] Vaccinated flocks

Table 2: Association between *M. synoviae* serological status and different shell quality parameters

Variables	Infected	Uninfected	Vaccinated
Number of flocks	11	5	3
Translucency	[a]2.4 ± 0.1	[a]2.3 ± 0.1	[b]3.1 ± 0.1
Shell reflectivity %	[a]31.5 ± 0.2	[b]28.0 ± 0.3	[c]29.7 ± 0.5
Egg weight (g)	[a]61.5 ± 0.4	[a]60.2 ± 0.9	[a]59.9 ± 0.5
Shell breaking strength (N)	[a]38.9 ± 0.5	[b]44.7 ± 0.7	[b]46.1 ± 1.3
Shell deformation (µm)	[a]295.8 ± 3.4	[b]343.5 ± 6.0	[a]310.8 ± 8.8
Egg shell weight (g)	[a]5.8 ± 0.1	[a]5.69 ± 0.1	[a]5.6 ± 0.1
% eggshell	[a]9.4 ± 0.1	[a]9.5 ± 0.1	[a]9.3 ± 0.1
Shell thickness (µm)	[a]390.9 ± 2.5	[a]385.6 ± 3.1	[a]383.9 ± 3.4

Means ± SE. Different superscripts across a row are statistically significantly different ($P<0.05$)

Chapter 6: Effects of egg shell quality and washing on *Salmonella* Infantis penetration (Samiullah, Masters Thesis)

Abstract

The egg industry in Australia is implicated in public health risks due to cases of egg product related food poisoning. The egg contents are an ideal growth medium for microorganisms which are hazardous to humans. Eggshell characteristics such as shell thickness, amount of cuticle present, thickness of individual layers can affect the ease with which bacteria can penetrate the eggshell. Egg washing could partially or completely remove the cuticle layer. The current study was conducted to study the effects of egg washing on cuticle cover and effects of egg shell quality and cuticle cover on *Salmonella* Infantis penetration across the egg shell. A higher incidence of unfavourable ultrastructural variables of the mammillary layer such as late fusion, Type B bodies, Type A bodies, poor cap quality, alignment, depression, erosion and cubics appeared to enhance bacterial penetration. The influence of egg washing on the ability of *Salmonella* Infantis on egg shell surface to enter the egg internal contents was also investigated. The results from the current study indicate that washing affected cuticle cover. There were no significant differences in *Salmonella* Infantis penetration of washed or unwashed eggs. Egg shell translucency may have effects on *Salmonella* Infantis penetration across the egg shell. The q-PCR assay, when used on enrichment broths, was more sensitive for detection of *Salmonella* Infantis from egg shell wash and internal contents than traditional direct agar culture. The agar egg and whole egg inoculation experiments indicated that *Salmonella* Infantis penetrated the eggshells but could not survive inside the egg, perhaps due to the antimicrobial properties of egg albumen.

Introduction

The egg industry in Australia is implicated in public health risks due to cases of egg product related food poisoning. The egg contents are an ideal growth medium for microorganisms which are hazardous to humans. An egg can be contaminated during its formation in the reproductive tract of a *Salmonella* spp. infected hen (vertical transmission). It can also be contaminated horizontally (after the shell has formed either during oviposition or following oviposition) depending upon the nature of the pathogen and architecture of the eggshell. Not all *Salmonella* serovars are transmitted vertically. *Salmonella* Enteritidis primarily follows the vertical transmission route (Barrow and Lovell, 1991; Humphrey, 1994; Keller *et al*., 1995) whereas *Salmonella* Infantis is transmitted horizontally (Okamura *et al.,* 2001). Cooking can destroy or reduce the levels of bacteria in eggs; however it is common practice to eat lightly cooked egg in most parts of the world including Australia. Cox *et al*. (2002) and Chousalkar and Roberts (Chapter 3) reported that *Salmonella* Infantis was the most common serovarin the Australian egg industry. It is difficult for bacteria to move across an intact good quality egg shell. However, earlier reports indicate that small defects in the egg shell may provide means for the predominant bacterial spp. on the egg shell to penetrate the shell and move into the egg contents (De Reu *et al*., 2006). Eggshell characteristics like shell thickness, amount of cuticle, thickness of individual layers (mammillary and palisade), pore distribution and mammillary ultrastructural variables have been shown to influence eggshell quality and the ease of microbial penetration (Berrang *et al.,* 1998; Nascimento *et al.,* 1992; Solomon, 1991). Translucency is reported as being an egg shell characteristic which results in irregular mammillary knobs probably due to the fusion of several mammillary cores during the early phases of eggshell formation (Bain *et al*., 2006). Egg translucency has also been shown to be positively correlated with microbial penetration (Chousalkar *et al.,* 2010). Disinfecting eggshells is a common and fundamental practice to eliminate or reduce populations of

pathogens on the surface of eggshells (Park *et al.,* 2005). Washing of eggs is practiced by some commercial layer farmers in order to remove most of the debris including some microbes from the outer surface of the eggshell. Some countries like Australia, the United States, Canada and Japan allow eggs to be washed and graded before being packaged for retail, whereas European Union countries are reluctant to allow the washing of grade A eggs (Hutchison *et al.,* 2004; Leleu *et al.,* 2011; Sparks and Burgess, 1993). In Australia, most commercially produced eggs are washed before marketing (Hutchison *et al.,* 2004). Egg washing can significantly lower numbers of *Enterobacteriaceae* on the outside of the eggs when compared to unwashed eggs (Musgrove *et al.* 2004a). Egg washing with chemicals such as alkaline solutions, sodium carbonate, and sodium hydroxides produced visually clean eggs but were hard enough to partially or completely remove the cuticle layers (Wang & Slavik, 1998). The current study was conducted to study the effects of egg washing on cuticle cover, as well as the effects of egg shell quality and cuticle cover on *Salmonella* Infantis penetration across the egg shell. The influence of egg washing on the ability of *Salmonella* Infantis on the egg shell surface to enter the egg internal contents was also investigated.

Materials and Methods

Fresh and visibly clean eggs were collected from a cage system at 42 and 44 wk of flock age. *Salmonella* Infantis strain isolated from egg shell wash, as reported in a previous chapter, was used for penetration experiments in this study.

Egg washing

Egg washing was conducted as described in Chapter 2.

Inoculum preparation

The inoculum was prepared as described in Chapter 2. Agar filled eggs or whole eggs were immersed for 1 minute in one of three serial dilutions of *Salmonella* Infantis strain N1/11 a control and approximately 10^5 and 10^3 colony forming units (CFU) per ml of phosphate buffered saline (PBS). Eggs from the control group were immersed in sterile PBS for 1 min.

Agar method for assessment of the egg shell penetration

The agar egg method is described in Chapter 2. Briefly, 64 fresh eggs were obtained from the cage front of 42 week old commercial Isa Brown laying hens. Eggs were divided into washed and unwashed groups (n=32). All eggs were candled before filling them with agar and translucency was scored as 0 (no translucency), 1 (mild translucency), 2 (moderate translucency) and 3 (severe translucency). Eggs from washed and unwashed groups were further divided into four treatment groups (n=8) based on the translucency score. Agar filled eggs from the treatment groups were immersed for 1 min in 10^5 CFU of *Salmonella* Infantis per ml of PBS. Eggs from the control groups were immersed in sterile PBS for 1 min. After inoculation, agar filled eggs were kept at 20°C for 21 days. The eggs were aseptically opened in a biosafety cabinet after 21 days to inspect for growth of typical colonies. Colonies seen near to the hole that had been made in the egg shell were not recorded as penetration. One sample analysis test (Statview® version 5.0.1 for windows, SAS Institute Inc. Copyright © 1992- 1998) was used to study the effect of egg shell translucency on percentage of penetrated egg shells.

Scanning electron microscopy (SEM) for studying translucency and cuticle scoring of penetrated egg shells.

Penetrated areas of egg shell were marked and correlated with egg translucency, the amount of cuticle present, shell thickness and mammillary layer ultrastructural scoring in treatment and control groups. All the penetrated points were marked and counted in a single ½ shell from which the percentage of penetration points through translucent spots was

calculated. For estimation of cuticle cover, eggshells were stained with MST cuticle blue dye, as described in Chapter 2 and scored for the amount of the cuticle present (based on Leleu, *et al.,* 2011) as shown in Table 1. Pieces of shell were prepared, observed under a scanning electron microscope and scored for ultrastructural features of the mammillary layer, as described in Chapter 2. Shell thickness was measured as described in Chapter 2.

Whole egg penetration assay

A whole egg penetration experiment, as described in Chapter 2, was performed to study the effects of egg washing on *Salmonella* survival on the egg shell surface and penetration into the internal contents of the egg. Ninety eggs were collected from 44 week old laying hens. These eggs were divided into two groups: washed (n=30) and unwashed (n=60). Eggs were further divided into 9 different treatment groups based on washing, dose of infection and incubation temperature. Eggs from the treatment groups were immersed for 1 minute in one of two serial dilutions for *Salmonella* Infantis strain N1/11 in approximately 10^5 and 10^3 CFU per ml of PBS. Eggs from the control groups were immersed in sterile PBS for 1 min. After inoculation, infected eggs were kept at 20°C or 37°C for 21 days.

Isolation of *Salmonella* Infantis from egg shell surface and egg internal contents

In order to recover *Salmonella* Infantis strain N1/11 after 21 post infection (p.i.), from eggshell surface and egg internal contents from the whole egg penetration study, eggs were washed in a sterile whirl pak bag (Nasco, USA) containing 20 mL of PBS. The eggs were gently rubbed for 90 seconds in order to detach any surviving bacteria and a 100 µL aliquot was plated on XLD, the plates were incubated overnight at 37˚C and checked for colony growth of *Salmonella* Infantis. For processing internal contents of the incubated eggs, eggs were dipped in 70 % ethanol for 30 seconds. Eggs were emptied into the whirl pack bags by opening the egg on the edge of a sterile container. The contents were mixed thoroughly and 2 mL was added into 18 mL PBS. 100 µL was plated on XLD plates and incubated overnight at 37˚C. Plates were examined for colony growth of *Salmonella* Infantis strain N1/11.

Real time PCR for quantification of *Salmonella* Infantis survival on egg shell surface and egg internal contents of washed and unwashed eggs

Salmonella enrichment and DNA extraction

Two mL of egg shell surface wash or egg internal contents from the whole egg inoculation experiment (2.6) were inoculated in 8 mL of buffered peptone water (Oxoid, Australia). The inoculated peptone water was incubated at 37˚C for 18 hrs. The DNA was extracted from overnight incubated peptone water samples using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, USA) according to the manufacturer's instructions.

Standard curves and quantification

A proportionality relationship between the CFUs and threshold cycle values (Ct) was determined by qPCR following the method proposed by Lyons *et al.* (2000). Briefly, the *Salmonella* Infantis strain used in the penetration experiment in this study was cultured on XLD agar at 37°C. The individual isolated colonies were then suspended in 2 mL of PBS, matched with 0.5 McFarland standards (bioMerieux Australia) and serial dilutions were performed to achieve 10^4 CFU/mL. The number of CFUs was determined after spreading serial dilutions on XLD agar plates. qPCR was performed on serial dilutions (1:10 to 1:100,000) of genomic DNA and a proportionality relationship was produced by plotting the Ct value against the logarithm CFU number (Lyons *et al.,* 2000). The regression line calculated from all the plots obtained was used as the standard reference line.

Real time PCR

Real time PCR was performed using a Rotor Gene 3000 real time PCR machine (Qiagen, Australia) and a TaqMan® *Salmonella enterica* detection Kit (Applied Biosystems, Australia).

Each reaction contained 9 µL of qPCR supermix and 6 µL of RNA template (12 ng) in a total reaction volume of 15 µL. The cycling parameters were 95^{o}C for 10 min, then 40 cycles at 95^{o}C for 15 s followed by 60^{o}C for 60s.

Results

Agar egg penetration assay

Penetration was observed in all egg shells from the washed group. For the unwashed eggs, 3 eggs each (out of 8 eggs) from translucency scores 2 and 1 showed no *Salmonella* Infantis penetration. All the control group eggs were negative.

Correlation of shell features with *Salmonella* Infantis strain N1/11 penetration

Cuticle cover tended to be more complete in unwashed (Figure 1) compared to washed eggs (Figure 2) and there was significant difference between the cuticle cover of washed and unwashed eggs (Table 2). There was no significant effect of shell thickness on the incidence of bacterial penetration. Shell thickness tended to be lower in the unwashed shells. A positive correlation was found between the presence of translucent areas in the eggshell and penetration points of bacteria into the egg. Most of the penetration points were through the translucent spots and were greater in eggs with a higher incidence of translucency for both washed and unwashed eggs. The exception to this trend was the unwashed eggs with "0" translucency score (Figure 3).

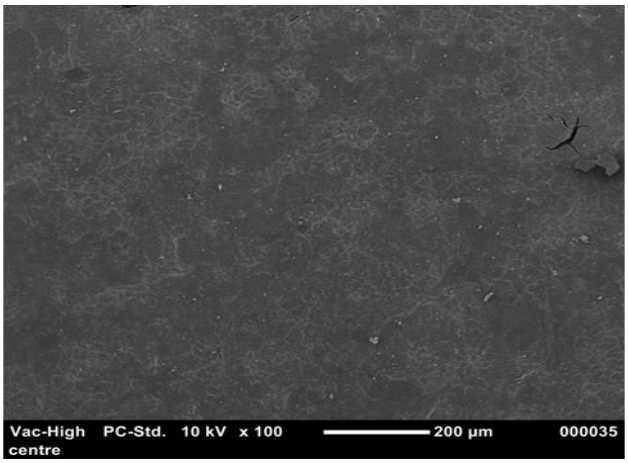


Figure 1: SEM image of eggshell with intact cuticle in unwashed eggs

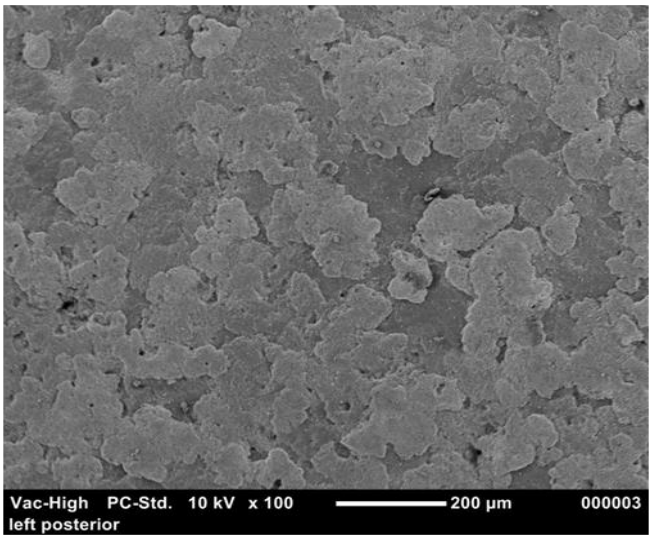


Figure 2: SEM image of eggshell with patchy cuticle in washed eggs

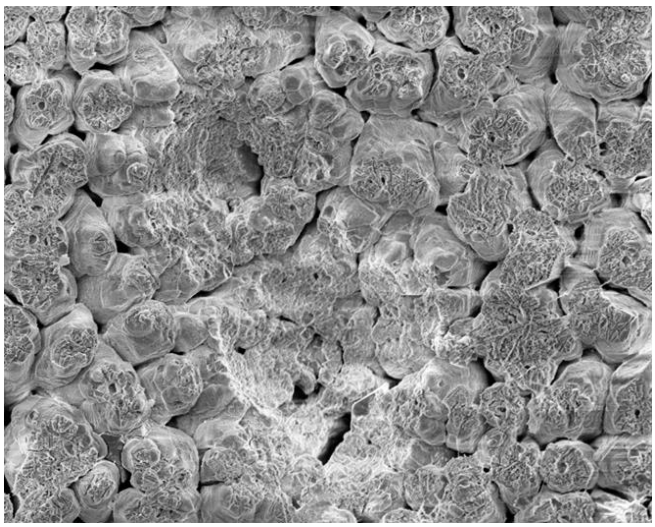


Figure 3: SEM of the mammillary layer of penetrated area showing depression. X200; Scale bar 100µm

There was no significant difference between the washed and unwashed groups of eggs for any of the mammillary layer ultrastructural variables except depression. A slightly higher incidence of depression was recorded in washed groups (Figure 4). SEM of the penetrated points in both groups (washed and unwashed) showed a higher incidence of alignment, late fusion, cubic, open pores, Type B bodies, Type A bodies, depression and erosion of the mammillary caps (Figure 5). The non penetrated areas in both groups (washed and unwashed eggs) showed good mammillary cap with higher incidence of confluence.

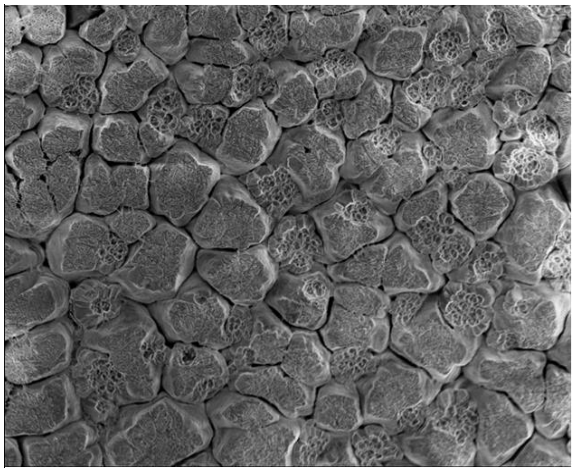


Figure 4: SEM of the mammillary layer of penetrated area showing eroded caps. X100; Scale bar 200µm

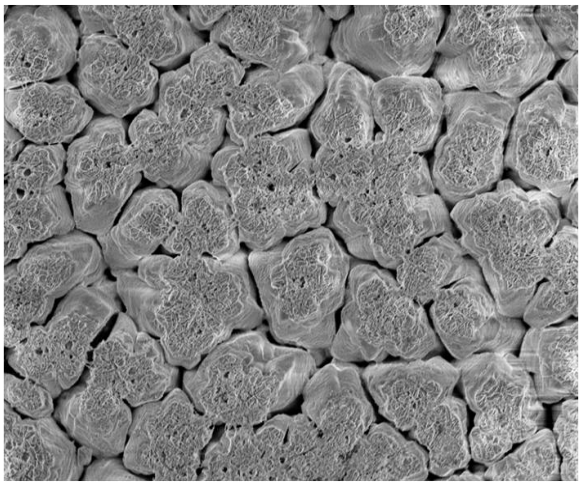


Figure 5: SEM of the mammillary layer of non-penetrated area showing good mammillary cap. X 170; Scale bar 100µm

Salmonella Infantis strain N1/11 survival in whole egg penetration assay

Of all samples incubated at 20˚C and 37˚C, *Salmonella* was isolated from only the shell surface of 1 sample incubated at 37˚C. The total number of colonies on the plate was 95 cfu. *Salmonella* was not isolated from shell surface of washed eggs. All eggs from control group were negative for *Salmonella* Infantis. *Salmonella* was not isolated from egg internal contents from washed or unwashed eggs.

Real time PCR assay

The log-linear standard curve generated from these plots showed high accuracy of the reaction with R^2 value= 0.990, y = -3.20 log (x) + 24.34. with various concentrations of bacterial DNA. The detection limit the assay performed during this experiment was 10 CFU/mL (C_T = 24.34 ± 0.01) hence the samples below this level were considered negative.

There was no significant difference between the *Salmonella* Infantis load on the egg shell surface of washed and unwashed eggs (Table 3). Also there were no significant differences between washed and unwashed eggs for the egg internal contents. However, the load of *Salmonella* Infantis strain N1/11 tended to be higher on the egg shell surface and in egg internal contents of washed eggs. There were no significant effects of temperature or dose

levels on survival or penetration of *Salmonella* Infantis strain N1/11 in washed or unwashed eggs.

In the washed egg group incubated at 20˚C and infected with 10^3 CFU*, Salmonella* Infantis strain N1/11 was detected on the shell surface of 8 out of 10 eggs and internal contents of 6 out of 10 eggs. In the unwashed egg group incubated at 20˚C and infected with 10^3 CFU*, Salmonella* Infantis strain N1/11 was detected on the shell surface of 8 out of 10 eggs and internal contents of 2 out of 10 eggs.

In the washed egg group incubated at 20˚C and infected with 10^5 CFU*, Salmonella* Infantis strain N1/11 was detected on the shell surface of all eggs and internal contents of 6 out of 10 eggs. In unwashed egg group incubated at 20˚C and infected with 10^5 CFU*, Salmonella* Infantis strain N1/11 was detected on the shell surface of 6 eggs and internal contents of 6 out of 10 eggs.

In unwashed egg group incubated at 37˚C and infected with 10^3 CFU*, Salmonella* Infantis strain N1/11 was detected on the shell surface of 8 out of 10 eggs and internal contents of 3 out of 10 eggs. In unwashed egg group incubated at 20˚C and infected with 10^5 CFU*, Salmonella* Infantis strain N1/11 was detected on the shell surface of 8 out of 10 eggs and internal contents of 6 out of 10 eggs.

Egg shell wash and internal contents from the control group tested negative for *Salmonella* Infantis strain N1/11.

Discussion

In Australia and most European countries, there is still debate about the benefits of washing eggs. However, research has also proved that wet washing can damage the cuticle layer (which prevents the entry of bacteria across the egg shell) thereby leaving pores exposed and potentiating bacterial penetration (Sparks and Burgess, 1993). Egg washing is widely used in many countries including Australia (Hutchison *et al*., 2004). European Union countries do not recommend washing grade A table eggs on the basis that the washing of eggs increases the likelihood of spoilage and moisture loss from the egg contents, due to destruction of the outer cuticle layer (Hutchison *et al*., 2004). In the present study, there was significant difference between the cuticle cover of washed and unwashed eggs. Trans-shell penetration tended to be higher in washed eggs compared to unwashed eggs, although the difference was not significant. A high incidence of penetration in the washed eggs might explain the importance of the cuticle in preventing microbial ingress, as the washed eggs had less cuticle compared to unwashed eggs. The hydrophobic proteinaceous outermost layer (cuticle) of the eggshell hinders bacterial penetration (Haigh and Betts, 1991). However, the function of the cuticle as a microbial defense tool has been questioned (Nascimento *et al.,* 1992). In the egg penetration studies of Messens *et al.* (2007), a high correlation was found between absence of cuticle and *Salmonella* penetration, but the amount of cuticle did not affect bacterial penetration in the studies of Messens *et al.* (2005b). Cuticle removal increased bacterial penetration from 20 to 60% in the studies of Alls *et al.* (1964). It is, however, essential to note that cuticle deposition could be variable in the same flock and that eggs were not divided based on the cuticle score for the penetration experiment during this study. Washing may have negative impacts on the cuticle; however this will depend up on techniques, chemicals or temperature used during washing.

In the present study, *Salmonella* Infantis strain N1/11 was isolated from only one shell wash sample by the agar culture method. *Salmonella* was not isolated from any egg internal contents. A high *Salmonella* Infantis strain N1/11 load was recorded on both the egg shell surface and in egg internal contents by Q-PCR method for eggs deliberately exposed to *Salmonella* Infantis strain N1/11. However, all the samples were pre enriched prior to the Q-PCR assay which may have improved *Salmonella* detection. The *Salmonella* Infantis strain N1/11 load was lower in the egg internal contents of washed and unwashed eggs. The agar egg inoculation experiment indicated that the *Salmonella* Infantis strain N1/11 used in this study was able to penetrate across the eggshell, but from the whole egg penetration

study, it appeared that *Salmonella* Infantis strain N1/11 survived poorly in the egg internal contents. This could be attributed to the antimicrobial properties of albumen. Also, a number of factors such as the pH of albumen, albumen viscosity and antimicrobial enzymes and proteins inhibit bacterial proliferation in egg contents (Mayes and Takeballi, 1983). Trans-shell penetration was confirmed using the agar moulding technique in which all the washed eggs were penetrated whereas the penetration rate in the unwashed group was 69.7± 8.1. Earlier studies have shown that *Salmonella* serovars other than *Salmonella* Infantis strain N1/11 penetrate the eggshell (Sauter and Peterson, 1974; DeReu *et al*., 2006). Murase *et al.* (2006) suggested that *Salmonella* serovars including *Salmonella* Infantis which enter the albumen are unlikely to show active motility through the albumen towards the yolk. Some albumen proteins like ovotransferrin have antimicrobial peptides (OTAP-92) that could kill Gram negative bacteria by crossing the outer membrane of bacteria and thus altering the biological function of its cytoplasmic membrane (Ibrahim *et al.,* 2000; Schoeni *et al.,* 1995). In the studies of Hughey and Johnson (1987), *Salmonella* Typhimurium was resistant to the antibacterial properties of albumen lysozyme but how lysozyme acts against *Salmonella* Infantis need to be investigated. In the present whole egg penetration study, washed eggs were only incubated at 20˚C as washed eggs are not used in the hatchery where incubation temperature is 37˚C. In this study, there was no effect of incubation temperature (20 vs 37˚C) on microbial penetration across egg shell.

Egg translucency had a positive correlation with microbial penetration in the present studies except for the unwashed translucency score “0” eggs. Higher translucency incidence may enhance microbial penetration across the eggshell (Chousalkar *et al.,* 2010). The high incidence of penetration of eggs from the unwashed group with translucency score of “0” might have been due to a translucency score which increased significantly following egg collection as the eggs were scored as soon as they were collected. Because translucency was not measured 21 days after inoculation/incubation in this experiment, further investigation is required to prove this hypothesis. Our finding regarding egg shell thickness is in agreement with earlier studies of De Reu *et al.* (2006) and Messens *et al.* (2005b) who found that eggshell characteristics such as shell pores, shell thickness, area of the shell did not affect bacterial penetration of whole eggs and agar eggs. Similarly, in the egg penetration studies of Williams *et al.* (1968), Messens *et al.* (2005b) and Smeltzer *et al.* (1979), bacterial penetration was independent of shell thickness. However, in the studies of Orel (1959) and Sauter and Peterson (1974), a significant effect of shell thickness on bacterial penetration was recorded. In the present studies, SEM of the penetrated areas of outer shell surface showed open pores not plugged by cuticle. Open pores with diameters larger than the bacterial size is the primary route of bacterial entrance to intact eggs (Tyler, 1956; Board and Halls, 1973). Nascimento *et al.* (1992) and Nascimento and Solomon (1991) reported bacterial penetration to be independent of pore numbers. Pores were highly penetrated areas in the studies of Haigh and Betts (1991) but, in the present study, bacterial penetration was observed equally in all regions of the shell. In the present study, a higher incidence of ultrastructural variables of the mammillary layer such as late fusion, Type B bodies, Type A bodies, poor cap quality, alignment, depression, erosion and cubic appeared to enhance bacterial penetration. Similarly, Solomon (1991) positively correlated all the ultrastructural variables that negatively affect mammillary layer quality with bacterial penetration. However, more research is needed to correlate the shell mammillary layer ultrastructural features with microbial penetration as, on the basis of a higher incidence of a single variable, a true baseline cannot be established. The eroded mammillary caps observed at penetration sites (Figure 5) are unusual. They may have been present in the shell and facilitated the entry of bacteria or the erosion may have resulted from the activity of the bacteria after they had penetrated the shell.

In the studies of Schoeni *et al.* (1995), *Salmonella* other than Infantis either remained stable or steadily increased in the egg albumen. Various phage types of *Salmonella* Typhimurium are often isolated from cases of egg product related food poisoning. Hence further research is essential to study the survival and egg shell penetration of different phage types of *Salmonella* Typhimurium isolated from layer industry in Australia. It is likely that the

motile and non-clustering properties of most of *Salmonella enterica* serovars enhance their penetration across the eggshell more frequently compared to other bacteria (De Reu *et al.,* 2006).

It can be concluded from the current experiments that *Salmonella* Infantis penetrated across the eggshells but could not survive well inside the egg, perhaps due to the antimicrobial properties of albumen. Research is needed to investigate the effect of the agar method on the properties of shell membranes as shell membranes play an important role in prevention of microbial entrance into the egg.

Table 1: Scoring sheet for cuticle quantification by SEM

Good intact cuticle (91-100%)	Less patchy cuticle (61-90%)	More patchy cuticle (11-60%)	Negligible or no cuticle (1-10%)
Score= 1	Score= 2	Score= 3	Score= 4

Table 2: Egg shell characteristics and % penetration in agar penetration experiment

	% penetration	Shell thickness (µm)	Cuticle score
Washed eggs	74.7 ± 7.3	407 ± 3.4[a]	3.9 ± 0.06[a]
Unwashed eggs	69.7 ± 8.1	383 ± 9.8[b]	3.3 ± 0.16[b]

Table 3: Q-PCR results of Salmonella Infantis survival on egg shell surface and internal contents of washed and unwashed eggs at 20^{0}C during whole egg penetration experiment

Treatment / Dose	Egg shell surface		Egg internal contents	
	10^3 CFU	10^5 CFU	10^3 CFU	10^5 CFU
Washed eggs (Incubated at 20 ^{0}C)	20.5 ± 0.9	18.5 ± 2.3	23.1 ± 2.2	22.3 ± 3.1
Unwashed eggs (Incubated at 20 ^{0}C)	14.3 ± 6.4	23.9 ± 1.7	22.5 ± 1.5	23.5 ± 1.4
Unwashed eggs (Incubated at 37 ^{0}C)	22.5 ± 1.1	18.2 ± 2.9	21.6 ± 2.9	18.4 ± 2.5

Q-PCR results presented in C_T values. CT values are mean ± SE obtained for 10 samples from each treatment group.

Chapter 7: Effect of egg washing and correlation between eggshell characteristics and egg penetration by various *Salmonella* Typhimurium phage types (Mr. V. Gole PhD Thesis)

Abstract

Salmonella is an important foodborne pathogen, causing an estimated 9,533 cases of infection in Australia per year. Egg or egg product related *Salmonella* poisoning is a major concern for the egg industry. *S.* Typhimurium is one of the most common serovars identified in *Salmonella* food poisoning cases throughout the world. The current study investigated the ability of *S.* Typhimurium phage types (ST PT 9, PT 44, PT 135, PT 170, PT 193) to penetrate washed and unwashed eggs using whole egg and agar egg penetration methods. All *S.* Typhimurium phage types were able to penetrate eggshells and survive in egg albumen (at 20°C) according to whole egg penetration results. Polymerase chain reaction results demonstrated that ST PT 44 (10^3 and 10^5 CFU/mL), and ST PT 193 (10^3 and 10^5 CFU/mL) egg penetration was significantly higher ($p<0.05$) in washed eggs when compared to unwashed eggs. Statistical analysis of the agar penetration experiment indicated that *S.* Typhimurium was able to penetrate washed eggs at a significantly higher rate when compared to unwashed eggs ($p<0.05$). When compared to unwashed eggs, washed eggs also had significantly damaged cuticles. Statistical analysis also indicated that eggshell penetration by *S*. Typhimurium was correlated with various eggshell ultrastructural features such as cap quality, alignment, erosion, confluence, Type B bodies and cuticle cover.

Introduction

Salmonella spp*.* have been one of the most important food poisoning pathogens throughout the last century and remains an unsolved conundrum in microbiology and public health (Hardy, 2004). It is estimated that 1.3 billion incidences of nontyphoidal salmonellosis occur throughout the world annually (Coburn *et al.,* 2007). The annual report of the OzFoodnet network (OzFoodnet, 2009) reported 9,533 cases of *Salmonella* infection in Australia. It is estimated that the annual loss due to all food borne illness in Australia is $1.2 billion (Hall *et al.,* 2005). The Australian egg industry is often blamed for cases of food poisoning due to salmonellosis. Salmonellosis can be acquired by the ingestion of raw or undercooked eggs. Cross contamination of ready-to-eat meals with *Salmonella* also plays a major role in food poisoning cases. Intact eggs can be contaminated by *Salmonella* using two possible routes: vertical transmission and horizontal transmission. Vertical transmission occurs as a result of *Salmonella* infection of the reproductive organs i.e. ovaries or oviduct hence also called the transovarian route. In the transovarian route, the egg yolk membranes or albumen surrounding are directly contaminated (Messens *et al.,* 2005a). The horizontal transmission is also called as trans-shell route in which *Salmonella* penetrates through the eggshell during or following oviposition (Miyamoto *et al.,* 1998). *S*. Enteritidis is a major cause of egg related *Salmonella* food poisoning cases and the most prevalent serovar in the layer industry across the world; however, it is not endemic in Australian layer flocks (Sergeant *et al.,* 2003). In Australia, *S.* Typhimurium is the most common serovar identified in human salmonellosis cases (OzFoodnet, 2009). Horizontal transmission is the most common route by which Salmonellae other than *S.* Enteritidis contaminate egg internal contents (Humphrey, 1994).

Important extrinsic factors such as the bacterial strain, temperature differential, moisture on the eggshell, the number of microorganisms in the inoculum and the storage

conditions may affect eggshell penetration by *Salmonella* spp*.* Intrinsic factors that may affect egg penetration include shell porosity, shell thickness and the extent of cuticle present on the shell (Messens *et al.,* 2005a). There is also some evidence to suggest that eggshell translucency is associated with greater microbial penetration (Chousalkar *et al*., 2010). However, there is a lack of substantial literature on the relationship between translucency, eggshell ultrastructure and the penetration of bacteria.

Faeces, water, caging material, nesting material, insects, hands, broken eggs, blood, soil or dust on the egg belt are the most common sources of egg shell contamination (Board and Tranter, 1995; Ricke *et al*., 2001). Egg washing can reduce the microbial load on the eggshell surface (Messens *et al.,* 2011) and thus may lower the rate of penetration of *Salmonella* across the eggshell and decrease the incidence of food poisoning. Egg washing is used to reduce eggshell contamination in many countries such as the United States, Australia and Japan (Hutchinson *et al.,* 2004). However, some researchers believe that egg washing chemicals can damage the cuticle layer of the eggshell (Wang and Slavik, 1998) which may result in moisture loss and deterioration of the internal quality of the egg. It is also possible that egg washing may favour the transmission of *Salmonella* across the eggshell particularly when the post-washing storage and drying conditions are substandard. As a result, there is currently a debate over the benefits of egg washing. Damage to the cuticle or alteration of the eggshell surface may change with different egg washing protocols (Wang and Slavik, 1998) and may result in variation in the penetration of bacteria across the eggshell. There is a lack of information in this area in the Australian context.

S. Typhimurium has been identified as the most prevalent serovar involved in cases of salmonellosis food poisoning in Australia (Ozfoodnet, 2009). Additionally, *S*. Typhimurium phage types (ST PT 9, ST PT 44, ST PT 135, ST PT 170 and ST PT 193) were frequently isolated/detected from egg products related to food poisoning cases in Australia (Ozfoodnet, 2009). However, there is a lack of information available regarding how well these ST PTs survive on the eggshell surface and penetrate the shell to contaminate the internal contents of the egg. A preliminary study suggested that eggshell ultrastructure may have an impact on eggshell penetration by *Salmonella* (Chapter 6) but there is a lack of direct evidence.

The objectives of this study were to examine the effect of egg washing on the survival of ST PTs on the eggshell surface, to investigate the penetration ability of different ST PTs, and to study the effect of egg washing on penetration of the eggshell by bacteria. The effect of egg washing on cuticle ultrastructure and the relationship between eggshell quality parameters and bacterial penetration were also investigated. Finally, the correlation between translucency and eggshell ultrastructure parameters and ease of bacterial penetration was studied. It was hypothesized that egg shell washing increases cuticle damage and also increases the rate of egg penetration by *S.* Typhimurium.

Materials and methods

Fresh and visibly clean eggs were collected from hens under 45 weeks old from a HyLine layer farm. All of the ST PTs (PT 9, 44, 135, 170 and 193), which had been initially isolated from Australian layer farms, were obtained from the Australian *Salmonella* Reference Centre in Adelaide, Australia.

Egg washing

Eggs were washed as described in Chapter 2.

Inoculum preparation

S. Typhimurium PTs stored at -80˚C in 80% glycerol were processed as described in Chapter 2. Agar filled eggs and whole eggs were immersed for 90 sec in one of three dilutions: PBS (control), ~10^3, and ~10^5 CFU/ mL *S.* Typhimurium inoculums.

Agar method for assessment of the eggshell penetration

The effects of washing, translucency, and eggshell quality on the bacterial penetration of the eggshell were assessed by the 'agar egg' method as described in Chapter 2. Fresh eggs were obtained from the cage front of layers. All eggs were candled, scored for translucency, and divided into two translucency groups based on candling score (n=32); where 1= low translucency, and 2= high translucency. Eggs from each group were then allocated to washed and unwashed groups (n=16 each) and subsequently allocated to inoculated (n=10) and control groups (n=6). Agar-filled eggs from each treatment group (washed and unwashed) were immersed for 90 sec in approximately 10^5 CFU/mL solution of *S.* Typhimurium. Eggs from the control groups (washed and unwashed) were immersed in sterile PBS for 90 sec. After inoculation, agar-filled eggs were incubated at 20°C for 21 days. After incubation, the eggs were aseptically opened and the penetration of *Salmonella* spp*.* was assessed by the blackening of the interior egg shell.

Scanning electron microscopy (SEM) for studying relationship of translucency and the effect of egg washing on eggshell ultrastructure and cuticle

Pieces of egg shell were prepared and observed under a scanning electron microscope as described in Chapter 2. In this study, eggshell ultrastructural features of the mammillary layer were scored as per the following criteria: Cap size: similar = 1, variable = 2; Confluence: low=1, high = 2; Cap quality: good = 1, poor = 2 ; Alignment: low = 1, high = 2, Type A bodies: low = 1, high = 2; Type B bodies: low =1 , high =2; Argonite : Absent =1, present =2; Erosions: absent =1, present =2; Depression: absent =1, present =2.

Assessment of the amount of cuticle present was conducted by electron microscopy, as described in Chapter 2 and shell thickness was measured as described in Chapter 2.

Statistical analysis of agar penetration experiment

All statistical analyses were performed with the statistical software R (R core team 2012). A logistic regression was used to assess the effects of washing, eggshell translucency and their interaction on eggshell penetration of inoculated eggs. Logistic regression was also used to explore the relationship between the overall egg shell structure and *S.* Typhimurium penetration. The overall eggshell structure was defined by the ultrastructural parameters of cap size, confluence, caps, alignment, the number of Type A and B bodies, the level of argonite, depression, erosion and shell thickness.

An ordered logistic regression was used to model the effects of washing, translucency and treatment on the cuticle score of all 64 eggs. In addition, the relationship between translucency score and egg ultrastructure parameters was investigated using logistic regression. Model fit was assessed using an Analysis of Variance (ANOVA) and based on a significance level of $p<0.05$, non-significant interactions were removed step-wise until only significant terms remained in the model.

Whole egg penetration experiment

The effects of egg washing on *S.* Typhimurium survival on the eggshell surface and penetration across the eggshell, as well as the survival of *S.* Typhimurium in the internal contents of the egg, were investigated using a 'whole egg penetration' approach as describef in Chapter 2. Ninety eggs were collected from HyLine Brown hens in early lay and were divided into two groups: washed (n=30) and unwashed (n=60). Washed eggs were divided into one control (PBS) and two treatment groups (10^3 and 10^5 CFU/mL) with 10 eggs each. All the washed eggs were incubated at 20°C after exposure to *S.* Typhimurium or the sham PBS treatment. Unwashed eggs were divided into two groups of 30 eggs. Group 1 was further divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. Eggs from group 1 were incubated at 20°C after exposure to *S.* Typhimurium or the sham PBS treatment. Group 2 was also divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. These unwashed eggs were incubated at 37°C. The reason that only unwashed eggs were incubated at 37°C is that washed eggs are not used

for hatching purposes in Australia. Each egg was dipped into 70% ethanol for 30 sec to sterilize the outer shell and allowed to air dry in a biosafety cabinet for 10-15 min. Eggs were then immersed for 90 sec in 10^3 CFU/mL or 10^5 CFU/mL of *S.* Typhimurium. After inoculation, eggs were incubated at 20˚C or 37˚C for 21 days.

Isolation of *S.* Typhimurium from eggshell surface and egg internal contents from whole egg penetration experiment

After incubation, each egg was placed in a Whirl-Pak bag (Nasco, USA) containing 10 mL of buffered peptone water (BPW; Oxoid, Australia) and each egg was massaged for 1 min. A 10 µL aliquot of the mixture was spread plated onto XLD plates and incubated overnight at 37˚C, and subsequently quantified.

To investigate the penetration and survival of *S.* Typhimurium in the internal contents of the egg, after the eggshell wash, eggs were dipped in 70% ethanol for 30 sec. Eggs were then aseptically opened, emptied into the Whirl-Pak bags and mixed. A 2 mL aliquot of the internal contents was transferred to 8 mL of BPW and 10 µL of this mixture was plated on XLD agar and incubated overnight at 37˚C. Plates were then observed for *Salmonella* identification. Slopes of suspected *Salmonella* isolates were sent to the Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia for confirmation. Statistical analysis was conducted using Fisher exact test (for internal contents samples) and t- test (for eggshell surface samples) of statistical software R (R core team, 2012).

Polymerase Chain Reaction amplification for detection of *S.* Typhimurium on eggshell surface and in internal contents of eggs from whole egg penetration experiment

Detection of *S.* Typhimurium on the eggshell surface and in internal contents was achieved using Polymerase Chain Reaction (PCR) of the *Salmonella*-specific *InvA* gene (Rahn *et al*., 1992). DNA was extracted and purified from BPW using Wizard® PlusMinipreps DNA purification system (Promega, Australia) as per manufacturer's instructions. Extracted DNA was suspended in nuclease free water, and stored at −20˚C until further use. Each PCR reaction mixture contained 10×reaction buffer (Fisher Scientific, Australia), 1 mM $MgCl_2$, 0.25 mM dNTPs, 5 µM forward *InvA* primer (5'-CTGGCGGTGGGTTTTGTTGTCTTCTCTATT-3'), 5 µM reverse *InvA* primer (5'-GTTTCTCCCCCTCTTCATGCGTTACCC-3'), 1.65 U Taq polymerase, and 10 ng DNA template made up to 20 µL with nuclease free water. Samples were amplified using a Kyratech automated thermal cycler (Adelab, Australia) with an initial denaturation step at 95˚C for 5 min, followed by 30 cycles of amplification (denaturation at 95˚C for 30 sec, annealing temperature 60˚C for 30 sec and extension at 72°C for 1 min 30 sec), with a final extension step at 72˚C for 5 min, followed by a holding temperature of 8˚C. PCR products were separated by 1.5% agarose gel electrophoresis in a Trisborate-EDTA (TBE) buffer. GelRed was used to visualize bands under ultra-violet light. The size of the PCR products was estimated using a 1 kb DNA ladder (Qiagen, Australia), with bands identified at 1062 bp.

The sensitivity of the agar culture method versus PCR was determined by using serial dilutions. *S.* Typhimurium stored at -80˚C in 80% glycerol were plated on xylose lysine deoxycholate (XLD) agar (Oxoid, Australia) and incubated overnight at 37˚C. Colonies were selected from XLD agar and resuspended in PBS to match the turbidity equivalent with a 0.5 McFarland standard (BioMerieux, Australia). Enumeration of viable bacteria was performed by serial dilution and spread plating 0.1 mL of suspension on XLD agar, and incubated overnight at 37˚C. DNA extraction and *Salmonella* specific PCR (as described above) was performed using enrichment broths inoculated with serial dilutions. Statistical analysis was conducted using Fisher's exact test of statistical software R (R core team, 2012).

Results

Whole egg penetration experiment

Survival of* Salmonella *Typhimurium on the eggshell surface after 21 days of incubation using agar culture method

As the penetration of bacteria across the eggshell is dependent on the survival of bacteria on the eggshell, we compared the survival of ST PTs on the eggshell surface of washed and unwashed eggs. Results indicated that there was no significant difference in the survival rate of ST PTs on the eggshell surface of washed and unwashed eggs.. The effect of temperature on the survival of *S.* Typhimurium on the eggshell surface of unwashed eggs was studied using two different temperatures (20˚C and 37˚C). The survival rate of ST PT 44 ($p=0.016$) and ST PT 193 ($p<0.05$) was significantly higher at 20˚C. For all ST PTs, the overall trend indicated that a temperature of 20˚C was more favourable for *S.* Typhimurium survival (Table 1). Using two different doses of infection (10^3 and 10^5 CFU/mL), the effect of dose on survival was studied and, as expected, results indicated that survival rate was higher in eggs infected with a 10^5 CFU/mL dose (Table 2). However, a significant difference was observed only in the case ST PT 170 ($p=0.016$) and ST PT 193 ($p<0.05$).

Penetration of eggs and contamination of internal contents by Salmonella Typhimurium using agar culture method

Statistical analysis (using Fisher's exact test) indicated that, at a dose of 10^5 CFU/mL, the penetration of ST PT 44 into washed eggs was significantly higher ($p=0.047$) compared to unwashed eggs. In contrast, ST PT 135 penetration at 10^5 CFU/mL was higher ($p=0.047$) in unwashed eggs (Table 3). For the other ST PTs (PT 9, 170 and 193), there was no significant difference in the *S.* Typhimurium penetration of washed and unwashed eggs. The effect of temperature on *Salmonella* Typhimurium egg penetration was studied at 20˚C and 37˚C. ST PT 135 penetration (infected with 10^5 CFU/mL) was significantly higher ($P=0.047$) at 20˚C. Temperature had no significant effect on the egg penetration of other ST PTs. The effect of dose on egg penetration was also investigated using two different doses (10^3 and 10^5 CFU/mL). At 10^5 CFU/mL, the penetration of PT 135 (at 20˚C) was significantly higher ($p=0.047$) as compared to 10^3 CFU/mL. However, for ST PTs, there was no significant effect of dose on egg penetration.

PCR amplification for detection of S. Typhimurium on eggshell surface and in internal contents of eggs from whole egg penetration experiment

PCR results indicated that, in case of all ST PTs, there was no significant difference in the number of *Salmonella* positive eggshells of washed and unwashed eggs. When the effect of incubation temperature on the detection of *Salmonella* on the eggshell surface was studied, it was observed ST PT 193 (10^5 CFU/mL) detection was significantly higher ($p=0.047$) at 20˚C as compared to 37˚C.

PCR results demonstrated that ST PT 44 (infected with 10^5 CFU/mL) and ST PT 193 (infected with 10^3 and 10^5 CFU/mL) penetration was significantly higher ($p<0.05$) in washed eggs than unwashed eggs (Table 3). The effect of temperature on the *S.* Typhimurium egg penetration was studied and results suggested that ST PT 135 (infected with 10^5 CFU/mL) penetration was significantly higher ($p=0.047$) at 20˚C as compared to 37˚C. When the effect of dose on egg penetration was studied, results showed that ST PT 135 (20˚C) egg internal content contamination was significantly higher ($p=0.047$) for eggs treated with 10^5 CFU/mL as compared to 10^3 CFU/mL. In other cases (except ST PT 9 at 20˚C), egg penetration tended to be higher at an infection dose of 10^5 CFU/mL as compared to 10^3 CFU/mL but the difference was not significant.

Comparison of agar culture method and non-selective enrichment/PCR

The sensitivity of the agar culture method versus non-selective enrichment/PCR was determined by using peptone water inoculated with serial dilutions. The limit of detection for

the agar culture method and PCR was 10 CFU/mL and less than 0.1 CFU/mL, respectively. After 21 days of incubation, using the agar culture method, 62% of the eggshells were reported positive for *Salmonella* whereas, using PCR, a significantly higher (p=0.0007) number of eggshells (82%) were observed *Salmonella* positive.

Using the agar culture method, it was observed that 16% of eggs were penetrated by *S.* Typhimurium but non-selective enrichment/PCR results indicated that 46.66% of egg internal contents were *Salmonella* positive. PCR detected a significantly higher (p=0.0001) number of positive egg internal contents as compared to the agar culture method.

At 20˚C and as per the agar culture method, 18% of washed eggs and 16% of unwashed eggs were penetrated by *S.* Typhimurium. However, non-selective enrichment/PCR results indicated a very different scenario where a significantly higher (p=0.0005) number of washed eggs (74%) were penetrated by *S.* Typhimurium as compared to unwashed eggs (38%).

Agar egg penetration experiment

Relationship of washing and translucency with egg penetration by S. Typhimurium

A summary of the results for the number of washed and unwashed eggs which were penetrated for each ST PT and translucency score is given in Table 4. An analysis of only inoculated eggs indicated that *S.* Typhimurium penetrations were significantly higher for washed eggs than for unwashed eggs {for ST PT 9 (p=0.016), ST PT 135 (p=0.002), ST PT 170 (P=0.0005) and ST PT 193 (P=0.015)}. All eggs –irrespective of their washing status or translucency score– were penetrated when infected with ST PT 44. For all ST PTs, 80-100% of washed eggs were penetrated while only 40-70% of unwashed eggs were penetrated. In most cases, there was no significant difference in the number of penetrated eggs between translucency scores 1 and 2, but translucency did have a significant relationship with the penetration of ST PT 193 (p=0.024).

Relationship of washing and translucency with egg cuticle score

The results of the ordered logistic regression identified egg washing as having a significant effect on the cuticle score for all ST PTs (Table 5). Fig. 1 and Fig. 2 show good quality cuticle of an unwashed egg and damaged cuticle/eggshell surface of a washed egg respectively. The interaction between washing and translucency was also significant for PT 135 (p=0.002). Depending on the ST PT, 60-100% of washed eggs have a cuticle score of 3 or 4 and for ST PT 135, 88% of washed eggs had a cuticle score of 4. This is compared to the cuticle scores of unwashed eggs being more evenly distributed between the four categories and 30-75% of unwashed eggs had cuticle scores of 3 or 4.

Relationship between eggshell structure and penetration by S. Typhimurium

To study the relationship between the eggshell structure and the susceptibility of the eggshell to penetration, the analysis considered only the inoculated eggs and identified correlation between penetration and certain ultrastructure parameters, depending on the phage type. With the exclusion of these correlated variables, the level of Type B bodies (fig. 3) was a significant linear predictor of ST PT 9 penetration (p=0.007). In the case of ST PT 135, cuticle (p=0.001), confluence (p=0.030) (fig. 4) and cap quality (p=0.0004) were significant predictors of eggshell penetration. Results indicated that the levels of confluence (p=0.007), alignment (p=0.040) and erosion (0.007) (Fig. 5) were significantly related to ST PT 170 eggshell penetration. It was also observed that ST PT 193 penetration was significantly related with eggshell ultrastructure parameters such as the level of confluence (p=0.008), cap quality (p=0.018), alignment (p=0.029) (Fig. 6) and erosions (p=0.009) (Table 6, 7). Statistical analysis also showed that, in most cases (except ST PT 135 (p=0.02)), shell thickness was not related to the eggshell penetration.

Relationship between eggshell ultrastructure parameters and translucency score

To investigate the relationship between translucency and eggshell ultrastructure parameters, the analysis identified different results for each ST PT. None of the ultrastructure parameters were significant for ST PT 9 and a significant relationship only existed between cap size and translucency for ST PT 170 ($p=0.038$). All eggs with high translucency (score 2) had a variable cap size (score 2) and approximately 9% of eggs with low translucency (score 1) had a similar cap size. For ST PTs 44 and 135, there were significant differences in erosion counts between eggs of differing translucency scores ($p=0.038$ and 0.004 respectively). The analysis of shell thickness indicated that eggs with low translucency have significantly thicker shells on average compared to eggs with high translucency for ST PT 44 ($p=0.017$) and ST PT 193 ($p=0.040$). In contrast, eggs with high translucency and inoculated with ST PT 135 have significantly thicker shells on average than eggs with low translucency ($p=0.020$).

Table 1: Survival of *Salmonella* Typhimurium phage types on eggshell surface of unwashed after 21 days of incubation: comparison between 20˚C and 37˚C

***Salmonella* Typhimurium phage type (ST PT)**	**Dose of infection (CFU/mL)**	**Temperature (˚C)**	**Eggshell contamination after incubation (Log CFU/ eggshell) Mean ± SE**	**p-value**
ST PT 9	10^3	20	2.511 ± 1.08	0.756
		37	3.039 ± 1.24	
	10^5	20	4.415 ± 0.16	0.287
		37	4.688 ± 0.18	
ST PT 44	10^3	20	3.582 ± 0.89	0.016
		37	0.000 ± 0.00	
	10^5	20	3.582 ± 0.89	0.016
		37	0.000± 0.00	
ST PT 135	10^3	20	0.895 ± 0.89	0.374
		37	0.000 ± 0.00	
	10^5	20	1.905 ± 1.17	0.178
		37	0.000 ± 0.00	
ST PT 170	10^3	20	1.564 ± 0.96	0.178
		37	0.000 ± 0.00	
	10^5	20	3.220 ± 0.85	0.052
		37	0.695 ± 0.69	
ST PT 193	10^3	20	2.508 ± 1.05	0.075
		37	0.000 ± 0.00	
	10^5	20	4.416 ± 0.20	< 0.05
		37	0.000 ± 0.00	

Table 2: Survival of *Salmonella* Typhimurium phage types on the eggshell surface after 21 days of incubation: comparison between different doses (10^3 and 10^5 CFU/mL) of infection

***Salmonella* Typhimurium phage type (ST PT)**	**Temperature (˚C)**	**Washing status**	**Dose of infection (CFU/mL)**	**Eggshell contamination after incubation (Log CFU/ eggshell) Mean ± SE**	**P-Value**
ST PT 9	20	Washed	10^3	3.087 ± 0.82	0.303
			10^5	4.087 ± 0.27	
		Unwashed	10^3	2.511 ± 1.08	0.152
			10^5	4.415 ± 0.16	
	37	Unwashed	10^3	3.039 ± 1.24	0.256
			10^5	4.688 ± 0.18	
ST PT 44	20	Washed	10^3	2.686 ± 1.09	0.175
			10^5	4.491 ± .013	
		Unwashed	10^3	3.582 ± 0.89	1.000
			10^5	3.582 ± 0.89	
	37	Unwashed	10^3	0.000 ± 0.00	NA
			10^5	0.0 ± 0.0	
ST PT 135	20	Washed	10^3	2.232 ± 0.92	0.389
			10^5	3.418 ± 0.92	
		Unwashed	10^3	0.895 ± 0.89	0.512
			10^5	1.905 ± 1.17	
	37	Unwashed	10^3	0.0 ± 0.0	NA
			10^5	0.0 ± 0.0	
ST PT 170	20	Washed	10^3	0.889 ± 0.89	0.016
			10^5	4.336 ± 0.21	
		Unwashed	10^3	1.564 ± 0.96	0.233
			10^5	3.220 ± 0.85	
	37	Unwashed	10^3	0.0 ± 0.0	0.374
			10^5	0.695 ± 0.69	
ST PT 193	20	Washed	10^3	1.673 ± 1.04	0.751
			10^5	2.120 ± 0.88	
		Unwashed	10^3	2.508 ± 1.05	<0.05
			10^5	4.416 ± 0.20	
	37	Unwashed	10^3	0.0 ± 0.0	NA
			10^5	0.0 ± 0.0	

NA: Not applicable

\Table 3: Relationship of washing and translucency with agar egg penetration by *Salmonella* Typhimurium

Salmonella **Typhimurium phage type (ST PT)**	**Number of penetrated/ Not-penetrated eggs**	**Low Translucency (Score 1)**		**High Translucency (Score 2)**		**p-value (Relationship of washing with agar egg penetration)**	**p-value (Relationship of translucency with agar egg penetration)**
		Unwashed	**Washed**	**Unwashed**	**Washed**		
ST PT 9	Not Penetrated	5	1	5	2	0.016	0.735
	Penetrated	5	9	5	8		
ST PT 44	Not Penetrated	0	0	0	0	1.000	1.000
	Penetrated	10	10	10	10		
ST PT 135	Not Penetrated	6	0	3	1	0.002	0.409
	Penetrated	4	10	7	9		
ST PT 170	Not Penetrated	6	1	6	1	0.0005	1.000
	Penetrated	4	9	4	9		
ST PT 193	Not Penetrated	2	0	6	2	0.016	0.024
	Penetrated	8	10	4	8		

Table 4: Effect of egg washing on cuticle score

Salmonella Typhimurium phage type (ST PT)	Washing status	Number of eggs	Average cuticle score (Mean ± SE)	p-value
ST PT 9	Washed	32	3.60 ± 0.09	0.0001
	Unwashed	32	2.75 ± 0.15	
ST PT 44	Washed	32	3.25 ± 0.15	
	Unwashed	32	2.72 ± 0.19	0.046
ST PT 135	Washed	32	3.50 ± 0.13	
	Unwashed	32	2.59 ± 0.14	<0.0001
ST PT 170	Washed	32	3.19 ± 0.16	
	Unwashed	32	2.59 ± 0.16	0.007
ST PT 193	Washed	32	2.81 ± 0.16	
	Unwashed	32	2.28 ± 0.15	0.014

Table 5: Relationship between eggshell ultrastructural parameters and penetration by *Salmonella* Typhimurium

Salmonella Typhimurium phage type (ST PT)	Number of penetrated/non-penetrated eggs	Alignment			Cuticle score					Confluence			Cap quality		
		Low	High	P-value	1	2	3	4	P-value	Low	High	P-value	Good	Poor	P-value
ST PT 9	Penetrated	4	23	0.437	1	4	6	16	0.095	21	6	0.512	9	18	0.971
	Non-penetrated	4	9		0	3	7	3		8	5		6	7	
ST PT 44	Penetrated	11	29	1.000	4	8	12	16	1.000	20	20	1.000	20	20	1.000
	Non-penetrated	0	0		0	0	0	0		0	0		0	0	
ST PT 135	Penetrated	11	19	0.832	0	5	9	16	0.001	22	7	0.030	5	25	0.0004
	Non-penetrated	3	7		0	8	1	1		6	4		3	7	
ST PT 170	Penetrated	4	22	0.040	1	4	11	10	0.094	23	3	0.007	6	20	0.355
	Non-penetrated	7	7		1	7	4	2		7	7		11	3	
ST PT 193	Penetrated	9	21	0.029	6	5	13	6	0.459	29	1	0.008	2	28	0.002
	Non-penetrated	4	6		1	4	4	1		7	3		7	3	

Table 6: Relationship between eggshell ultrastructural parameters and penetration by *Salmonella* Typhimurium

Salmonella Typhimurium phage type (ST PT)	Number of penetrated/non-penetrated eggs	Type A bodies			Type B bodies			Erosion			Argonite			Depression		
		Low	High	P Value	Low	High	P Value	Absent	Present	P Value	Absent	Present	P Value	Absent	Present	P Value
ST PT 9	Penetrated	25	2	0.391	13	14	0.007	15	12	0.075	27	0	NA	26	1	0.353
	Non-penetrated	12	1		4	9		6	7		13	0		13	0	
ST PT 44	Penetrated	37	3	1.000	24	16	1.000	25	15	1.000	31	9	1.000	39	1	1.000
	Non-penetrated	0	0		0	0		0	0		0	0		0	0	
ST PT 135	Penetrated	30	0	NA	3	27	0.640	13	17	0.093	30	0	NA	30	0	NA
	Non-penetrated	10	0		2	8		8	2		10	0		10	0	
ST PT 170	Penetrated	23	3	0.550	11	15	0.767	8	18	0.007	22	4	0.070	26	0	NA
	Non-penetrated	14	0		7	7		6	8		14	0		14	0	
ST PT193	Penetrated	24	6	0.38	10	20	0.298	6	24	0.010	30	0	NA	30	0	NA
	Non-penetrated	10	0		5	5		2	8		10	0		10	0	

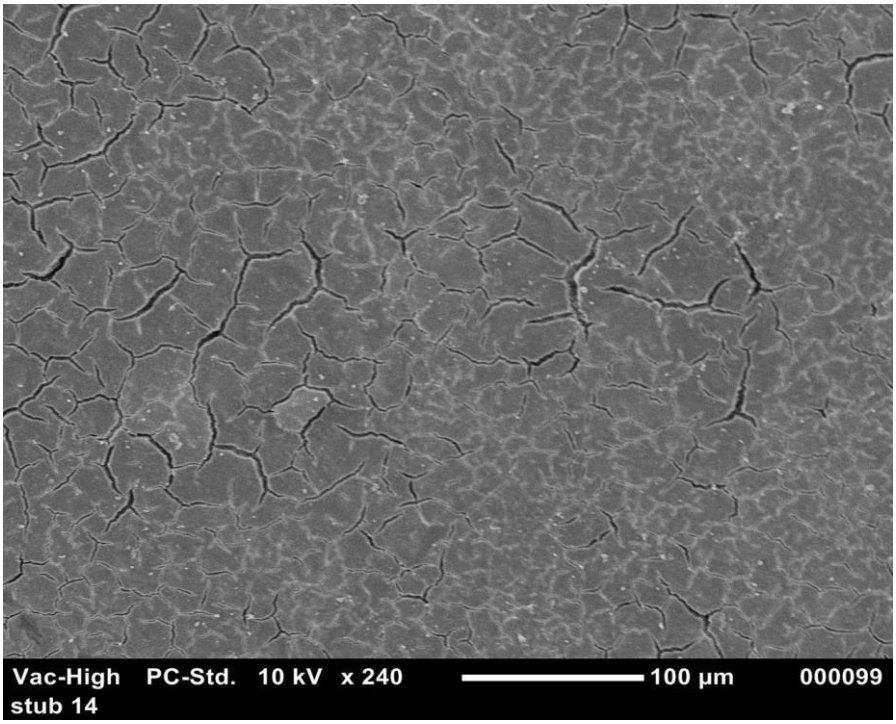


Figure 1: SEM image of good quality cuticle in unwashed eggs with no eggshell pores exposed

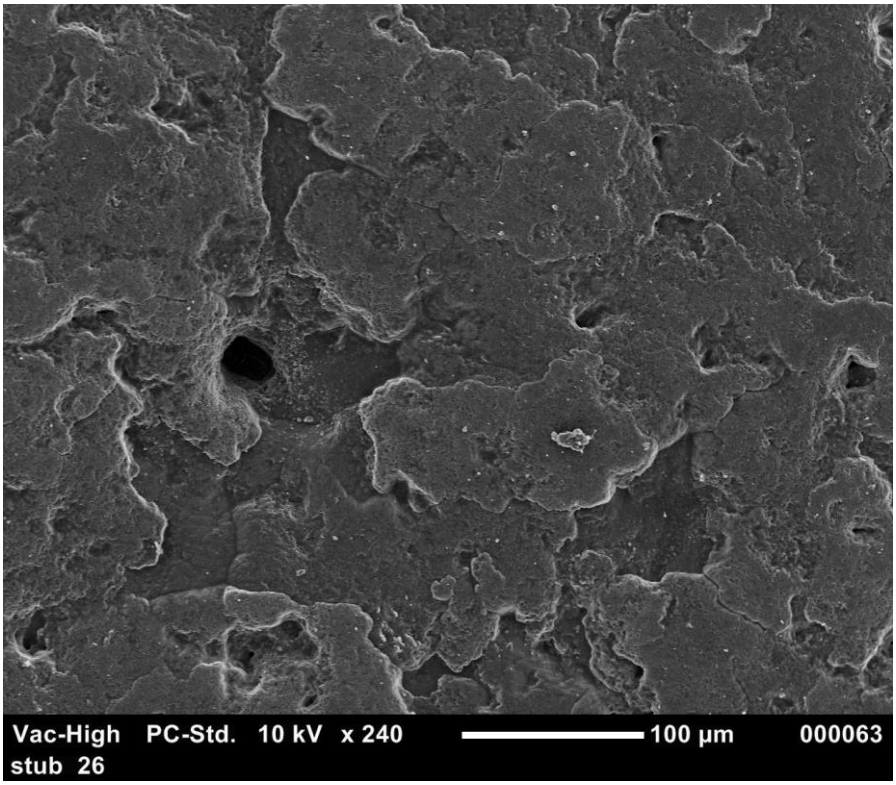


Figure 2: SEM image of damaged eggshell surface and exposed eggshell pore in washed eggs

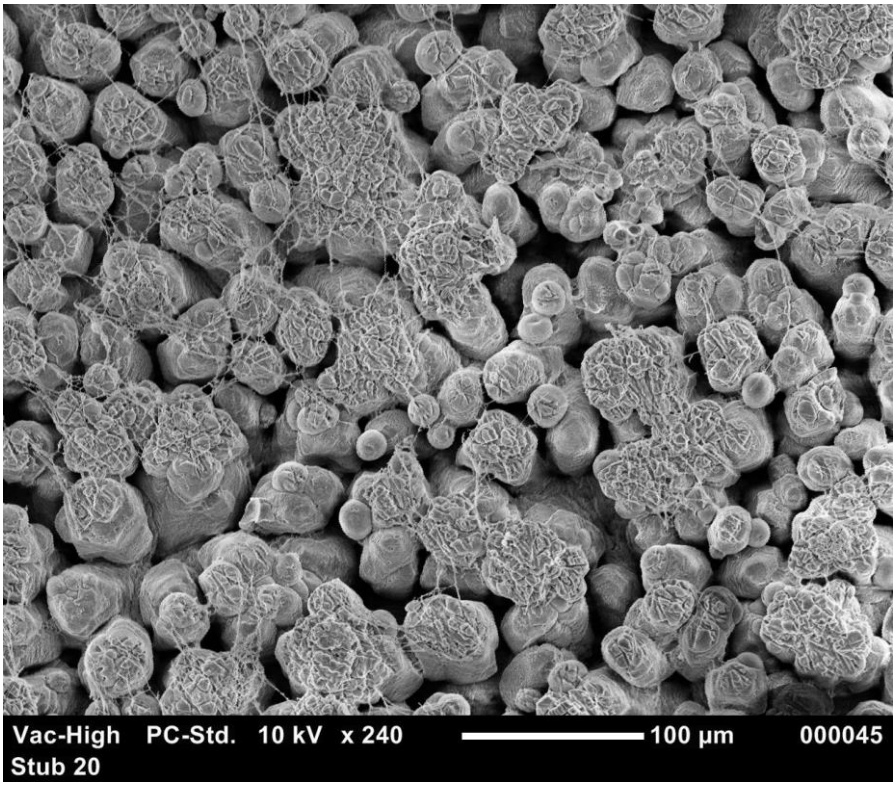


Figure 3: SEM image showing large number of Type B bodies in eggshell

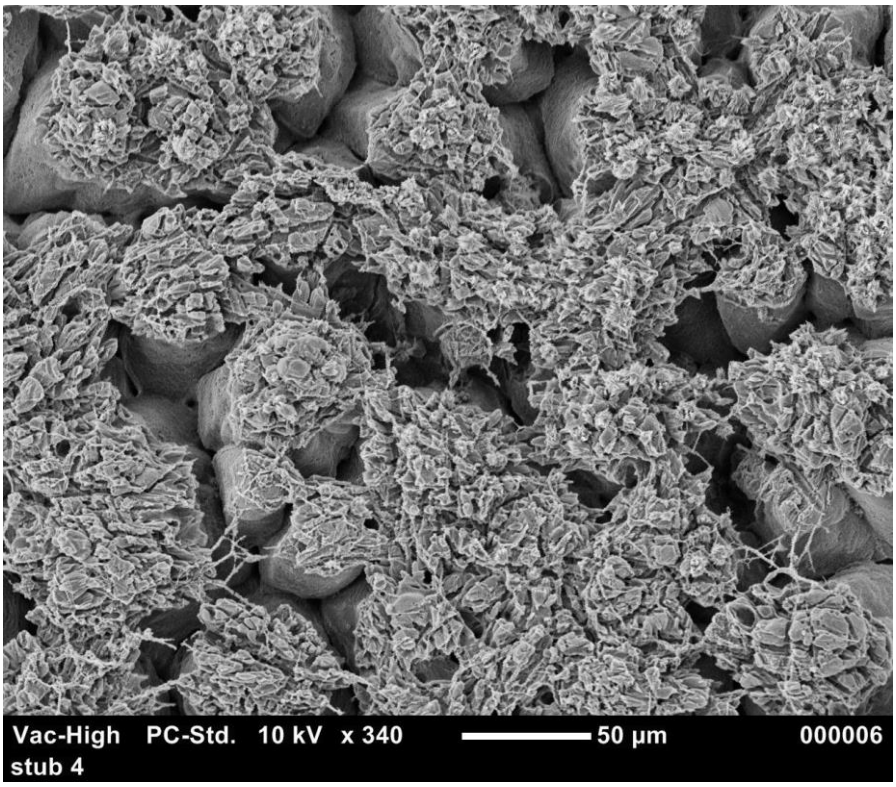


Figure 4: SEM image of good quality mamillary caps with better confluence

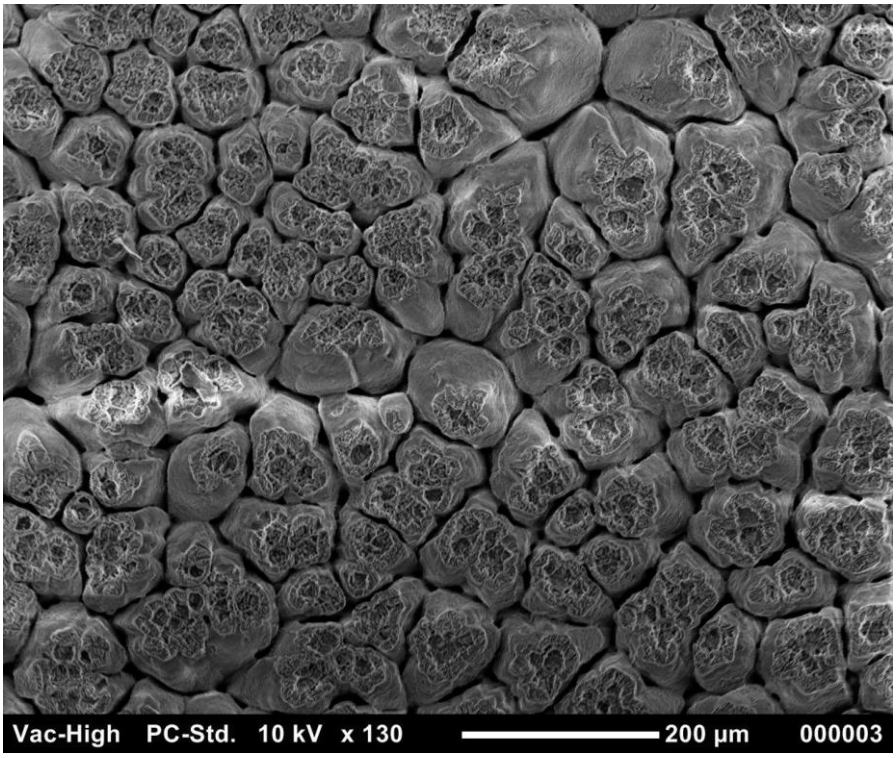


Figure 5: SEM image of extensive erosions throughout the eggshell

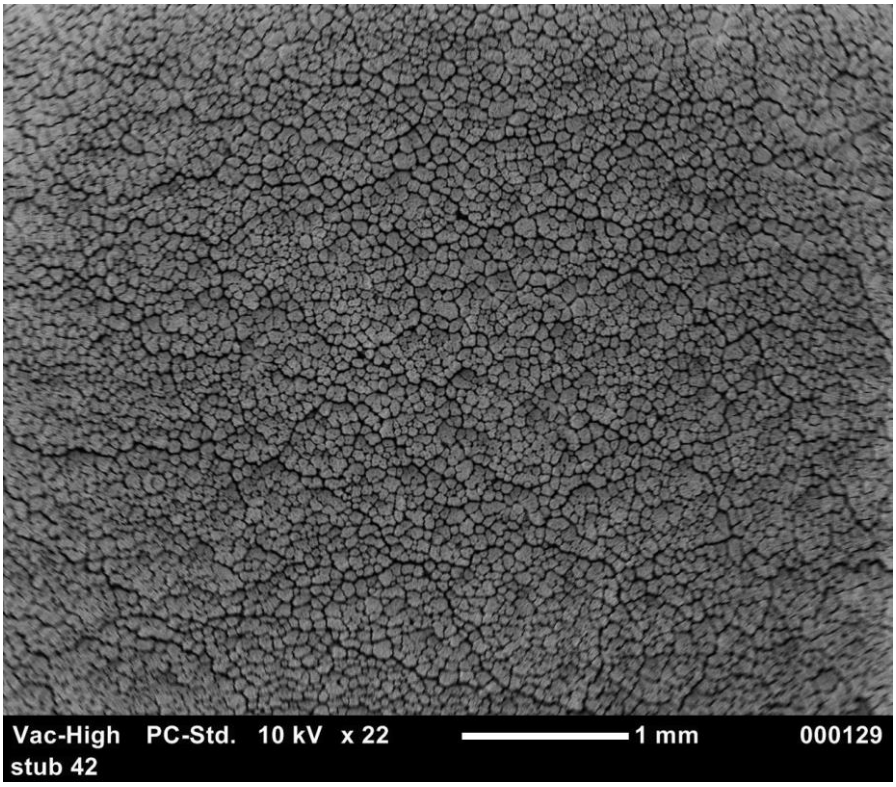


Figure 6: SEM image showing alignment in the mamillary layer

Discussion

It is important to study the survival ability of *S.* Typhimurium on the eggshell surface during storage, not only because *S.* Typhimurium can contaminate egg internal content through horizontal transmission, but it also poses a risk to the health of an individual who is handling the eggs. Contaminated eggs may result in cross contamination of other food items. However, there is limited information available on the survival ability of different ST PTs on the eggshell surface. Our results indicated that all of the ST PTs used in the present study were capable of surviving on the eggshell surface after 21 days of incubation (at 20˚C) following contamination.

In Australia, a large proportion of commercially produced eggs are washed. The penetration of bacteria across the eggshell is dependent on the survival of bacteria on the eggshell surface and egg storage conditions. Hence, we compared the survival rate of *S.* Typhimurium on the eggshell surface of washed and unwashed eggs stored at different temperatures. Results indicated that there was no significant difference in the survival rate of *S.* Typhimurium on the eggshell surface of washed and unwashed eggs. The pattern of survival of *S.* Typhimurium on the eggshell surface varied greatly and there was no uniformity.

PCR is rapid, reliable and sensitive technique for detecting *Salmonella* (Rahn *et al.,* 1992). Hence, in the present study, PCR was used to detect *Salmonella* positive eggshells as well as egg internal contents in the whole egg penetration experiment. The limit of detection for the agar culture method and PCR was 10 CFU/mL and less than 0.1 CFU/mL, respectively, which clearly suggests that PCR was more sensitive in detecting *Salmonella.* PCR results also indicated that, in case of all ST PTs, there was no significant difference in the number of *Salmonella* positive eggshells of washed and unwashed eggs. It seems that the egg washing does not affect the survival ability of *S.* Typhimurium on the eggshell surface. There is no information available in the literature with which to compare these findings.

When the effect of temperature (20˚C and 37˚C) on the survival of *S.* Typhimurium on the eggshell surface was studied, the overall trend indicated that a temperature of 20˚C is more favourable for *S.* Typhimurium survival on the eggshell surface. This may be due to the reduced rate of metabolism by *Salmonella* at a lower temperature induced by the unfavourable growth conditions of a dry eggshell surface (Radowski, 2002a). These findings are in agreement with previous experiments which also reported better survival of *Salmonella* on eggshell surface at lower temperatures (Baker, 1990; Radowski, 2002b; Botey-Salo *et al.,* 2012); however, there were differences in the incubation temperatures and type of *Salmonella* serovar used in the previous study (*S.* Enteritidis). Results from PCR indicated that *S.* Typhimurium positive eggshells were more frequently observed when eggs were incubated at 20˚C.

The present study showed that, if eggs are stored at 20˚C or 37˚C, *S.* Typhimurium can survive on the eggshells for three weeks following contamination. For all ST PTs, after 21 days of incubation, 62% of the eggshells were positive for *S.* Typhimurium. These findings are in agreement with (De Reu *et al.,* 2006) who found a high survival rate of *S.* Enteritidis on the eggshell surfaces after 21 days of infection.

In order to study the egg penetration ability of ST PTs, a whole egg penetration/infection assay was used in the current study as compared to some other studies where *Salmonella* culture was artificially inoculated into the albumen using a syringe. Results indicated that all ST PTs used in the present study (at 20˚C) were capable of penetrating the eggshells and surviving in the egg albumen which is considered to be a hostile environment for the survival of bacteria. In the current study, it was found that ST PT 44 penetration was significantly higher in washed eggs than unwashed eggs. For other ST PTs (PT 9, 170 and 193), there was no significant difference in the *S.* Typhimurium penetration of washed and unwashed eggs. Even though the results of the traditional method showed that ST PT 135 penetration (at infection of 10^5 CFU/mL) was higher in

unwashed eggs, PCR results indicated that there was no significant difference. PCR results also indicated that ST PT 44 (10^5 CFU/mL) and ST PT 193 (10^3 and 10^5 CFU/mL) egg penetration was significantly higher ($P<0.05$) in washed eggs than unwashed eggs, which could be due to damage to the cuticle by egg washing chemicals. These findings are in agreement with a previous study (Wang and Slavik, 1998) whose authors observed that the washing of the eggs with alkaline sodium carbonate solution resulted in damage to the eggshell surface increased *S.* Enteritidis egg penetration. At 20˚C and as per the agar culture method, 18% of washed eggs and 16% of unwashed eggs were penetrated by *S.* Typhimurium. However, PCR results indicated a very different scenario where significantly higher ($p=0.0005$) number of washed eggs (74%) were penetrated by *S.* Typhimurium as compared to unwashed eggs (38%).

The effect of temperature on *S.* Typhimurium penetration was studied at 20˚C and 37˚C. ST PT 135 penetrations (at infection of 10^5 CFU/mL) was significantly higher at 20˚C. For other ST PTs, there was no significant effect of incubation temperature on egg penetration. Similar results were observed when samples were tested by PCR. In previous studies (Schoeni *et al.,* 1995; Wang and Slavik, 1998; Braun *et al*., 1999), there was no consistency in the results. It was previously observed that storage temperature did not significantly affect the penetration of *S*. Enteritidis across the eggshell (Wang and Slavic, 1998). However, other studies (Schoeni *et al.,* 1995; Braun *et al.,* 1999), reported that, with the increase in temperature, the egg penetration of *Salmonella* was also increased. It is difficult to compare findings of these previous studies with the results of present experiment as in all these previous studies (Schoeni *et al.,* 1995; Wang and Slavik, 1998; Braun *et al.,* 1999), eggs were incubated at 4˚C and 20˚C whereas in the present experiment 20˚C and 37˚C temperatures were used to incubate eggs.

The effect of dose on egg penetration was also investigated using two different doses (10^3 and 10^5 CFU/mL). At 10^5 CFU/mL, the penetration of ST PT 135 (at 20˚C) was significantly higher ($p=0.047$) as compared to 10^3 CFU/mL. PCR results also confirmed that the egg penetration by ST PT 135 was dependent on the dose of infection. These findings are consistent with a number of previous studies which indicated that the rate of contamination of eggs is directly proportional to the number of *Salmonella* in the culture used for infecting the eggs (Schoeni *et al*., 1995; Miyamoto *et al.,* 1998; Braun *et al.,* 1999), After the 20 days of storage at 15˚C, the higher inoculation dose resulted in 10 times (10.1%) more contamination of egg internal contents as compared to the lower inoculation dose (1%) (Braun *et al.,* 1999). Similar findings were reported by Schoeni *et al.* (Schoeni *et al.,* 1995) where it was observed that the contents and membranes were more frequently contaminated when faeces containing a high dose of *Salmonella* were used to infect the eggs.

Using the agar culture method, it was observed that, overall, 16% of eggs were penetrated by *S.* Typhimurium but PCR results indicated that 46.66% egg internal contents were *Salmonella* positive. PCR detected a significantly higher ($p=0.0001$) number of positive egg internal contents as compared to the traditional method.Using the whole egg penetration approach, out of all the eggs tested, 16% of internal contents were observed to be positive for *S.* Typhimurium. This is due to the antimicrobial properties of albumen. Hence, in order to get a better idea of the relationship between egg washing and translucency and eggshell penetration by *S.* Typhimurium, the agar egg penetration approach was used. Statistical analysis showed that ST PTs penetration of washed eggs was significantly higher ($p<0.005$) than unwashed eggs. This may be due to the damage of cuticle by egg washing chemicals. To evaluate this further, the effects of washing on cuticle deposition was investigated using SEM. Results from the ordered logistic regression indicated that, for all ST PTs, egg washing had a significant effect on the cuticle score with washing resulting in damage to the cuticle. These findings were not in agreement with a previous experiment where it was observed that egg washing had no significant effect on the quality of the cuticle (Leleu *et al.,* 2011). The difference in the findings may be due to the variation in the age of laying hens and the difference in the protocol of egg washing. In the present study, eggs were collected from

younger hens (< 45 weeks) in contrast to the previous experiment where eggs were collected from old laying hens (> 54 weeks) (Leleu *et al*., 2011). It was previously observed that increasing age of laying hens has a negative impact on cuticle thickness (Sparks and Board, 1984; European Food Safety Authority, 2005). The variation in results of different experiments might result from to the difference in the egg washing protocol. Wang and Slavik (1998) reported that different chemicals used in the egg washing protocol can result in different degrees of cuticle damage. In the present study, in case of ST PT 135, cuticle quality was observed as a significant linear predictor of *Salmonella* eggshell penetration. The mature cuticle closes the pores on the eggshell and protects the egg from the water and bacterial invasion (Berrang *et al.,* 1999) and the removal of cuticle can result in three fold increase in bacterial penetration (Alls *et al.,* 1964). De Reu *et al*. (2006) observed that penetrated eggs had a significantly lower deposition of the cuticle as compared to non-penetrated eggs.

In the present study, using the agar approach, the relationship of translucency with the *S.* Typhimurium eggshell penetration was studied. Results indicated that, in most cases (except for PT 193), there was no significant relationship between translucency and eggshell penetration. However, Chousalkar *et al.* (2010) reported a significant correlation between egg shell translucency and eggshell penetration by *S.* Infantis and *E. coli*. It is also essential to note that, in these two experiments, different bacterial strains were used to study eggshell penetration. These differences might also be due to variation in the method of scoring of egg translucency. In the present study, a quantitative approach was used to score the translucency of eggs.

The SEM results were also analysed to study the relationship of eggshell quality parameters with the eggshell penetration. A higher incidences of alignment, erosions, poor cap quality, Type A mammillary bodies, Type B mammillary bodies may result in the weakening of the eggshell (Solomon, 1992a). Confluence is required for a stronger eggshell region (Roberts and Brackpool, 1994). Our results indicated that, for ST PT 135 (p=0.030), ST PT 170 (p=0.007) and ST PT 193 (p=0.008), eggshell penetration was negatively correlated with the level of confluence. Results also indicated that, for PT 170 and PT 193, eggshell penetration was positively correlated with a higher incidence of alignment and erosion. In case of ST PT 135 and ST PT 193, eggshell penetration was negatively correlated with good cap quality. All these results are in agreement with the previous findings of Solomon (1992a) who reported that good mammillary caps and confluence can resist bacterial penetration whereas alignment, erosion and Type B bodies assist bacterial penetration. However, in the present study it was not clear as to why Type B bodies were negatively correlated with the incidence of ST PT 9 eggshell penetration. Statistical analysis also showed that, in most cases, shell thickness was not related to the eggshell penetration. Similarly, a number of studies have observed that the shell thickness did not affect whole egg and agar egg penetration (Messens *et al*., 2005b; De Reu *et al.,* 2006).

When the relationship between translucency and eggshell ultrastructure parameters was investigated, the analysis identified different results for each ST PT. ST PT 170 analysis indicated that a high translucency score was significantly correlated with a variable cap size. For ST PTs 44 and 135, there were significant differences in erosion counts between eggs of differing translucency scores (P=0.038 and 0.004 respectively). There is no information available in the literature with which to compare these findings. In conclusion, the whole egg penetration experiment results indicated that all the ST PTs used in the present study were capable of penetrating the eggshells and able to survive in the egg albumen which is considered to be a hostile environment for the survival of bacteria. It was also observed that, after 3 weeks of incubation following contamination, all ST PTs still survived on the dry eggshell surface. This finding underlines the importance of proper storage and careful handling of eggs in the food industry and the domestic environment. Egg washing can reduce the level of *Enterobacteriaceae* (up to 4 $\log_{10}$) on the eggshell surface very efficiently (May *et al.,* 2013) but, in the present study, results from agar penetration experiment indicated that the trans-shell penetration was higher in washed eggs than unwashed eggs. Hence, appropriate attention is essential to make sure eggs are kept at appropriate storage

and drying conditions so that they will not come in contact with *Salmonella* after washing. In one study, swabs taken from multiple premises of grading machinery were reported positive for *Enterobacteriaceae* (May *et al.,* 2013); such situation could pose a higher risk of contamination of washed eggs. Hence, regular cleaning of the egg washing machine and grading equipment is essential to avoid recontamination of eggs once they are washed. All these measures will help the egg industry to reduce egg related *Salmonella* food poisoning cases. Only in case of ST PT 44, was 100% eggshell penetration observed in washed and unwashed eggs. This suggests that ST PT 44 may have more capacity for trans-shell penetration as compared to other ST PTs used in this experiment. However, it is essential to keep in mind that, in the present study, only one isolate per ST PT was used; hence further investigation using multiple isolates of same ST PT is essential in order to confirm the variation in penetration ability of different phage types. There was no significant relationship observed between translucency and eggshell penetration. It is possible that egg translucency may have increased during the incubation period, after infecting the eggs with *S*. Typhimurium, which may have resulted in no relationship being observed between the translucency and *S*. Typhimurium eggshell penetration. Further experiments are essential to confirm this hypothesis. Eggshell penetration of *S.* Typhimurium was related to different eggshell ultrastructure parameters such as alignment, poor cap quality, erosion and poor confluence. Hence, studies are essential to determine the factors which are responsible for these eggshell ultrastructural changes.

Chapter 8: Eggshell characteristics and egg penetration by *Salmonella* Agona (A. Ray Honours Thesis)

Abstract

The ability of *Salmonella* Agona to penetrate egg shells was investigated using the whole egg preparation and the agar egg model. *S.* Agona showed some capacity for penetration of eggshells. For the whole egg preparation, four out of 60 eggs had *S.* Agona in the egg contents and there was no difference between washed and unwashed whole eggs for the incidence of penetration. However, for the agar eggs, 22 out of 48 eggs were penetrated and unwashed eggs were penetrated more often (87.5%) than washed eggs (50%). There was no clear relationship between translucency score and the incidence of penetration by *S.* Agona.

Introduction

This study investigated the ability of *Salmonella* Agona to penetrate the egg shell. *S.* Agona is not a particularly pathogenic serovar but it does provide a model for other *Salmonella* serovars.

Salmonella Agona is not as virulent as *Salmonella* Typhimurium or *Salmonella* Enteritidis; however, Foley *et al.* (2008) list it as the cause of many clinical infections. Clinical presentation of *Salmonella* Agona infection includes diarrhoea, headaches, abdominal pain and fever and some patients have also reported vomiting and giddiness (Synnott *et al.,* 1998).

There have been a number of recorded outbreaks of *Salmonella* Agona; 135 isolates were found in England and Wales in 1996 (Synnott *et al.,* 1998). In 1971, *Salmonella* Agona was the second most common *Salmonella* serotype isolated in the UK and Synnott *et al.* (1998) blamed this on the importation of feed for poultry and pigs. *Salmonella* Agona became widespread in broiler chickens and pork sausages. In egg and chicken products, Agona represents <1.5% of clinical cases and 1.9% of non-clinical cases in the United States (Foley *et al.*, 2008). Foley *et al.* (2008) also states that *S.* Agona is in the top 10 of *Salmonell*a serotypes causing both clinical and non-clinical presentations for *Salmonella* infections from swine and turkey products.

Materials and Methods

Eggs were sourced from poultry on the University of Adelaide Roseworthy campus. All microbiological work was conducted in laminar flow hoods to prevent unwanted bacterial contamination.

Whole Egg Penetration by *Salmonella* Agona

The whole egg penetration study aimed to determine how frequently the shell and internal contents are penetrated by *Salmonella* Agona and at what concentrations penetration occurs.

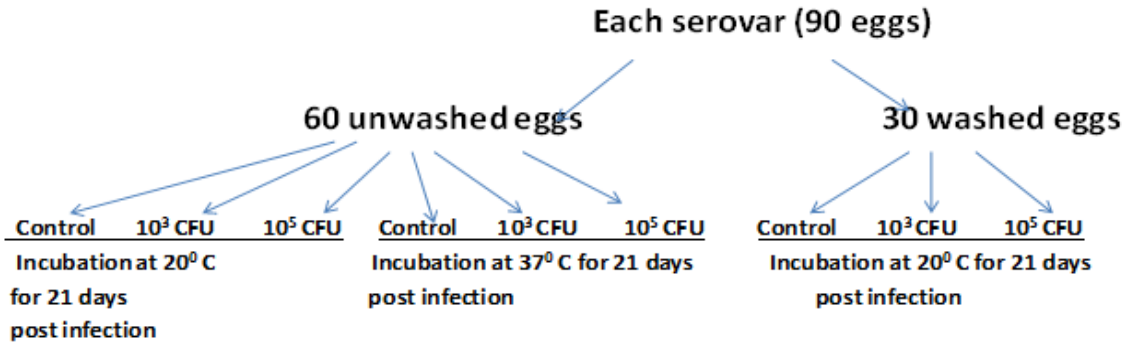


Figure 1: Experimental design for the whole egg salmonella penetration study.

Ninety eggs were weighed and scored for translucency. The eggs were then divided into 3 groups, 1 washed and 2 unwashed. These groups were further divided into 3 subgroups of 10 as shown in Figure 1 and Table 1. Eggs were washed described in Chapter 2. Bacterial suspensions were prepared as described in Chapter 2 and doses of 10^2 and 10^4 CFU/mL were used in this experiment.

Table 1: Group treatments for whole egg penetration studies.

Group	Exposure	Incubation	Washed
1	Control	20°C	Yes
2	10^3 CFU	20°C	Yes
3	10^5 CFU	20°C	Yes
4	Control	20°C	No
5	10^3 CFU	20°C	No
6	10^5 CFU	20°C	No
7	Control	37°C	No
8	10^3 CFU	37°C	No
9	10^5 CFU	37°C	No

Salmonella Agona was streaked on XLD media and incubated overnight at 37°C. Individual colonies were selected and inoculated in 20 mL of PBS to enable turbidity matching to a 0.5 McFarland standard. Dilutions were prepared with 2ml of infected PBS into 18 mL of fresh PBS. By colony counts 10^2 and 10^4 CFU/mL doses were prepared in 150ml of PBS.

Eggs were soaked in 70% ethanol for 30 seconds to remove any bacteria present on the shell. Eggs were then dipped into 10^2 and 10^4 CFU/mL bacterial suspensions for 60 seconds before being allowed to dry in cardboard cartons. Eggs were stored at 20°C and 37°C for 21 days. Eggs were washed in the presence of 10 mL of buffered peptone water and a 2 mL sample was removed from each shell wash for *Salmonella* isolation. Eggs were opened aseptically and 10 mL of internal contents were homogenised, then a 2 mL sample was removed for *Salmonella* isolation. A 10 µL aliquot of each sample collected from shell wash and internal contents was plated on an XLD plate and incubated at 37°C overnight, colonies were counted and stored for future serotyping.

Agar Egg Penetration by *Salmonella* Agona

Salmonella Agona was streaked on XLD media and incubated overnight at 37°C. Individual colonies were selected and inoculated in 20 mL of PBS to make turbidity matching to a 0.5 McFarland standards. Dilutions were prepared with 2 mL of infected PBS into 18 mL of fresh PBS. By colony counts 10^5 CFU/mL doses were prepared in 150 mL of PBS.

Forty-eight eggs were selected, 12 from each of the scores 0-3. Six eggs of each group were washed in an experimental egg washing machine designed to simulate commercial equipment. A small hole was made in the top (small end) of the egg using a 16 gauge needle on a 10 mL syringe. The needle was used to remove the contents of the egg and the inside of the egg was washed out with >10mL of BPW (Buffered Peptone Water). The eggs were allowed to dry before being filled with > 36mL of molten XLD agar media. The hole at the top of the shell was sealed with cellotape and the shell was wiped with a 70% ethanol solution. Eggs were stored overnight in a cardboard carton under UV light to ensure sterility while the agar solidified.

The eggs were partially submerged (with the hole remaining dry) in 10^5 CFU/mL of *Salmonella* Agona suspended in a 10^5 CFU/mL bacterial suspension or PBS (control) for 90 seconds. The eggs were then incubated at 20°C for 21 days. After 21 days eggs were opened in such a manner as to preserve large shell sections and remove the agar. Blackened shells denoted penetrated eggs.

Scanning electron microscopy of penetrated eggshells

Pieces of eggshell were selected from sites where the bacteria had penetrated the shell and prepared for scanning electron microscopy as described in Chapter 2.

Results

Whole Egg Penetration by Salmonella Serotypes

Detection of *Salmonella* Agona in either the egg contents or shell wash proved rare. Of the 60 eggs exposed to the bacteria, nine shell washes and four internal contents showed positive *Salmonella* results. Only a single egg produced both a positive shell wash and internal contents result.

Positive shell wash samples were more common in eggs incubated at 20°C than 37°C, (7/40 [17.5%] at 20°C, 2/20 [10%] at 37°C). In contrast, positive internal contents samples were more common at 37°C than 20°C, (1/40 [2.5%] at 20°C, 3/20 [7.5%] at 37°C). Washed eggs and unwashed eggs had similar penetration rates for both internal contents and shell wash (internal contents 1/20 [5%] washed, 3/40 [7.5%] unwashed) (shell wash 3/20 [15%] washed, 6/40 [15%] unwashed). None of these results were significantly different among treatments. The 30 eggs in the three control groups showed no *Salmonella* Agona.

Egg weights were similar for all groups ranging from 62.89 g in group nine to 67.45 g in group four, the differences between the groups were determined not to be statistically significant.

Agar Egg Penetration

A total of 48 eggs were included in this study and a total of 22 (45.8%) were penetrated by *Salmonella* Agona. 12 (50%) washed eggs were penetrated and 14 (87.5%) unwashed eggs were penetrated and these results are statistically significantly different between groups. None of the 16 control eggs were infected in this study.

Table 3.4.3 shows four (33.33%) of eggs with a zero translucency score were infected, seven (58%) of eggs with a one translucency score were infected. Seven (58.33%) of eggs with a two translucency score were infected and four (33.33%) of eggs with a three translucency score were infected.

SEM of Penetrated Eggshells

Examination of the penetrated shells from experiment 2 showed no consistent structural features associated with bacterial penetration. One penetrated shell had a region of mammillary depressions, others had regions with high number of type B mammillary bodies in the area of bacterial penetration. All penetrated shells had mammillary body erosions near the site of penetration.

Table 2: Experimental preparations and results for whole egg bacterial penetration study

Group	Exposure	Incubation	Washed	Shell wash positive	Total Number of Colonies	Internal contents positive	Total Number of Colonies
1	Control	20°C	Yes	0		0	
2	10^3	20°C	Yes	3 (33.33%)	123	1 (10%)	82
3	10^5	20°C	Yes	0		0	
4	Control	20°C	No	0		0	
5	10^3	20°C	No	3 (33.33%)	296	0	
6	10^5	20°C	No	1 (10%)	1	0	
7	Control	37°C	No	0		0	
8	10^3	37°C	No	1 (10%)	1	3 (33.33%)	Many*
9	10^5	37°C	No	1 (10%)	1	0	

'Many*' indicates plates that had too many colonies to accurately count (>500).

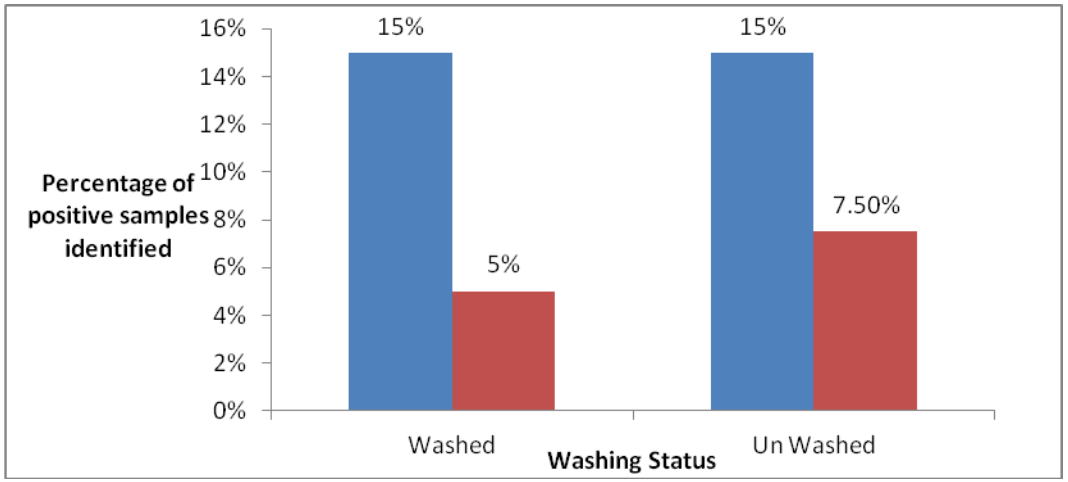


Figure 2: Bar graph showing rates of *Salmonella* Agona identification in shell wash (Blue) and internal contents (Blue) divided by washing status.

Table 3: Experimental preparations and results for agar egg bacterial penetration study.

Translucency score	Status	Total	Number Penetrated	% Penetrated
0	Washed	4	1	25%
0	Unwashed	4	3	75%
0	Control	4	0	0%
1	Washed	4	3	75%
1	Unwashed	4	4	100%
1	Control	4	0	0%
2	Washed	4	3	75%
2	Unwashed	4	4	100%
2	Control	4	0	0%
3	Washed	4	1	25%
3	Unwashed	4	3	75%
3	Control	4	0	0%
Total		48	22	46%
Total	Washed % Penetrated			50%
Total	Unwashed % Penetrated			87.5%

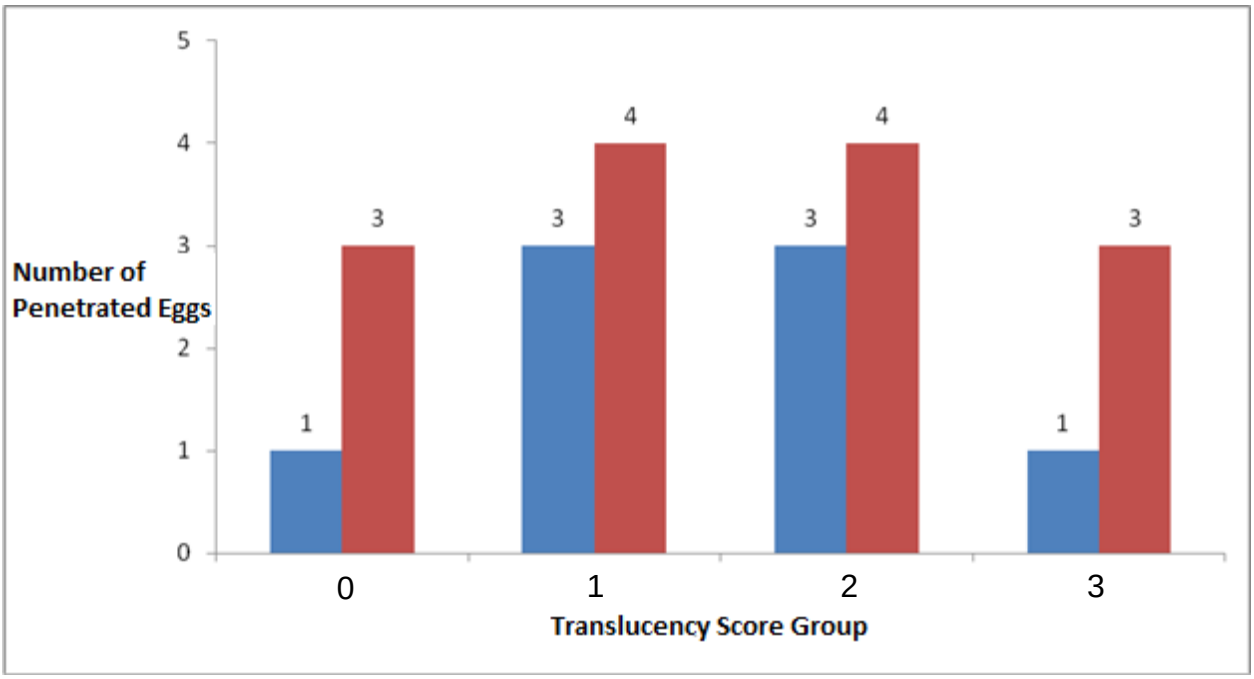


Figure 3: Number of agar eggs penetrated by *Salmonella* Agona, divided by translucency score group. Blue bars indicate washed egg group and red bars indicate unwashed egg group.

SEM of Penetrated Eggshells

SEM analysis of penetrated shells provides a useful tool in determining if there is an underlying structural feature associated with bacterial penetration. Although my images failed to reveal any constant structural abnormality of the four shell samples analysed, one

showed a region of mammillary depressions and another showed increased numbers of type B mammillary bodies. The localized features that may be related with bacterial penetration may also be involved in the appearance of translucency.

SEM of non penetrated eggshells showed that five of the ten, 4 translucency score samples showed either b type mammillary bodies or cubic formations while the scans of the one translucency score group were unremarkable. There were no significant differences between the size or number of mammillary bodies between the groups

One noticeable effect of shell penetration by bacteria is the erosion of mammillary caps near the site of penetration. These erosions were not visible on the control shells nor the adjacent areas removed for SEM.

Discussion

Whole Egg Contamination by *Salmonella* Agona

Salmonella Agona is not known as a particularly pathogenic serovar and clinical cases in humans are rare. *Salmonella* Agona has shown the ability to penetrate eggshells in this experiment being found in both shell wash isolates and internal contents isolates. Overall, positive samples were rare, with all groups showing 15% or lower identification. This may be caused by the reduced pathogenicity of *Salmonella* Agona when compared to other *Salmonella* serovars such as Typhimurium or Enteritidis. However, there is a lack of literature on *Salmonella* Agona and horizontal eggshell transmission.

There was little variation between the washed and unwashed eggs, which is inconsistent with the results achieved in the remainder of the study (Chousalkar, Chapter 7). It was expected that there would be greater penetration in the washed group. Washing has been implicated in weakening the shell's defences to bacteria and there have been a number of possible mechanisms proposed. Washing may act to spread bacteria horizontally between eggs, although modern commercial washing procedures are designed to prevent this. Washing may act to remove some or all of the gram positive bacteria colonizing the shell removing competition for *Salmonella* serovars. Washing eggs soon after oviposition may have a negative effect on the still drying cuticle, removing or reducing it thus decreasing the organic protection of the pores.

Salmonella Agona showed increased presence in the shell wash at 20°C than 37°C; however, at 37°C, there was increased penetration of the shell and colonization of the internal contents. This may be explained by the defence mechanisms of the egg contents. At 37°C, *Salmonella* Agona may be more resistant to the bactericidal effects of the albumen, while at 20°C, the external shell may provide a more suitable environment. These results are similar to those of Gast *et al.* (2007), who showed increased yolk penetration at 30°C as compared with 20°C in a number of *Salmonella* strains; however, *Salmonella* Agona was not part of their study. Messens *et al.* (2004) proposed that, during long term storage at non refrigerated temperatures, there may be a leak of nutrients out of the yolk and into the albumen which may act to negate the effects of the albumen's anti-bacterial role. However, these authors achieved these results at 20°C. These effects may be amplified at 37°C and this is something that should be investigated in future experiments.

Agar Egg Contamination by Salmonella Agona

The agar egg approach is a good method of comparing eggshell penetration abilities of different bacteria. However, no conclusion can be made about the infection of whole eggs as the protective actions of egg albumen, yolk and associated internal structures are not examined (De Reu *et al.,* 2006). Unwashed eggs were more readily penetrated then washed eggs (87.5% vs. 50%). It is possible that this is a feature of *Salmonella* Agona but, unfortunately, there is little literature on this serovar.

There was no clear pattern concerning penetration and translucency, translucency scores 1 and 2 had increased penetration as compared with the 0 and 3 scores. In this experiment, egg age (post oviposition) was not constant and, in some eggs, translucency

was not given time to develop before measuring or beginning experiments. Because of this, no conclusions can be drawn about translucency and bacterial penetration from this experiment. It is difficult to compare studies in this area as each researcher uses different flock ages and different experimental procedures, also few researchers publish data on egg storage times prior to inoculation. The current experiment should be repeated and bacterial penetration rates should be compared with initial translucency score, final translucency score and total change in translucency score. If this is not possible, egg age should be standardized throughout the study.

Using the agar moulding technique, Messens *et al.* (2005b) found that between 30 and 45% of eggs were infected, and De Reu *et al.* (2006), after storing eggs for 21 days to better replicate normal storage conditions, found *Salmonella* Enteritidis penetration rates of approximately 33%.

Chousalkar *et al.* (2010), using the same agar egg procedure, incubated eggs at 20°C and 37°C and found that there was a positive correlation between increasing translucency and increased bacterial penetration. However, *Salmonella* Agona was not used in the study, and there was no information provided concerning egg age at time of candling. Berrang *et al.* (1998), state that "It is likely that factors other than just shell quality are involved in bacterial penetration of eggshells". The one consensus in the literature relating to bacteria and eggs is that there should be prompt transfer to refrigerated storage immediately after laying.

SEM of Penetrated Eggshells

SEM analysis of penetrated shells provides a useful tool in determining if there is an underlying structural feature associated with bacterial penetration. Although my images failed to reveal any constant structural abnormality of the four shell samples analysed, one showed a region of mammillary depressions and another showed increased numbers of type B mammillary bodies. The localized features that may be related with bacterial penetration may also be involved in the appearance of translucency.

SEM of non penetrated eggshells in the agar egg study showed that five of the ten, 4 translucency score samples showed either b type mammillary bodies or cubic formations while the scans of the one translucency score group were unremarkableThere were no significant differences between the size or number of mammillary bodies between the groups

One noticeable effect of shell penetration by bacteria is the erosion of mammillary caps near the site of penetration. These erosions were not visible on the control shells nor the adjacent areas removed for SEM.

Chapter 9: Evaluation of incidence of microcracks before and after commercial egg processing and grading

Abstract

The incidence of microcracks before and after commercial automated egg processing was evaluated. There was no difference in the translucency score before and after processing but the incidence of microcracks following processing was significantly lower than prior to processing. Commercial crack detectors are able to detect and reject microcracks found in commercial eggs.

Introduction

Most commercial eggs in Australia are processed using sophisticated Such equipment may include a light candling box but detection of cracked and defective eggs is often automated. Commercial crack detectors can be set to reject cracks of different resonance.

This small trial investigated the incidence of microcracks in eggs from a flock both before and after processing to provide an indication of the extent to which commercial automated crack detectors remove eggs from the egg processing chain.

Materials and Methods

A total of three hundred and sixty eggs were collected from the grading floor of the commercial egg farm in South Australia. One hundred and eighty eggs were collected before the processing/egg grading and the remaining 180 were collected after egg processing/grading. The eggs were brought to the laboratory at Roseworthy and candled for scoring translucency and number of micro cracks. The eggs were then stored in the cool room for two weeks. All the eggs were again scored for translucency and number of micro cracks. Effects of storage on translucency and micro cracks in graded and non graded eggs were investigated.

Results and Discussion

Effects of grading/egg processing on egg shell translucency

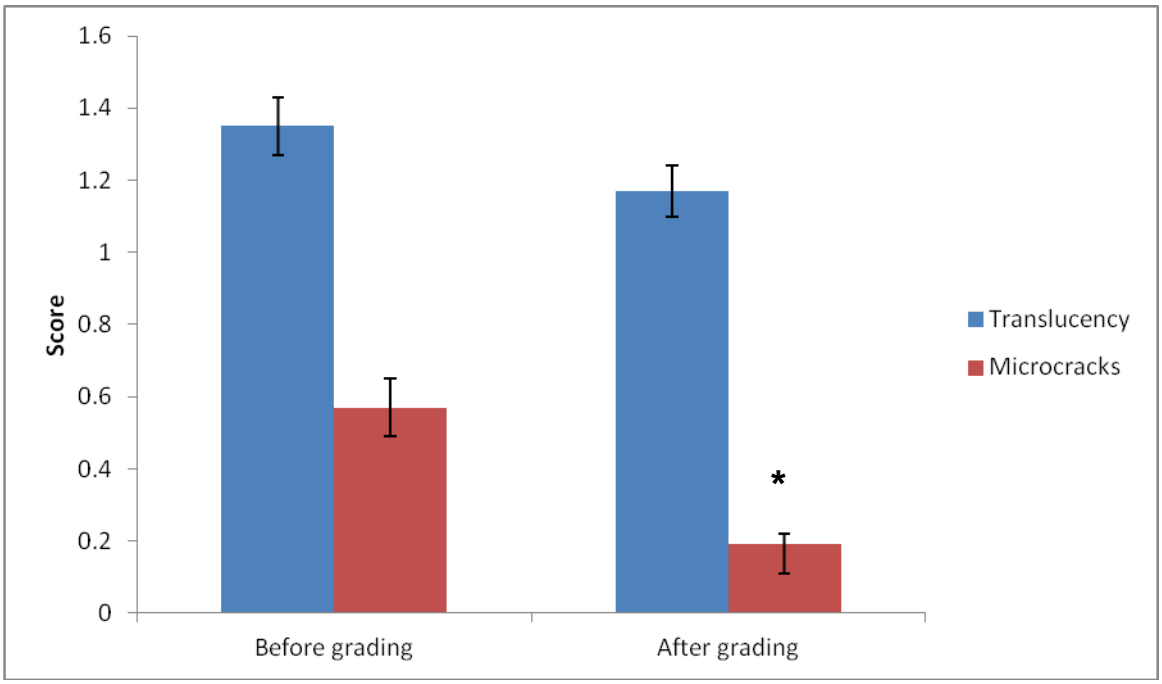


Figure 1: Effects of grading/egg processing on egg shell on microcracks score.

There were no significant differences in the translucency score of the eggs collected before and after processing (P=0.11). However a significant difference was recorded in the microcrack score before and after egg grading (P<0.0001) with grading resulting in a reduction in the number of microcracks.

Commercial crack detectors are able to detect and reject microcracks found in commercial eggs.

Chapter 10: Effect of production system and flock age on egg quality and total bacterial load in commercial laying hens (Samiullah Masters Thesis)

Abstract

The shell of the egg is essential in providing shape to the egg and ensuring safe packaging of the internal contents; however, shell defects have been shown to increase the risk of microbial contamination of eggs. Eggs were collected from cage and free range flocks at the ages of 25, 35, 45, 55, 65 and 75 wk. From each collection per flock, 30 eggs were processed for the eggshell and egg internal quality determination, 30 eggs for cuticle estimation, 30 eggs for mammillary layer ultrastructural features scoring and 60 eggs for egg microbial enumeration. Translucency score and shell reflectivity were significantly higher in free range eggs and increased with flock age in both production systems. Egg weight, shell weight, percentage shell, shell thickness, albumen height, Haugh unit and yolk color were higher for cage eggs. The amount of cuticle was higher in cage eggs and fluctuated with flock age in both production systems. For the mammillary layer ultrastructural variables, there was a significant effect of production system and flock age for early fusion, type A body, and type B body whereas aragonite, depression, erosion and hole were rarely observed. Variability of mammillary cap size, the incidence of poor mammillary cap quality, incidence of late fusion, alignment, Type A bodies, Type B bodies and cubic cone formation were greater in free range versus cage system and increased with flock age in both production systems. The incidence of confluence and early fusion were greater in cage eggs and decreased with age in both production systems. There was significantly lower total microbial load for cage eggs compared to free range eggs but the overall bacterial load recorded in this study was low. It can be concluded that the cage eggs were better in overall quality when directly compared to the free range eggs.

Introduction

In Australia, conventional cage and free range are the main commercial egg production systems, with an estimated retail share of the Australian egg production market in 2011 of 55% and 34%, respectively (AECL Annual Report, 2011-2012). Free range production is increasing as its market share value in the year 2011 (34%) was higher than for 2010 (28%) (AECL Annual Report, 2010-2011). Eggs obtained from conventional cage systems have been reported as being cleaner and having better overall egg quality compared to free range (Dukic-Stojcic *et al.,* 2009).

In the present study, the effect of two production systems (cage vs free range) and of increasing flock age was evaluated for overall egg quality, the amount of cuticle cover on the eggshell, the incidence of various mammillary layer ultrastructural features and the total bacterial load on the eggshell surface, in shell crush and egg internal contents.

Materials and Methods

Eggs were collected at 25, 35, 45, 55, 65, and 75 wk of age from one conventional cage (CC) flock and one free range (FR) flock of Hy-Line brown laying hens. The two flocks were 4 weeks apart in age. Although the feed was not identical for both flocks, diets were formulated according to HyLine Australia specifications and contained wheat (or wheat plus sorghum), soybean meal, meat meal, vegetable oil, limestone and yolk color pigment as major components. The CC flock was in an environmentally-controlled conventional cage system with six hens in each cage (550 cm^2 per bird). The free range flock size was 16000

hens with free access to an outside confined range (10 birds/m^2 in the poultry house, 4 birds/m^2 on the range).

During the study period, a total of 900 eggs were processed from each production system (150 eggs per collection per flock). Out of 900 eggs from each flock, 540 eggs (90 per collection per flock) were processed for the determination of traditional egg external and internal quality parameters, amount of cuticle estimation and the scoring of eggshell ultrastructural features. The remaining 360 eggs (60 per collection per flock) were processed for egg microbiology in which the total bacterial load on eggshells, in eggshell crush and internal contents was recovered and enumerated.

Eggshell and Egg Internal Quality Measurements

Eggshell quality and egg internal quality were measured as described in Chapter 2.

Estimation of the Amount of Cuticle

The amount of cuticle present on the eggshells was determined as described in Chapter 2.

Ultrastructural Scoring of Shell Mammillary Layer

The ultrastructural features of the mammillary layer were scored using a scanning electron microscope (SEM) (JCM-5000 NeoScope). Samples of shell were prepared and viewed as described in Chapter 2.

Egg Microbial Enumeration

Enumeration of the total bacterial count (TBC) and total *Enterobacteriaceae* count (TEC) on the surface of the eggshell was conducted as described in Chapter 2.

Data Analysis

Data were analyzed using Statview Software (SAS Institute Inc., Version 5.0.1.0). A two way analysis of variance (ANOVA) was conducted taking production system and flock age as independent variables and all other variables as dependent. Level of significance was indicated by probability of less than 5%. The Fishers LSD test was used to differentiate levels of significance between mean values.

Results and Discussion

Eggshell and Egg Internal Quality Measurements

For the eggshell quality parameters, there was a significant main effect of both production system and flock age and interaction between the two for translucency score, shell reflectivity (%) (a measure of shell color lightness), egg weight (g), shell weight (g) ($P<0.0001$) and shell thickness (µm) ($P=0.0088$) (Table 1). Shell breaking strength (N), shell deformation (µm) and percentage shell were not significantly affected by production system although there was a significant interaction between production system and flock age. For the egg internal quality, there was a significant main effect of both production system and flock age and significant interaction between the two for albumen height (mm), Haugh unit and yolk color.

Translucency score ($P<0.0001$) and shell reflectivity ($P=0.0001$) were higher in the free range (FR) flock and increased with flock age in both systems whereas egg weight, shell weight, percentage shell and shell thickness were higher in the cage production system (CC) ($P<0.0001$) and generally increased with flock age, irrespective of production system. The egg internal quality parameters; albumen height, Haugh unit and yolk color were higher in the CC production system as compared with FR ($P<0.0001$) and decreased with flock age except for the yolk color.

Translucency develops when moisture escapes from the egg albumen through the shell membranes into the ultrastructure of the mammillary layer. Most freshly laid eggs show relatively few translucent spots, and translucency develops within the first 24 hr after laying

(Holst *et al.,* 1931; Solomon 1986; Talbot and Tyler, 1974). The incidence of translucency increases with the passage of time until 6 to 7 days after the egg is laid. The incidence of translucency varies from egg to egg and is affected by both storage time and factors such as flock age. It ranges from small pin points to hair like lines and circular spots of approximately 1 mm^2. The higher translucency in FR eggs might be due to the time lapse between collection and processing. The lower shell reflectivity (darker shell color) in cage eggs versus eggs from free range birds and increasing shell reflectivity with flock age in both systems has been reported previously (Sekeroglu *et al.,* 2010).

In the current study, shell reflectivity generally increased to a greater extent in the free range flock, as flock age increased. Shell reflectivity varies with the amount of pigment (protoporphyrin IX) deposited in the eggshell. The lighter the shell color, the higher the shell reflectivity and vice versa (Samiullah and Roberts, 2013). Shell reflectivity can be used to measure stress conditions in brown shelled laying hens (Mills *et al.,* 1991; *Nys et al*., 1991)

Egg weight in the conventional cage flock was significantly higher than for the free range birds at all flock ages. In the free range flock, egg weight (g) increased between 25 and 35 weeks and then remained relatively constant. In contrast, in the cage production system there was a consistent slight increase in egg weight with increasing flock age. These observations are in comparison to the findings of previous studies (Van Den Brand *et al*., 2004; Wang *et al.,* 2009) in which a greater increase in egg weight was observed in free range eggs. A slightly higher egg weight in a cage flock compared to a free range flock has been reported previously (Pavlovski *et al.,* 2004; Varguez-Montero *et al.,* 2012) which is similar to the current findings.

Shell weight and shell thickness were higher for cage eggs versus free range in the present study. No significant difference was recorded for shell weight between cage and floor pen and litter system eggs in previous studies (Petek *et al.,* 2009; Banga-Mboko *et al.,* 2010; Tumova *et al.,* 2011). In the studies of Abrahamsson and Tauson (1998), higher shell weight was recorded for cage eggs compared to an aviary system. Shell thickness was not significantly different between free range and cage systems in several previous studies (Van Den Brand *et al.,* 2004; Wang *et al.,* 2009; Tumova *et al.,* 2011), contrary to the findings of the present study. The current results for higher shell thickness in cage eggs cannot be compared directly with previous research findings as most of the authors compared cage eggs with production systems that were different from the free range production system of the present study.

Housing system had little effect on shell breaking strength in the present study. Breaking strength was higher for free range than for cage at 45 and 55 weeks but the reverse was the case at 65 wk. Higher shell strength in conventional cage eggs compared to all other systems has been reported (Hidalgo *et al.,* 2008) and a higher value for shell breaking strength was recorded in cage eggs than for a litter system (Tumova *et al.,* 2011). An increase in shell strength towards peak production, followed by a subsequent decrease with flock age in cage and outdoor flocks has been reported (Tumova and Ledvinka, 2009). Shell deformation (µm) showed a similar pattern to shell breaking strength, with no overall difference between production systems, indicating that housing system had little effect on shell elasticity or fragility. There was a significant interaction ($P<0.0001$) between the production system and flock age for shell deformation which was higher for cage than free range at 45 and 55 wk. Shell deformation was greater in conventional cage eggs versus all other systems in a previous study (Hidalgo *et al*., 2008) and higher shell deformation was recorded for cage eggs compared to aviary system (Abrahamsson and Tauson, 1998). However, another study reported no significant effect of production system on shell deformation (Tanaka and Hurnik, 1992).

In the current study, percentage shell was not significantly affected by production system which is similar to other published data (Sekeroglu *et al.,* 2010). A higher percentage shell has been recorded in cage system eggs versus other systems by Hidalgo *et al.* (2008). A slightly higher percentage shell was recorded for free range egg versus cage system in other studies (Van Den Brand, 2004; Wang *et al.,* 2009) which is in contradiction to the current findings.

Albumen height (mm) was statistically significantly different (P≤0.05) between the two production systems at different ages. However, there was a longer time interval between egg collection and egg analysis of the free range eggs which would explain much of this difference. A variable albumen height in cage eggs with increasing hen age, with a linear decrease in outdoor hen eggs, has been recorded previously (Wang *et al.,* 2009). A relatively higher albumen height was recorded in free range eggs compared with cages, with an overall decrease with flock age (Van Den Brand, 2004) although there was no significant effect of production system on albumen height (Van Den Brand, 2004; Varguez-Montero *et al.*, 2012).

A statistically significant effect (P≤0.05) of production system and interaction between production system and flock age was found for Haugh unit and this followed a similar pattern to albumen height. Haugh unit decreased with flock age more in the free range whereas, in the cage system, it followed a consistent decline. A higher Haugh unit in cage eggs versus other systems was reported previously (Roland, 1979; Tumova and Edeid, 2005; Lichovnikova and Zeman, 2008; Tumova *et al.,* 2011). A slight decrease and then increase in Haugh unit occurred in an outdoor production system at the same time as a linear decrease occurred in a cage production system (26-50 wk flock age)(Wang *et al.,* 2009). Free range eggs had the lowest Haugh unit scores compared to other systems (Hidalgo *et al.,* 2008). In other studies (Mostert *et al.,* 1995; Petek *et al.,* 2009; Sekeroglu *et al.,* 2010), there was no significant effect of production system on Haugh unit and one study reported a higher Haugh unit in free range compared to cage and deep litter systems (Pavlovski *et al.,* 2004). Higher albumen height in cage eggs showed that these eggs were better in internal quality compared to free range eggs.

Yolk color was generally more consistent for the cage production system. A significantly higher yolk color in free range compared to cage eggs was recorded in some previous studies (Sencic *et al*., 2006; Petek *et al.,* 2009; Varguez-Montero *et al.,* 2012). In other studies (Sekeroglu *et al.,* 2010), there was no significant difference in yolk color between free range and cage eggs. A higher yolk color for floor flock versus cage system eggs was recorded (Singh *et al.,* 2009). It can be concluded that yolk color varies more with flock nutrition than age or production system and will be dependent on both the amount of pigment added to the feed and, in the case of free range production, how much vegetation is consumed by the birds.

Estimation of the Amount of Cuticle

There was a significant main effect of production system, flock age and a significant interaction between production system and flock age for L* and b* components of the L*a*b* space system as shown in Table 2. There was a significant main effect of production system and flock age for a* components of the L*a*b* space system but no significant interaction between the two.

The higher values of L* for free range compared to cage eggs indicated less pigment in free range compared to cage system eggs. From the current spectrophotometric measurements of stained eggs, it can be concluded L* values are less significantly affected by MST blue stain. The a* is the most important component in the L*a*b* space system as it shows the amount of the MST stain acquired by the cuticle. The more negative values in the cage system versus free range indicated more cuticle present in the cage eggs. The lack of significant interaction between production system and flock age indicated that flock age had similar effects on the amount of cuticle deposition in both production systems. The b* component of the L*a*b* color space system is the grading between blue and yellow where yellow is towards the positive end of the scale and blue is towards the negative end. There were significant main differences between production systems with free range flocks being higher.

Ultrastructural Scoring of Shell Mammillary Layer

Scanning Electron Microscopic (SEM) observations indicate that no two eggs from the same flock at the same age possess the same ultrastructural characteristics which, in turn, reflect a high rate of variation in the secretion of eggshell precursors in the shell gland of the hen's reproductive system. Thus, oviducal malfunction may result in the breaching of the shell's inherent defence mechanisms (Nascimento *et al.,* 1992). Analysis of the uterine fluid proteins (Gautron *et al.,* 1997) at various stages of shell formation and *in vitro* crystallization experiments (Gautron *et al.,* 1996) have revealed egg specific proteins that influence the process of calcification by modifying and modulating crystal growth (Nys *et al*., 2004). Shell quality decreases with increased flock age and old hen eggs are reported to be more prone to microbial penetration, as compared to eggs from younger flocks (Solomon, 1992a).

For the ultrastructural variables of the mammillary layer, a significant main effect of production system was recorded for: mammillary cap size, mammillary cap quality, early fusion, Type A bodies, Type B bodies, aragonite, cuffing and erosion (Table 3). A significant interaction between production system and flock age ($P<0.05$) was found for mammillary cap size, late fusion, alignment, Type A bodies, Type B bodies, cubic cone formation, cuffing, and changed membrane. There was no significant effect of production system or interaction between production system and flock age for the incidence of confluence, cubics, depression or hole. Variability of mammillary cap size, the incidence of poor mammillary cap quality, incidence of late fusion, alignment, Type A bodies, Type B bodies and cubic cone formation were greater in free range (FR) versus cage system (CC) and increased with flock age in both production systems. The incidence of confluence and early fusion were greater in CC and decreased with age in both production systems. More variable mammillary cap size leads to poor membrane attachment and poor cap quality which can affect the overall quality of the shell ultrastructure. Overall, cap quality was poorer in free range compared to cage eggs. Good caps quality cones show higher affinity for membrane attachment, thus making the shell stronger. A higher incidence of early fusion has a positive impact on shell strength and vice versa (Solomon, 1991). The overall incidence of early fusion was slightly higher in cage eggs versus free range. A higher incidence of early fusion increases the effective thickness of palisade columns (Solomon, 1991). and is a positive feature of the mammillary layer. Increased incidence of cuffing has a positive effect on the mammillary layer, and its higher incidence in cage eggs suggests better ultrastructural quality (Solomon, 1991).

Late fusion, alignment, cubics, cubic cone formation, changed membrane, depression and hole negatively affect mammillary layer quality. Their incidence was not significantly different between the two production systems. The higher incidence of mammillary layer variables like Type A bodies, Type B bodies and aragonite in free range eggs compared to cage eggs indicates better quality of cage eggs.

Egg Microbial Enumeration

A significant main effect ($P<0.05$) of production system and flock age and interaction between the two was recorded only for total bacterial count (TBC) on the eggshell surface. There was a significant main effect of flock age on TBC in shell crush but no significant effect of production system or interaction between production system and flock age as shown in Table 4. The total *Enterobacteriaceae* count (TEC) on shell was significantly affected by flock age and interaction between production system and flock age, whereas TEC in shell crush was only significantly affected by flock age. None of the egg internal contents were positive for bacteria.

Understanding external contamination of shells is important in order to evaluate the shelf life and food safety of commercial eggs. In most cases, egg internal contamination results from penetration of bacteria deposited on the shell surface as it is being laid or after it has been laid (Schoeni *et al*., 1995). *Salmonella* poisoning related to egg products has attracted the attention of food safety authorities in Australia and all over the world. The shell wash method used in the current study has been used successfully by other workers for microbial enumeration from the eggshell surface (De Reu *et al.,* 2005). In the current study, the TBC on shell surface was significantly higher in free range eggs compared to cage eggs.

On average, 20-30 times more bacteria have been isolated previously from the surface of litter floor eggs compared to a wire floor (Quarles *et al.,* 1970). There is a greater chance of eggshell contamination in litter and free range systems compared to cages as freshly laid eggs can be contaminated when coming in contact with contaminated surfaces (Harry, 1963; Gentry and Quarles, 1972). A 15 times greater bacterial load on the eggshell surfaces of deep litter eggs compared to cage eggs has been reported (Harry, 1963). Total aerobic flora were higher (more than 1.0 log) on eggs from an aviary housing system compared to conventional and furnished cage systems (De Reu *et al.,* 2005). In another study (Wall *et al*., 2008), the total bacterial load and *Enterobacteriaceae* on the eggshell were significantly higher for furnished cage eggs compared to conventional cage eggs. In the present study, the TBC in shell crush was not significantly different between production systems and remained below 2 log cfu/mL of rinsate throughout production in both systems. The TEC on the shell and shell crush was not affected by the production system and compared to TBC, was quite low. Bacterial contamination of the eggshell is greatly affected by factors such as diet (Smith *et al*., 2000) and poultry house environment (De Reu *et al.,* 2005). A significantly higher *Enterobacteriaceae* count on eggshells from furnished cage production (12.3%) compared to conventional cages (5.8%) was recorded but the egg internal contents from both production systems were free from bacteria (Wall *et al*., 2008). In the present study, egg internal contents from both production systems were free from bacteria.. Comparing different housing systems, previous studies (Quarles *et al.,* 1970; Smeltzer *et al.,* 1979; De Reu *et al.,* 2005; Mallet *et al*., 2006; Wall *et al.,* 2008) have shown that eggs produced by cage systems have generally lower bacterial counts compared to other production systems but the differences found between the cage and non cage system eggs in terms of contamination are less pronounced under commercial conditions (De Reu *et al.,* 2008). The lack of differences in total microbial load between the cage and free range production system could be attributed to proper hygienic measurements followed on the farms during the study period.

Conclusions

The eggshell quality variables; egg weight, shell weight and shell thicknesses increased with flock age and were higher in the conventional cage flock versus the free range flock.
The egg internal quality variables albumen height and Haugh units decreased with flock age and were higher in cage flock compared to free range whereas yolk color was higher in cage eggs and fluctuated with flock age in both production systems.
The amount of cuticle present on the eggshell varied significantly with flock age and was significantly higher in cage eggs versus free range.
The increased incidence of late fusion, alignment, type A body, and type B body with increasing flock age indicates that shell quality decreases as hen age increases. In the present study, a significantly higher incidence of poor mammillary cap quality, incidence of late fusion, alignment, Type A bodies, Type B bodies and cubic cone formation was recorded in free range versus cage eggs.
The total bacterial and *Enterobacteriaceae* load was relatively low and was significantly higher in free range eggs compared to cage eggs.

Table 1: Eggshell and egg internal quality measurements

Variables	Flocks age (weeks)												P value		
	Conventional Cages						Free Range								
	25	35	45	55	65	75	25	35	45	55	65	75	P	A	P*A
Translucency score	2.23 ±0.20	1.17 ±0.18	1.03 ±0.19	2.40 ±0.21	1.63 ±0.26	1.43 ±0.21	2.77 ±0.27	*2.40 ±0.25	*3.97 ±0.17	*3.13 ±0.19	*2.97 ±0.18	*3.10 ±0.17	<0.0001	0.0002	<0.0001
Shell reflectivity (%)	27.10 ±0.67	30.33 ±0.41	28.87 ±0.59	30.17 ±0.82	29.70 ±0.67	29.40 ±0.57	*29.67 ±0.79	*32.57 ±0.92	*33.37 ±0.52	*37.63 ±1.30	*38.70 ±0.98	*33.97 ±1.2	<0.0001	<0.0001	0.0001
Egg weight (g)	60.35 ±0.87	63.90 ±0.75	63.27 ±0.89	62.97 ±0.78	64.47 ±0.82	68.60 ±0.82	*51.68 ±0.59	62.57 ±0.85	64.30 ±0.58	61.73 ±0.84	*61.97 ±0.85	*63.84 ±1.0	<0.0001	<0.0001	<0.0001
Shell breaking strength (N)	40.07 ±1.4	41.83 ±0.96	39.55 ±0.90	38.19 ±0.99	40.22 ±1.60	36.67 ±1.70	41.31 ±1.60	38.45 ±1.50	*42.74 ±1.30	*41.50 ±1.30	*35.52 ±1.50	37.49 ±1.50	0.9182	0.0173	0.0112
Deformation unit (µm)	328.3 ±12.0	307.3 ±5.45	298.0 ±5.37	299.0 ±5.04	294.0 ±7.62	320.0 ±15.8	334.6 ±7.55	316.6 ±6.58	*322.6 ±8.01	*321.3 ±6.57	294.0 ±12.0	261.6 ±8.04	0.8885	<0.0001	<0.0001
Shell weight (g)	5.40 ±0.07	5.88 ±0.07	5.85 ±0.08	5.67 ±0.08	5.99 ±0.12	6.14 ±0.13	*4.73 ±0.09	*5.20 ±0.11	5.81 ±0.06	5.51 ±0.12	5.70 ±0.09	5.94 ±0.12	<0.0001	<0.0001	0.0009
Percentage shell	9.02 ±0.09	9.21 ±0.09	9.26 ±0.11	9.03 ±0.14	9.29 ±0.13	8.96 ±0.17	9.15 ±0.15	*8.33 ±0.15	9.05 ±0.09	8.92 ±0.13	9.22 ±0.14	9.33 ±0.15	0.0853	0.0048	0.0002
Shell thickness (µm)	370.1 ±3.49	389.4 ±3.82	378.0 ±3.85	376.9 ±5.13	392.4 ±6.02	389.0 ±5.86	*354.8 ±5.41	*352.5 ±5.74	385.3 ±3.55	381.5 ±6.01	387.3 ±4.60	389.3 ±4.72	0.0088	<0.0001	<0.0001
Albumen height (mm)	11.04 ±0.14	10.65 ±0.18	10.24 ±0.21	9.39 ±0.20	10.07 ±0.20	9.95 ±0.28	*9.04 ±0.18	*9.33 ±0.23	*8.53 ±0.20	*6.76 ±0.29	*7.32 ±0.23	*4.81 ±0.24	<0.0001	<0.0001	<0.0001
Haugh Unit	103.5 ±0.62	101.0 ±0.77	99.23 ±0.92	95.40 ±0.96	98.60 ±1.03	96.67 ±1.57	*96.50 ±0.89	*95.17 ±1.14	*90.77 ±1.11	*80.13 ±1.98	*84.20 ±1.38	*63.33 ±2.31	<0.0001	<0.0001	<0.0001
Yolk color	10.07 ±0.09	10.90 ±0.16	11.03 ±0.13	10.50 ±0.15	11.70 ±0.13	11.40 ±0.16	*7.73 ±0.31	*10.03 ±0.15	*9.80 ±0.11	10.07 ±0.19	*9.37± 0.19	11.80± 0.21	<0.0001	<0.0001	<0.0001

Mean ± SE

* Indicates significant difference from cage system at the same age;

P- Production system; A- Age; P*A- Production system & Flock age interaction

Table 2: Spectrophotometry (L*a*b*) values of stained eggshells

Variables	Flocks age (weeks)												P value		
	Conventional Cages						Free Range								
	25	35	45	55	65	75	25	35	45	55	65	75	P	A	P*A
L*	51.06 ±0.49	54.76 ±0.61	56.47 ±1.23	54.28 ±0.86	55.09 ±0.53	53.09 ±0.86	*56.12 ±0.75	*57.09 ±0.53	*59.79 ±0.65	*60.44 ±0.93	*62.08 ±0.96	*60.96 ±0.73	<0.0001	<0.0001	0.0029
a*	-2.67 ±0.75	0.26 ±0.78	-3.84 ±1.20	-4.83 ±1.14	-1.05 ±0.66	-4.61 ±1.01	*3.15 ±0.98	2.52 ±0.83	-0.85 ±0.92	-2.15 ±1.02	-1.35 ±0.97	-2.58 ±1.24	<0.0001	<0.0001	0.0699
b*	32.31 ±0.30	32.4 ±0.41	28.46 ±0.64	29.44 ±0.29	31.92 ±0.43	28.97 ±0.42	*34.47 ±0.42	*34.67 ±0.50	*32.42 ±0.45	*33.65 ±0.88	30.76 ±0.58	*31.29 ±0.54	<0.0001	<0.0001	<0.0001

Mean ± SE

* Indicates significant difference from cage system at the same age

P- Production system; A- Age; P*A- Production system & Flock age interaction

L* is between white (100) and black (0).

a* is between green (-ve) and red (+ve)

b* between blue (-ve) and yellow (+ve)

Table 3: Scores for ultrastructural features of shell mammillary layer

Variables	Flocks age (weeks)												P value		
	Conventional Cages						Free Range								
	25	35	45	55	65	75	25	35	45	55	65	75	P	A	P*A
Mammillary cap size	1.48 ±0.11	1.70 ±0.11	2.33 ±0.12	2.13 ±0.18	2.00 ±0.09	1.90 ±0.10	*1.83 ±0.09	*2.10 ±0.12	2.03 ±0.14	2.13 ±0.12	2.27 ±0.11	*2.20 ±0.09	0.0067	<0.0001	0.0090
Confluence	3.00 ±0.11	2.83 ±0.11	2.37 ±0.13	2.23 ±0.14	1.77 ±0.14	1.83 ±0.16	2.70 ±0.14	2.73 ±0.11	2.33 ±0.14	2.30 ±0.17	1.87 ±0.16	*1.40 ±0.12	0.1401	<0.0001	0.3149
Cap quality	1.48 ±0.11	1.67 ±0.15	2.07 ±0.14	2.57 ±0.11	2.37 ±0.18	2.23 ±0.11	1.60 ±0.12	2.03 ±0.18	*2.57 ±0.18	2.73 ±0.16	2.43 ±0.16	2.40 ±0.12	0.0070	<0.0001	0.6687
Early fusion	3.21 ±0.11	2.80 ±0.10	2.77 ±0.08	2.20 ±0.13	2.37 ±0.09	2.40 ±0.09	3.00 ±0.09	*2.37 ±0.10	*2.37 ±0.11	2.13 ±0.12	2.20 ±0.12	2.33 ±0.09	0.0003	<0.0001	0.3285
Late fusion	2.45 ±0.14	3.40 ±0.12	2.67 ±0.09	3.00 ±0.07	3.07 ±0.08	3.07 ±0.07	2.73 ±0.13	3.13 ±0.08	*2.97 ±0.09	3.03 ±0.03	2.97 ±0.03	3.00 ±0.05	0.5407	<0.0001	0.0062
Alignment	2.17 ±0.17	2.00 ±0.14	2.30 ±0.11	2.87 ±0.13	2.77 ±0.12	2.47 ±0.12	1.97 ±0.12	2.33 ±0.10	*2.77 ±0.09	2.77 ±0.10	2.67 ±0.11	*2.87 ±0.12	0.0600	<0.0001	0.0111
Type A body	1.17 ±0.07	1.37 ±0.12	1.23 ±0.08	1.50 ±0.09	1.57 ±0.09	1.47 ±0.09	*1.00 ±0.00	1.27 ±0.08	1.30 ±0.08	1.77 ±0.10	1.70 ±0.11	*1.90 ±0.09	0.0440	<0.0001	0.0082
Type B body	1.52 ±0.12	1.83 ±0.14	2.00 ±0.14	2.13 ±0.12	1.87 ±0.09	1.90 ±0.11	1.40 ±0.12	1.97 ±0.11	2.20 ±0.16	*2.53 ±0.10	*2.63 ±0.14	*2.80 ±0.18	<0.0001	<0.0001	0.0006
Aragonite	1.00 ±0.00	1.10 ±0.06	1.03 ±0.03	1.13 ±0.09	1.07 ±0.05	1.00 ±0.00	1.00 ±0.00	1.20 ±0.11	1.17 ±0.09	1.33 ±0.10	1.07 ±0.05	1.03 ±0.03	0.0381	0.0032	0.5660
Cubic	1.10 ±0.06	1.13 ±0.06	1.17 ±0.08	1.13 ±0.08	1.13 ±0.06	1.07 ±0.05	1.03 ±0.03	1.03 ±0.03	1.20 ±0.07	1.07 ±0.05	1.03 ±0.03	1.10 ±0.07	0.4327	0.1947	0.7456
Cubic cone formation	1.00 ±0.00	1.37 ±0.09	2.90 ±0.12	1.90 ±0.12	2.87 ±0.12	1.93 ±0.13	1.00 ±0.00	*2.10 ±0.15	*2.57 ±0.11	2.13 ±0.11	*2.40 ±0.13	2.13 ±0.13	0.3520	<0.0001	<0.0001
Cuffing	2.86 ±0.12	2.83 ±0.09	1.93 ±0.11	1.47 ±0.12	1.37 ±0.09	1.30 ±0.09	2.90 ±0.14	*2.13 ±0.14	1.97 ±0.11	1.27 ±0.08	1.17 ±0.07	1.20 ±0.07	<0.0001	0.0024	0.0069
Changed membrane	1.86 ±0.22	1.20 ±0.07	1.23 ±0.10	1.40 ±0.10	1.10 ±0.06	1.10 ±0.06	*2.63 ±0.17	*1.53 ±0.10	1.10 ±0.07	*1.00 ±0.00	1.17 ±0.08	1.00 ±0.00	0.1350	<0.0001	<0.0001
Depression	1.07 ±0.05	1.03 ±0.03	1.00 ±0.00	1.10 ±0.06	1.13 ±0.06	1.00 ±0.00	1.00 ±0.00	1.20 ±0.09	1.10 ±0.06	1.07 ±0.05	1.10 ±0.05	1.20 ±0.10	0.0854	0.5658	0.0577
Erosion	1.00 ±0.00	1.00 ±0.00	1.07 ±0.05	1.07 ±0.05	1.10 ±0.06	1.03 ±0.03	1.00 ±0.00	1.03 ±0.03	1.07 ±0.05	1.23 ±0.08	1.10 ±0.06	1.17 ±0.07	0.0393	0.0131	0.2626
Hole	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.03	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.03 ±0.00	1.03 ±0.00	1.00 ±0.03	1.00 ±0.00	1.00 ±0.00	0.5527	0.9999	0.3104

Mean ± SE; * Indicates significant difference from cage system at the same age; P- Production system; A- Age; P*A- Production system & Flock age interaction

Table 4: Bacterial load in cfu per eggshell of whole shell and shell crush

Variables	Flocks Age (weeks)												P Value		
	Conventional Cages						Free Range								
	25	35	45	55	65	75	25	35	45	55	65	75	P	A	P*A
TBC on eggshell	3.42 ±0.08	1.55 ±0.52	3.09 ±0.35	3.48 ±0.59	3.29 ±0.22	4.07 ±0.11	*3.81 ±0.12	*2.50 ±0.33	2.89 ±0.20	3.47 ±0.29	*3.79 ±0.15	*4.39 ±0.09	<0.0001	<0.0001	0.0012
TBC in shell crush	0.35 ±0.35	1.56 ±0.53	1.31 ±0.45	1.19 ±0.41	1.96 ±0.55	0.86 ±0.48	0.34 ±0.22	1.46 ±0.37	1.20 ±0.28	1.26 ±0.26	1.88 ±0.33	1.47 ±0.40	0.6112	0.0332	0.5984
TEC on eggshell	1.63 ±0.45	0.58 ±0.39	1.79 ±0.52	3.08 ±0.36	1.57 ±0.44	1.68 ±0.47	2.10 ±0.38	0.84 ±0.27	1.42 ±0.31	*2.06 ±0.35	*1.26 ±0.29	1.32 ±0.29	0.0671	0.0265	0.0133
TEC in shell crush	0.00 ±0.00	1.51 ±0.41	1.71 ±0.38	0.20 ±0.20	0.20 ±0.20	0.43 ±0.29	0.05 ±0.03	1.74 ±0.38	1.23 ±0.26	0.20 ±0.13	0.64 ±0.22	0.91 ±0.29	0.2111	<0.0001	0.0709

Mean ± SE

TBC- total bacterial count; TEC- total *Enterobacteriaceae* count; cfu is colony forming units;

* Indicates significant difference from cage system at the same age

P- Production system; A- Age; P*A- Production system & Flock age interaction

Chapter 11: CT scanning of eggshells to elucidate underlying causes of translucency (A. Ray Honours Thesis)

Abstract

The use of computed tomography to examine the phenomenon of translucency was investigated. Successive experiments determined the optimal settings for visualising the pores within the egg shell. A very high resolution was required to enable the pores to be seen clearly and to be categorised into straight, branching on the inside of the egg shell or branching on the external side of the egg shell. Doping of shells with iodine solutions did not improve the resolution. The most common pore type was straight pores passing directly through the egg shell. However, there was a correlation between a high incidence of translucency and a higher percentage of pores that branched towards the outside of the egg shell.

Introduction

Computed Tomography or CT has, to our knowledge, never been used to analyse egg shells. CT allows us to view high resolution cross sections of the shell. CT involves placing the sample between the X-ray source and a detector; this allows a 2D density map to be taken, the sample is then rotated and further density maps are taken. These multiple images are used by reconstruction software to produce a 3D image of the object (Hausherr *et al.,* 2006). CT is non destructive allowing the sample to be used again for further testing. CT is also a rapid process when compared to the preparations required for SEM.

CT allows us to artificially colour objects of different densities which allows better comparison of objects that are of similar densities. Discussing carbon compounds, Hausherr *et al.* (2006) comment how CT can be more effective then SEM when characterizing similar materials.

Samples for CT scanning may be exposed to an iodine “doping” agent; this solution would enter the specimen and invade the same spaces as water. However, due to the higher density of the iodine solution any areas invaded by the doping agent should be readily identifiable. This would, potentially, allow detailed measurements of pores and spaces within the palisade layer of the shell.

One limit of CT is that the resolution or maximum magnification is linked to the size of the object being imaged, the larger the sample size the lower the resolution, providing lower magnification.

Methods

A GE Phoenix CT scanner was used in addition to the Neoscope JM-5000 SEM to investigate shell structure in relation to translucency. Shell fragments were broken by hand into approximately 5cm square samples and the membrane was removed manually by rubbing under running water. After an initial scan, translucent features were marked on the outside of the shell sample using a Dremel tool and small engraving attachment.
The initial scans were completed using the following settings,

Phoenix GM V-tomex Standard tube 80 kV
X-ray: 80 kV
Microamps: 130
Modus: 1
Images per scan: 800
Timing: 800 ms
Voxel size (resolution): 35.27 microns
Power: 14.4; 0.86 watts
Filter: 0.5 Cu

Analysis of the images involved reconstituting the individual X-ray images into a 3D figure. To standardize the individual samples, a 5mm sphere was isolated from each image, and the greyscale histogram of each image was standardized to a similar pattern to produce the best view of the pores. In order to produce a volume measure, a region of shell was selected with a tolerance of 2000 units of variance. These scans provided a good starting point; however they proved to be of limited use due to their low resolution. In subsequent CT scans, higher resolution scans and doping agents were investigated.

The initial CT studies failed to show any internal structures of the shell; in order to optimise the results of iodine doping the nanotube X-ray tube was used to achieve a higher resolution. The following CT settings where used.

Phoenix GM V-tomex Nano tube 180 kV
X-ray: 80 kV
Microamps: 180
Modus: 1
Images per scan: 800
Timing: 800 ms
Voxel size (resolution): 5.64 microns
Power: 14.4; 0.86 watts
Magnification: 70.974

And

Phoenix GM V-tomex standard tube 180 kV
X-ray: 80 kV
Microamps: 130
Modus: 0
Images per scan: 800
Timing: 800 ms
Voxel size (resolution): 35.27 microns
Power: 14.4; 0.86 watts
Magnification: 11.342

Initial CT studies provided low resolution images that were inadequate to determine the internal structures of the shell. In order to view the mammillary bodies and the internal pores, samples were scanned at a higher resolution. In order to scan samples at a higher resolution, the sample size had to be reduced. In order to achieve a resolution of ~5 um the sample size was reduced to ~5 mm/~5 mm square. Samples were scanned using the two available X-ray tubes. The following scan settings were used.

Phoenix GM V-tomex Nano tube 180 kV
X-ray: 80 kV
Microamps: 180
Modus: 1
Images per scan: 800

Timing: 800 ms
Voxel size (resolution): 5.64 microns
Power: 14.4; 0.86 watts
Magnification: 70.974

And

Phoenix GM V-tomex standard tube 180 kV
X-ray: 80 kV
Microamps: 130
Modus: 0
Images per scan: 800
Timing: 800 ms
Voxel size (resolution): 35.27 microns
Power: 14.4; 0.86 watts
Magnification: 11.342

In order to standardize the area observed for all samples, a 1 mm radius sphere was isolated and digitally extracted. Volume of the shell within the 1mm radius sphere was recorded using the volume metric within the software and a tolerance of 2000 units of variance. The mammillary layer of the shell was examined for any distinctive features which were then recorded. The number and type of pore (non-branching, internally-branching or externally-branching) were recorded on the XY axis. 3D reconstruction was conducted with VG Studio Max Version2.0.

Results

The initial CT scans produced images of insufficient resolution to visualize the internal structures of the shell. Pore counts were attempted, however reliable results were not achieved. Figure 1 shows a low resolution CT image. Doped CT samples failed to provide increased visibility of pores and internal structures than the high resolution CT studies. The Doped CT scans were not used for analysis. Figure 2 shows a comparison between a doped CT image, a low resolution CT scan and a high resolution CT scan. The high resolution CT scans allowed visualization of both the mammillary bodies and the internal structures of the shell. The high resolution CT scans revealed no apparent structural cause for translucency.

Pore counts were possible in these high resolution scans and their branching status was visible. Pore count averages are shown in Table 1 and Figure 4. Eggs with translucency score of 1 showed 57 straight pores per 3.14 mm^2 from 13 samples with an average of 4.38 pores per 3.14 mm^2. Translucency score 3 showed 40 pores per 3.14 mm^2 from 7 samples with an average of 5.71 pores per 3.14 mm^2 and translucency score 4 showed 66 pores per 3.14 mm^2 from 12 samples with an average of 5.5 pores per 3.14 mm^2. Straight pore counts per unit area were statistically different between translucency score groups.

Externally branching pores were found to be more common in the translucency score 4 group with an average of 1.917 externally branching pores per 3.14 mm^2. The translucency score 3 group had an average of 0.429 externally branching pores per 3.14 mm^2 and the one translucency score group had an average of 0.091 externally branching pores per 3.14 mm^2 . The result for the translucency score 4 group was found to be significantly different from the other groups for externally branching pores per 3.14 mm^2.

The internally branching pore counts were similar for all groups, with no statistically significant differences among translucency score categories. The translucency score 1 group had an average internally branching pore count of 0.820 pores per 3.14 mm^2, the translucency score 3 group had an average internally branching pore count of 0.787 pores per 3.14 mm^2 and

the translucency score 4 group had an average internally branching pore count of 0.674 pores per 3.14 mm^2.

The volume measurement completed using VG Studio Max Version2.0 showed increasing volume with increasing translucency score (Figure 5). The average volume, of translucency score group 1 eggs was 0.893 mm^3. The average volume of translucency score 3 group eggs was 0.903 mm^3. And the average volume of translucency score four group eggs was 0.954 mm^3. The difference in volume between the three scores was not significant.

Discussion

The initial CT images were a starting point to determine the settings that needed to be used for studying egg shell structure; however the results from these scans were not suitable for identifying which if any internal structures were related to the appearance of translucency. A second attempt involved marking out translucency features with a Dremel engraving tool to allow their identification through the CT; however, this failed to show anything significant. In another preliminary experiment, samples were divided on the basis of their 'type' of translucency (pole, region & feature). In this experiment and previous experiments there was no difference between the groups and in future experiments the translucency score was used to divide groups.

With the addition of a “doping” agent came hopes of viewing the precise location of water within the shell; however the doped CT samples were not as clear as the un-doped samples in the high resolution CT study. While only a small number of CT samples were doped with iodine doping agents, it was clear that there was no advantage in doping shell samples. The doped samples were of lower resolution and provided lower visibility of internal structures then the non-doped nano-tube scanned shell samples. There were two iodine doping solutions prepared and both produced similar results. Samples where allowed 48-72 hours to soak in two different iodine solutions, the 48-72 hour timeline was used to simulate the saturation time of shells to show full translucent features. This provided enough time for the iodine solution to fully enter the gaps in the shell that are occupied by water in a translucent shell.

As the results of the CT are dependent on the relative difference in the electron density of adjacent objects, a doping agent must increase this relative difference. The objects in an un-doped sample are air and the shell calcium carbonate. When a sample doped with iodine solution is used, the relative objects become shell calcium and iodine which occupies the gaps within the shell. If a doping agent was required for future CT images it should be of a far greater electron density then iodine. It appeared that the air present in the pores of the egg shell provided a better contrast with the calcium carbonate of the egg shell than did the iodine doping agents.

High resolution CT scans provided much greater image quality and resolution than previous scans. Images from the CT nano-tube were used for analysis as they provided better quality images and greater magnification than the standard X-ray tube. While the higher resolution scans do limit the size of the sample area being examined, the advantages here outweigh the negatives. When examining the mammillary bodies or internal structures of the shell, the area viewable (5 mm^2) is adequate, even when that area is further reduced to ensure a standard area for all samples. One mm spheres were used in this experiment and they contained a sufficient number of mammillary bodies and pores for analysis. The nature of CT allows two-dimensional frame by frame analysis of the internal structures of the shell. It is using these transverse cuts though the shell that allowed the identification of pores and their branching structures.

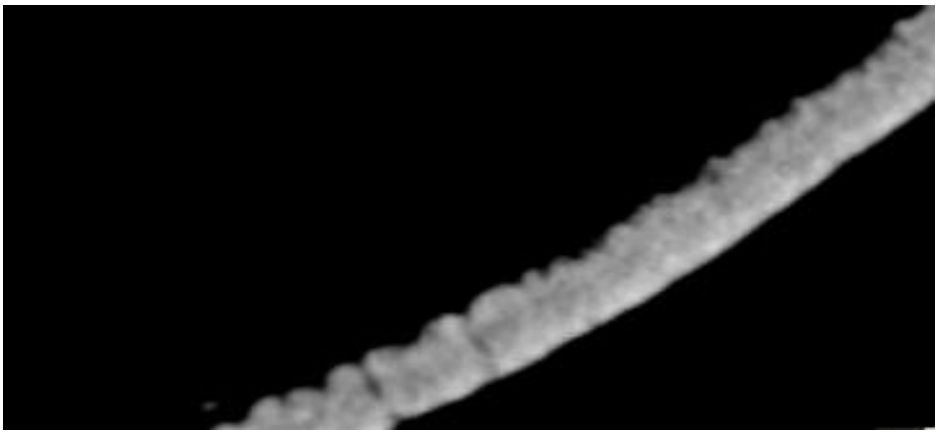


Figure 1: Low resolution CT scan showing possible pore structures.

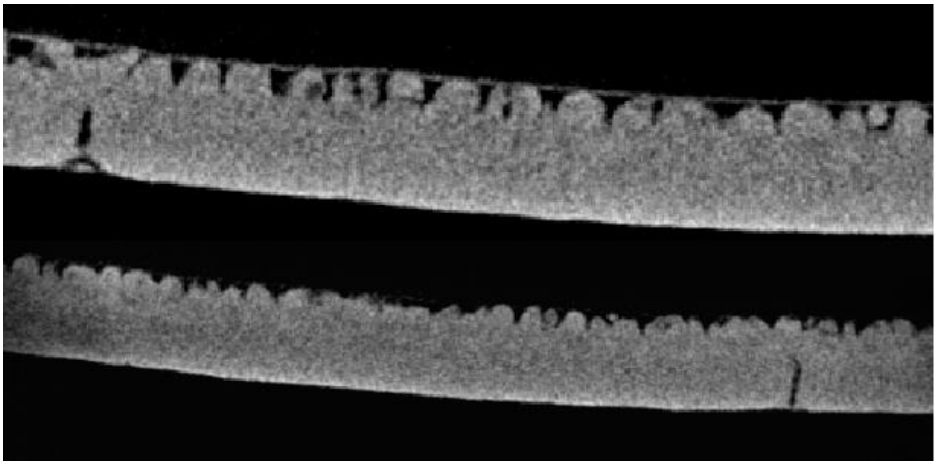


Figure 2: Doped high resolution CT scans, (top IKI, bottom I2E).

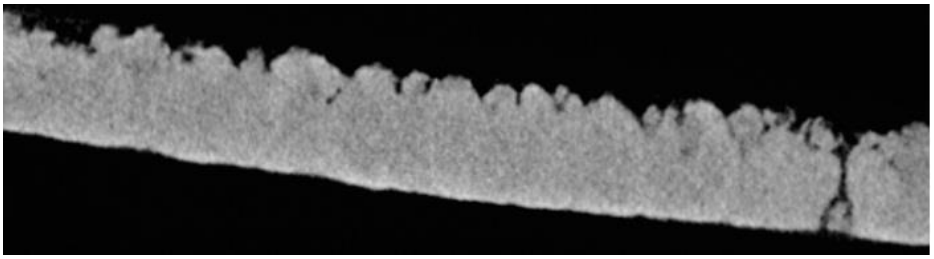


Figure 3: High resolution CT scan showing externally branching pore.

Table 1: Pore counts and volume measurements for translucency score groups one, three and four. Values with different superscripts are significantly different

Translucency Score Group	Average number of Pores per sample			Volume (mm3) per 5mm sphere
	Straight	Internal	External	
1	[a]4.55	0.55	[b]0.09	0.89
3	[b]5.86	0.57	[b]0.43	0.90
4	[c]3.08	0.50	[a]1.92	0.95

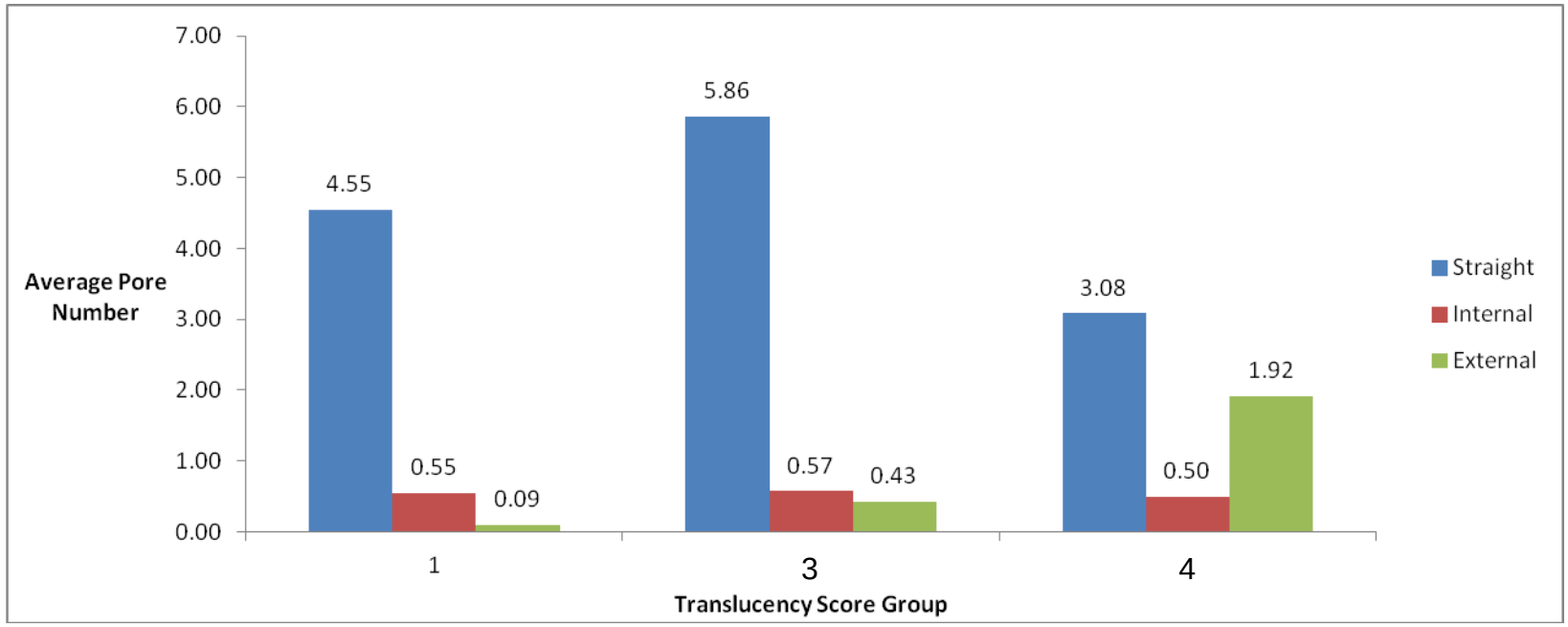


Figure 4: Average pore counts for translucency score groups one, three and four, divided into straight, internally branching and externally branching pores.

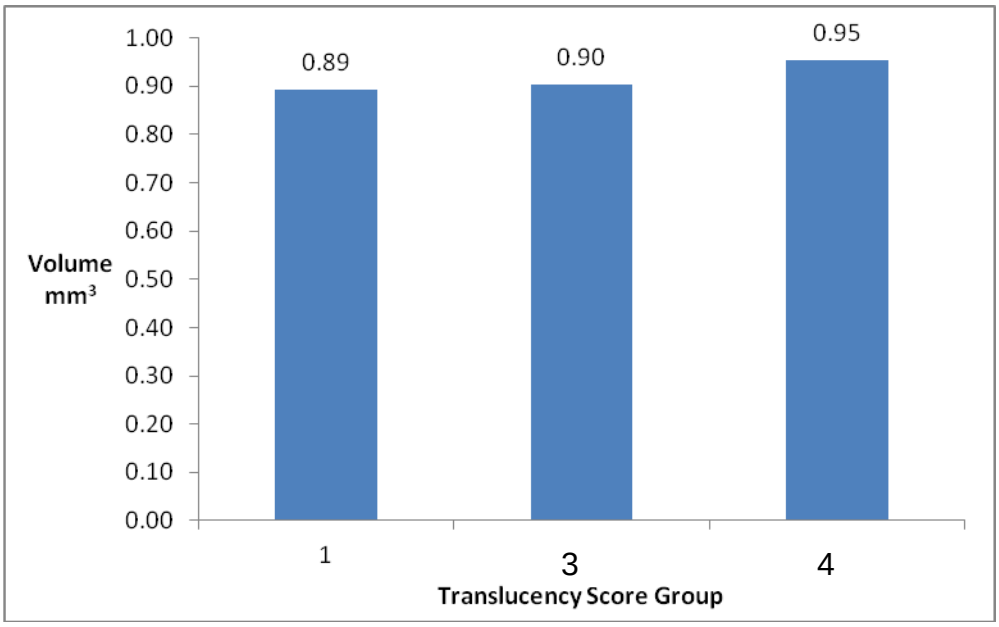


Figure 5: Bar graph showing volume of shell (mm^3) of a 4.19 mm^3 sphere (1 mm radius) of shell from translucency score groups one, three and four.

In all translucency score groups, the number of internally branching pores remained similar as did the total number of normal (un-branched) pores. However, as the translucency score increased, the number of externally branching pores increased. The difference in externally branching pores was found to be significantly higher in the translucency score 4 group, although not all samples in this group featured externally branching pores. It is possible that not all pores were visible with the high resolution CT; some pores may not have been counted because of their small size or direction. The nature of the transverse cuts may prevent viewing of pores that are moving perpendicular to the field of view.

The identification of differently branching pores is not consistent with the work of Tullet (1978), who states that, in domestic fowl, the pores transverse the shell radially without branching. Tullet (1978) may not have observed internally or externally branching pores due to the method used. In order to view pore structures through SEM, shells must be randomly broken and a single cut is viewed per sample. In all groups in the present study, there are far more normal (unbranched) pores than branching pores. Also, the branches of the internally and externally branching pores were, in some cases, more difficult to observe indicating smaller structures. It was the variation in the pore counts between translucency groups that led to the investigation of the relationship between conductance, direct pore counts and translucency.

The volume of shell measure is the volume of calcium carbonate in a digitally extracted region. The volume present in the analyzed area showed a consistent increase with translucency score. Although the difference between groups was small and not statistically significant, the trend observed may indicate a relationship. The accuracy of the volume measurement may also be questioned as the resolution may not have been high enough to fully capture the intricate shell structure. There have been no previous measures with which to compare these findings. However, in the future, increasing the size of the sample groups may provide more reliable results. In the future, higher resolution results may be possible using a synchrotron.

General Discussion

Eggs will continue to be viewed as a risk for human *Salmonella* infections, as evidenced by the recent OzFoodNet Working Group reports (2009, 2010).

Ensuring good egg quality is a first line of defence against movement of bacteria into eggs. The results of the current study confirm previous reports that egg shell quality (as measured by traditional quality measures as well as shell ultrastructure) and egg internal quality decline with increasing flock age. However, the extent of cuticle cover, which is widely accepted as being of significance in preventing bacterial ingress into the egg did not vary significantly across the laying life of commercial flocks. The use of cuticle blue dye was verified by scanning electron microscopy as being a reliable indicator of the extent of cuticle cover on egg shells. This provides a simple means by which cuticle cover can be visualized by commercial egg producers.

The enumeration of total bacteria on and in eggs and the enumeration and identification of *Enterobacteriaceae* associated with eggs indicated that the levels of bacteria on the outside of commercial eggs in Australia compare favourably with those from other developed countries such as the United States and Europe. Fortunately, in Australia the serovar which is most commonly involved in vertical transmission, *Salmonella* Enteritidis, is not endemic in commerical layer flocks. However, the presence of bacteria on the outside of eggs poses a threat to consumers as these bacteria can contaminate eggs during the food preparation process. The horizontal study (different flocks at different ages - Chapter 3) found that there was no difference in the levels of *Enterobacteriaceae* among different flock ages. However, the vertical study (same flock sampled at different ages) on one cage and one free range flock reported in Chapter 10 found that there were some differences with flock age for the free range flock. This suggests that monitoring of free range flocks may be even more important than for cage flocks, particularly if the egg collection system does not separate eggs quickly from the birds and/or permits contact between eggs and contaminated surfaces.

The identification of bacteria reported in Chapter 4 indicated that the most prevalent serovar of *Salmonella* on eggs was *Salmonella* Infantis, consistent with previous reports from Australia and overseas *Salmonella* Infantis has been demonstrated to cause food-borne illness in humans and therefore needs to be monitored in Australia layer flocks. Also the sample size used for this study was small and hence a nation-wide prevalence study to investigate *Salmonella* transmission (bird-environment-egg movements) is required. The *Salmonella* transmission from bird-environment-egg in cage farm is currently being investigated through another Poultry CRC project.

The studies on the prevalence of *Mycoplasma synoviae* (MS) in layer flocks was estimated by measuring antibodies in egg yolk. As shown in Chapter 5, the prevalence of antibodies for MS is variable across different farms but can be quite high. However further experimental studies are essential to study the possible effects of Australian strains of *Mycoplasma synoviae* on egg quality and production.

Several studies were conducted into the ability of different serovars of *Salmonella* to penetrate the egg shell of both washed and unwashed eggs. Chapter 6 reports on *Salmonella* Infantis, Chapter 7 on various *Salmonella* Typhimurium phage types and Chapter 8 reports on *Salmonella* Agona. Egg washing, as conducted in these studies, appeared to result in removal of the cuticle. For *S.* Infantis, there was no difference in the incidence of bacterial penetration of washed and unwashed eggs and this serovar was not isolated from egg contents of either washed or unwashed eggs. In contrast, the phage types of *S.* Typhimurium were all able to penetrate the shell of both washed and unwashed eggs with the incidence of penetration of washed eggs being higher than that of unwashed eggs. *S.* Typhimurium was also able to survive in eggs (externally and internally) incubated at 20°C. *S.* Agona showed some capacity for penetration of eggshells although there was no difference between washed and unwashed whole eggs for the incidence of penetration. However, for the agar eggs, unwashed eggs were penetrated more often than washed eggs.

For all these penetration studies, there were correlations between the incidence of penetration or points at which penetration occurred and egg shell features (ultrastructure, cuticle cover) which are associated with poorer quality egg shells.

The project investigated a number of potential causes of egg shell translucency. There were correlations between translucency score and some ultrastructural features of the mammillary layer which are usually associated with poorer quality shells. A study using CT scanning found that there appears to be a correlation between translucency score and the incidence of egg shell pores that branch towards the outside of the shell. Eggs with higher translucency score also tended to have higher porosity.

A small study was conducted to evaluate the incidence of microcracks before and after commercial egg processing. Microcracks almost certainly contribute to the appearance of translucency. However, they are difficult to identify with certainty. Following commercial egg processing the incidence of microcracks was significantly lower than before processing. This indicates that the commercial egg processing crack detectors are identifying and removing some of the eggs with microcracks.

Implications

The results of this series of studies indicate that egg shell quality is important in determining the ease with which bacteria are able to pass across egg shells. Bacteria are always present on the outside (and to a lesser extent in the pores) of egg shells and there is a risk that bacteria will enter the egg contents either by passing through the shell (via pores or shell defects) or during handling of eggs in domestic or commercial food preparation. The fact that eggs are regularly consumed either raw or undercooked, adds to the potential risk. The findings from the limited range of serovars and phage types of *Salmonella* tested in this series of studies suggests that some serovars and phage types penetrate the egg shell more efficiently than others. A better understanding of this process would inform the management of food safety in the egg industry. The underlying causes of egg shell translucency are still being investigated, supported by a Poultry CRC Postgraduate Scholarship. However, some causes have been identified. A high incidence of translucency, along with poor shell quality, appears to increase the risk of bacterial ingress into the egg.

Recommendations

The investigators recommend that the Australian Egg Industry continues to support research, development and extension in the area of food safety, with *Salmonella* serovars and phage types being the main target. The Australian Egg Industry needs to proactive, and to be seen to be proactive in addressing risks of food borne disease associated with eggs.

Extension activities arising from the outcomes of this sub-project could include seminars and workshops at which the issues could be addressed. This would be particularly helpful if the findings of Dr. Margaret Sexton's egg washing project (Sub-project 3.2.1) were also included.

Acknowledgements

All studies reported here were funded by the Australian Poultry CRC, established and supported under the Australia Government's Cooperative Research Centres Program. We thank the University of New England, Charles Sturt University and the University of Adelaide for their support for the studies. The assistance of Mr Rowly Horn is gratefully acknowledged. The support and cooperation of members of the Australian Egg Industry was invaluable to the sub-project and is very much appreciated.

References

Abate G, Qureshi S, Mazumder SA (2011) *Cedecea davisae* bacteremia in a neutropenic patient with acute myeloid leukemia. *Journal of Infection* **63**, 83-85.

Abrahamsson P, Tauson R (1998) Performance and egg quality of laying hens in an aviary system. *Journal of Applied Poultry Research* **7,** 225-232.

AECL Annual Report 2010/2011. Retrieved from http://www.aecl.org/system/attachments/484/original/Annual%20Report%202010.2011.pdf?1321843838.

AECL Annual Report 2011/2012. Retrieved from http://www.aecl.org/system/attachments/583/original/Annual%20Report%202012.pdf?1353999180.

Alls AA, Cover MS, Benton WJ, Krauss WC (1964) Treatment of hatching eggs for disease prevention - factors affecting permeability and a visual inspection of drug absorption. *Avian Diseases* **8,** 245-246.

Bain MM, MacLeod N, Thomson R, Hancock JW (2006) Microcracks in eggs. *Poultry Science* **85,** 2001-2008.

Baker RC (1990) Survival of *Salmonella* enteritidis on and in shelled eggs, liquid eggs and cooked egg products. *Dairy, Food and Environ Sanitation* **10**, 273-275.

Barnhart HM, Dreesen DW, Bastien R, Pancorbo OC (1991) Prevalence of *Salmonella* Enteritidis and other serovars in ovaries of layer hens at time of slaughter. *Journal of Food Protection* **54,** 488-491.

Barrow PA, Lovell MA (1991) Experimental infection of egg-laying hens with *Salmonella* Enteritidis phage type 4. *Avian Pathology* **20,** 335-348.

Banga-Mboko H, Mabas JS, Adzona PP (2010) Effect of housing system (battery cages versus floor pen) on performance of laying hens under tropical conditions in Congo Brazaaaville. *Research Journal of Poultry Science* **3,** 1-4.

Berrang ME, Frank JF, Buhr RJ, Bailey JS, Cox NA, Mauldin J (1998) Eggshell characteristics and penetration by *Salmonella* through the productive life of a broiler breeder flock*. Poultry Science* **77,** 1446–1450.

Berrang ME, Cox NA, Frank JF, Buhr RJ (1999) Bacterial penetration of the eggshell and shell membranes of the chicken hatching egg: A Review. *Journal of Applied Poultry Research* **8,** 499-504.

Board RG (1966) The course of microbial infection of the hen's egg. *Journal of Applied Microbiology* **29,** 319-341.

Board RG, Halls NA (1973) The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *British Poultry Science* **14,** 69-97.

Board RG, Tranter HS (1995) The microbiology of eggs. In: Stadelman WJ, Cotterill OJ, editors. Egg science and technology. Food Products Press, NY. pp. 81-103.

Botey-Saló P, Anyogu A, Varnam AH, Sutherland JP (2012) Survival of inoculated Salmonella on the shell of hens' eggs and its potential significance. *Food Control* **28**, 463-469.

Braun P, Fehlhaber K, Wicke A (1999) *Salmonella* enteritidis invades the egg through the shell. *World Poultry Special*: 23-24.

Chousalkar KK, Flynn P, Sutherland M, Roberts JR, Cheetham BF (2010) Recovery of *Salmonella* and *Escherichia coli* from commercial shell eggs and effect of translucency on bacterial penetration in eggs. *International Journal of Food Microbiology* **142,** 207-213

Coburn B, Grass GA, Finlay BB (2007) *Salmonella*, the host and disease: a brief review. *Immunology and Cell Biology* **85,** 112-118.

Courtney S, Mossoba ME, Hammack TS, Keys C, Al-Khaldi SF (2006) Using PCR amplification to increase the confidence level of Salmonella Typhimurium DNA microarray chip hybridization. *Molecular and Cellular Probes* **20,** 163-171.

Cox NA, Davis BH, Watts AB, Colmer AR (1973) Salmonella in the laying hen. *Poultry Science* **52,** 661-666.

Cox JM, Woolcock JB, Sartor AL (2002) The significance of *Salmonella*, particularly *Salmonella* Infantis, to the Australian egg industry. Report for the Rural Industries Research and Development Corporation, Australia. Retrieved from: http://www.aecl.org/assets/www.aecl.org/outputs/UQ-19EA-Final-Report.pdf

Davies RH, Breslin M (2003) Investigation of *Salmonella* contamination and disinfection in farm egg-packing plants. *Journal of Applied Microbiology* **94,** 191-196.

Daughtry B, Sumner J, Hooper G, Thomas C, Grimes T, Horn R, Moses A, Pointon A (2005) National food safety risk profile of eggs and egg products. A report submitted to Australian Egg Corporation Limited, Sydney, Australia.

De Reu K, Grijspeerdt K, Heyndrickx M, Zoons J, De Baere K, Uyttendaele M, Debevere J, Herman L (2005) Bacterial eggshell contamination in conventional cages, furnished cages and aviary housing systems for laying hens. *British Poultry Science* **46,** 149-155.

De Reu K, Grijspeerdt K, Messens W, Heyndrickx M, Uyttendaele M, Debevere J, Herman L (2006) Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including Salmonella Enteritidis. *International Journal of Food Microbiology* **112,** 253-260.

De Reu K, Messens W, Heyndrickx M, Rodenburg TB, M. Uyttendaele M, Herman L (2008) Bacterial contamination of table eggs and the influence of housing systems. *World's Poultry Science Journal* **64,** 5-19.

De Reu K, Rodenburg TB, Grijspeerdt K, Messens W, Heyndrickx M, Tuyttens FAM, Sonck B, Zoons J, Herman L (2009) Bacteriological contamination, dirt, and cracks of eggshells in furnished cages and noncage systems for laying hens: An international on-farm comparison. *Poultry Science* **88,** 2442-2448.

Dominquez-Vera J M, Gautron J, Garcia-Ruiz J M, Nys Y (2000) The effect of avian uterine fluid on the growth behaviour of calcite crystals. *Poultry Science* **79,** 901-907.

Dukic-Stojcic M, Peric L, Bjedov S, Milosevic N (2009) The quality of table eggs produced in different housing systems. *Biotechnology in Animal Husbandry* **25,** 1103-1108.

Dunn I (2011) Poultry breeding for egg quality: traditional and modern genetic approaches. In 'Improving the safety and quality of eggs and egg products' Vol 1. (Eds Y. Nys, M. Bain, F. Van Immerseel) pp. 245-260. (Woodhead Publishing Limited: Cambridge, U.K.).

Dunn IC, Rodriguez-Navarro AB, Mcdade K, Preisinger R, Waddington D, Wilson PW, Bain MM (2011) Genetic variation in eggshell crystal size and orientation is large and these traits are correlated with shell thickness and are associated with eggshell matrix protein markers. *Animal Genetics* **43,** 410-418.

European Food Safety Authority (2005) Opinion of the scientific panel on biological hazards on the request from the commission related to the microbiological risks on washing of table eggs. *European Food Safety Authority Journal* **269,** 1-39. Retrieved from: http://www.efsa.europa.eu/en/efsajournal/doc/269.pdf.

Feberwee A, Morrow CJ, Ghorashi SA, Noormohammadi A H, Landman WJ (2009a) Effect of a live *Mycoplasma synoviae* vaccine on the production of eggshell apex abnormalities induced by a *M. synoviae* infection preceded by an infection with infectious bronchitis virus D1466. *Avian Pathology* **38,** 333-340.

Feberwee A, De Wit JJ, Landman WJ (2009b) Induction of eggshell apex abnormalities by *Mycoplasma synoviae*: field and experimental studies. *Avian Pathology* **38,** 77-85.

Foley S, Lynne A, Nayak (2008) Salmonella challenges: Prevalence in swine and poultry and potential pathogenicity of such isolates. *Journal of Animal Science*. **86,** 149-162.

Gassama-Sow A, Wane AA, Canu NA, Uzzau S, Kane AA, Rubino S (2006) Characterization of virulence factors in the newly described *Salmonella enterica* serotype Keurmassar emerging in Senegal (sub-Saharan Africa). *Epidemiology and Infection* **134,** 741-743.

Gast RK, Guraya R, Guard-Bouldin J, Holt PS (2007) In vitro penetration of egg yolks by *Salmonella* Enteritidis and *Salmonella* Heidelberg strains during thirty six hour ambient temperature storage. *Poultry Science* **86,** 1431-1435.

Gautron J, Hincke MT, Nys Y (1997) Precursor matrix proteins in the uterine fluid change with stages of eggshell formation in hens. *Connective Tissue Research* **36,** 195-210.

Gautron J, Bain M, Solomon S, Nys Y (1996) Soluble matrix of hen's eggshell extracts changes *in vitro* the rate of calcium carbonate precipitation and crystal morphology. *British Poultry Science* **37,** 853-866.

Gentry R F, Quarles CL (1972) The measurement of bacterial contamination on eggshells. *Poultry Science* **51,** 930-933.

Gilmore MS, Ferretti JJ (2003) The thin line between gut commensal and pathogen. *Science*. **299,** 1999–2002.

Greiner M, Sohr D, Gobel P (1995) A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. *Journal of Immunological Methods* **185,** 123-132.

Guesdon V, Faure JM (2004) Laying performance and egg quality in hens kept in standard or furnished cages. *Animal Research* **53,** 45-57.

Hagan JC, Ashton NJ, Bradbury JM, Morgan KL (2004) Evaluation of an egg yolk enzyme-linked immunosorbent assay antibody test and its use to assess the prevalence of Mycoplasma synoviae in UK laying hens. *Avian Pathology* **33,** 93-97.

Haigh T, Betts WB (1991) Microbial barrier properties of hen eggshells. *Microbios* **68,** 137-146.

Hall G, Kirk MD, Becker N, Gregory JE, Unicomb L, Millard G, Stafford R, Lalor K, the OzFoodNet Working Group (2005) Estimating Foodborne Gastroenteritis, Australia. *Emerging Infectious Disease* **11,** 1257-1264.

Hardy A (2004) Salmonella: a continuing problem. *Postgraduate Medical Journal* **80,** 541-545.

Harry EG (1963) The relationship between egg spoilage and the environment of the egg when laid. *British Poultry Science* **4,** 91-100

Hausherr, J., Fischer, F., Krenkel, W., Altstädt, V. (2006). Material characterisation of C/SiC: comparison of computed-tomography and scanning electron microscopy. Conference on Damage in Composite Materials 2006, September, Stuttgart, Germany. Retrieved from: http://www.ndt.net/article/cdcm2006/papers/hausherr.pdf

Hensel M (2004) Evolution of pathogenicity islands of Salmonella enterica. International *Journal of Medical Microbiology* **294,** 95-102.

Hess B, Burchett A, Huntington MK (2008) *Leclercia adecarboxylata* in an immunocompetent patient. *Journal of Medical Microbiology* **57,** 896-898.

Hidalgo AM, Rossi M, Clerici F, Ratti SM (2008) A market study on the quality characteristics of eggs from different housing systems. *Food Chemistry* **106,** 1031–1038.

Hinke MT, Gautron J, Mann K, Panheleux M, McKee M D, Bain M, Solomon SE, Nys Y (2003) Purification of ovocalyxin- 32, a novel chicken eggshell matrix protein. *Connective Tissue Research* **44,** 16-19.

Hinz KH, Legutko P, Schroeter A, Lehmacher W, Hartung M (1996) Prevalence of motile salmonellae in egg-laying hens at the end of the laying period. *Journal of Veterinary Medicine* **43,** 23-33.

Holst WF, Almquist HJ, Lorenz FW (1932).A study of shell texture of the hen's egg. *Poultry Science* ***11*****,** 144-149.

Hughes LA, Shopland S, Wigley P, Bradon H, Leatherbarrow AH, Williams NJ, Bennett M, Pinna ED, Lawson B, Cunningham AA, Chantrey J (2008) Characterisation of *Salmonella enterica* serotype Typhimurium isolates from wild birds in northern England from 2005 – 2006. *BMC Veterinary Research* **4,** 4-14.

Hughey VL, Johnson EA (1987) Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Applied and Environmental Microbiology* **53,** 2165-2170.

Humphrey TJ (1994) Contamination of eggshell and contents with *Salmonella* Enteritidis: A review. *International Journal of Food Microbiology* **21,** 31-40.

Huneau-Salaün A, Michel V, Huonnic D, Balaine L, Le Bouquin S (2010) Factors influencing bacterial eggshell contamination in conventional cages, furnished cages and free-range systems for laying hens under commercial conditions. *British Poultry Science* **51,** 163-169.

Hutchison ML, Gittins J, Sparks AW, Humphrey TJ, Burton C, Moore A (2004) An assessment of the microbiological risks involved with egg washing under commercial conditions. *Journal of Food Protection* **67,** 4-11.

Ibrahim HR, Sugimoto Y, Aoki T (2000) Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochimica et Biophysica Acta* (BBA) - General Subjects **1523,** 196-205.

Jones DR, Musgrove MT, Northcutt JK (2004) Variations in external and internal microbial populations in shell eggs during extended storage. *Journal of Food Protection* **67,** 2657-2660.

Jones DR, Musgrove MT (2007) Pathogen prevalence and microbial levels associated with restricted shell eggs. *Journal of Food Protection* **70,** 2004-2007.

Jones FT, Rives DV, Carey JB (1995) Salmonella contamination in commercial eggs and an egg production facility. *Poultry Science* **74,** 753-757.

Jones MA, Hulme SD, Barrow PA, Wigley P (2007) The Salmonella pathogenicity island 1 and Salmonella pathogenicity island 2 type III secretion systems play a major role in pathogenesis of systemic disease and gastrointestinal tract colonization of *Salmonella* enterica serovar Typhimurium in the chicken. *Avian Pathology* **36,** 199-203.

Jordan FTW (1975) Avian mycoplasma and pathogenicity- A review. *Avian Pathology* **4,** 165-174.

Kapetanov M, Orlic D, Potkonjak D, Velhner M (2010) Mycoplasma in poultry flocks in the year 2009 compared to the year 2000 and significance of the control measures in Serbia. *Lucrări Ştiiniifice Medicină Veterinără* **18,** 249-253.

Keller LH, Benson CE, Krotec K, Eckroade RJ (1995) *Salmonella* Enteritidis colonization of the reproductive tract and forming and freshly laid eggs of chickens. *Infection and Immunity* **63,** 2443-2449.

Kleven SH, Bradbury JM (2008) Avian mycoplasmosis (mycoplasma gallisepticum, M. synoviae). In: OIE Standards Commission eds. OIE manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). *Office International des Epizooties*, Paris, 482-496.

Kornacki JL, Johnson JL (2001) *Enterobacteriaceae*, coliforms and *Escherichia coli* as quality and safety indicators. Pages 69–80 in Compendium of Methods for the Microbiological Examination of Foods. 4th ed. F. P. Downes and K. Ito,ed. Am. Public Health Assoc., Washington, DC.

Leleu S, Messens W, De Reu K, De Preter S, Herman S, Heyndrickx M, DE Baerdemaeker J, Michiels CW, Bain M (2011) Effect of egg washing on the cuticle quality of brown and white table eggs. *Journal of Food Protection,* **74,** 1649–1654.

Lichovnikova M, Zeman L (2008) Effect of housing system on the calcium requirements of laying hen and eggshell quality. *Czech Journal of Animal Science* **53,** 162-168.

Lierz M, Hagen N, Harcourt-Brown N, Hernandez-Divers SJ, Luschow D, Hafez HM (2007) Prevalence of mycoplasmas in eggs from birds of prey using culture and a genus-specific mycoplasma polymerase chain reaction. *Avian Pathology* **36,** 145-150.

Lostroh CP, Lee CA (2001) The Salmonella pathogenicity island-1 type III secretion system. *Microbes and Infection* **3,** 1281-1291.

Lott BD, Drott JH, Vardhman TH, Reece FN (1978) Effect of *Mycoplasma synoviae* on egg quality and egg production of broiler breeders. *Poultry Science* **57,** 309-311.

Lyons SR, Griffen AL, Leys EJ (2000) Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. *Journal of Clinical Microbiology* **38,** 2362–2365.

Macowan KJ, Atkinson MJ, Bell MA, Brand TF, Randall CJ (1984) Egg transmission of a respiratory isolate of *Mycoplasma synoviae* and infection of the chicken embryo. *Avian Pathology* **13,** 51-58.

Malik MW, Ayub N, Qureshi IZ (2006) Passive immunization using purified IgYs against infectious bursal disease of chickens in Pakistan. *Journal of Veterinary Science* **7,** 43-46.

Mallet S, Guesdon V, Ahmed AMH, Nys Y (2006) Comparison of eggshell hygiene in two housing systems: Standard and furnished cages. *British Poultry Science* **47,** 30-35.

Marcus SL, Brumell JH, Pfeifer CG, Finlay BB (2000) Salmonella pathogenicity islands: big virulence in small packages. *Microbes and Infection* **2,** 145-156.

May D, Sexton M, Holds G, Kiermeier A (2013) Egg washing- Trials and tribulations. *Proceedings of the Australian Poultry Science Symposium* **24,** 256-259.

Mayes FJ, Takeballi MA (1983) Microbial contamination of the hen egg: a review. *Journal of Food Protection* **46,** 1092-1098.

Messens W, Duboccage L, Grijspeerdt K, Heyndrickx M, Herman L (2004) Growth of *Salmonella* serovars in hens' egg albumen as affected by storage prior to inoculation. *Food Microbiology*, 21, 25-32.

Messens W, Gittins J, Leleu S, Sparks N (2011) Egg decontamination by egg washing. In: Nys Y, Bain M, Van Immerseel, F. Editors. Improving the safety and quality of eggs and egg products. Woodhead Publishing Limited Cambridge UK. pp. 163-180.

Messens W, Grijspeerdt K, Herman L (2005a) Eggshell penetration by *Salmonella*: A review. *World's Poultry Science Journal* **61,** 71–84.

Messens W, Grijspeerdt K, Herman L (2005b) Eggshell characteristics and penetration by *Salmonella enterica* serovar Enteritidis through the production period of a layer flock. *British Poultry Science* **46,** 694-700.

Messens W, Grijspeerdt K, De Reu K, De Ketelaere B, Mertens K, Bamelis F, Kemps B, De Baerdemaeker J, Decuypere E, Herman L (2007) Eggshell penetration of various types of hens eggs by *Salmonella* enterica serovar Enteritidis. *Journal of Food Protection* **70,** 623-628.

Mills AD, Nys Y, Gautron J, Zawadski J (1991) Whitening of brown shelled eggs: individual variation and relationships with age, fearfulness, oviposition interval and stress. *British Poultry Science* **32,** 117-129.

Miyamoto T, Horie T, Baba E, Sasai K, Fukata T, Arakawa A (1998) *Salmonella* penetration through eggshell associated with freshness of laid eggs and refrigeration. *Journal of Food Protection* **61,** 350-353.

Moats WA (1980) Classification of bacteria from commercial egg washers and washed and unwashed eggs. *Applied and Environmental Microbiology* **40,** 710-714.

Moats WA (1981) Factors affecting bacterial loads on shells of commercially washed eggs. *Poultry Science* **60,** 2084-2090.

Mohammed HO, Yamamoto R, Carpenter TE, Ortmayer HB (1986a) A statistical model to optimize enzyme-linked immunoabsorbent assay parameters for detection of *Mycoplasma gallisepticum* and *M. synoviae* antibodies in egg yolk. *Avian Diseases* **30,** 389-397.

Mohammed HO, Yamamoto R, Carpenter TE, Ortmayer HB (1986b) Comparison of egg yolk and serum for the detection of *Mycoplasma gallisepticum* and *M. synoviae* antibodies by enzyme linked immunoabsorbent assay. *Avian Diseases* **30,** 398-408.

Mostert BE, Bowes EH, Van der Walt JC (1995) Influence of different housing systems on the performance of hens of four laying strains. *South African Journal of Animal Science* **25,** 80-86.

Murase T, Fujimoto K, Nakayama R, Otsuki K (2006) Multiplication and motility of *Salmonella* enterica serovars Enteritidis, Infantis and Montevideo in *in vitro* contamination models of eggs. *Journal of Food Protection* **69,** 1012-1016.

Murray CJ (1994) Salmonella serovars and phage types in humans and animals in Australia 1987–1992. *Australian Veterinary Journal* **71,** 78-81.

Musgrove MT, Jones DR, Northcutt, JK, Cox NA, Harrison MA (2004a) Identification of *Enterobacteriaceae* from washed and unwashed commercial shell eggs. *Journal of Food Protection* **67,** 2613-2616.

Musgrove MT, Jones DR, Northcutt JK, Curtis PA, Anderson KE, Fletcher DL, Cox NA (2004b) Survey of shell egg processing plant sanitation programs: Effects on non-egg-contact surfaces. *Journal of Food Protection* **67,** 2801-2804.

Musgrove MT, Jones D, Northcutt JK, Harrison MA, Cox NA, Ingram KD, Hinton AJ (2005a) Recovery of *Salmonella* from commercial shell eggs by shell rinse and shell crush Methodologies. *Poultry Science* **84,** 1955-1958.

Musgrove MT, Jones DR, Northcutt JK, Cox NA, Harrison MA (2005b) Shell rinse and shell crush methods for the recovery of aerobic microorganisms and *Enterobacteriaceae* from shell eggs. *Journal of Food Protection* **68,** 2144-2148.

Nascimento VP, Cranstoun S, Solomon SE (1992) Relationship between shell structure and moment of *Salmonella* Enteritidis across the eggshell wall. *British Poultry Science* **33,** 37-48.

Nascimento VP, Solomon SE (1991) The transfer of bacteria (*Salmonella* Enteritidis) across the eggshell wall of eggs classified as “poor” quality. *Animal Technology* **42,** 157-166.

Nys Y (1986) Relationship between age, shell quality and individual rate and duration of shell formation in domestic hens. *British Poultry Science* **27,** 253-259.

Nys Y, Gautron J, Garcia-Ruiz JM, Hincke MT (2004) Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. *Computes Rendus Palevol* **3,** 549-562.

Nys Y, Zawadzki J, Gautron J, Mills AD (1991) Whitening of brown-shelled eggs: mineral composition of uterine fluid and rate of protoporphyrin deposition. *Poultry Science* **70,** 1236-1245.

Okamura M, Kamijima Y, Miyamoto T, Tani H, Sasai K, Baba E (2001) Differences among six *Salmonella* serovars in abilities to colonize reproductive organs and to contaminate eggs in laying hens. *Avian Diseases* **45,** 61-69.

Orel V (1959) The Pseudomonas spoilage of eggs laid by individual hens. *Poultry Science* **38,** 8-12.

OzFoodNet Working Group (2009) Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: Annual Report of the OzFoodNet network. 2009. *Communicable Diseases Intelligence* **34,** 396–426 (2010).

OzFoodNet Working Group (2010) Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the OzFoodNet network, 2009. *Communicable Diseases Intelligence* **36,** 213-241 (2012).

Park CM, Hung YC, Lin CS, Brackett RE (2005) Efficacy of electrolyzed water in inactivating *Salmonella* Enteritidis and *Listeria monocytogenes* on shell eggs. *Journal of Food Protection* **68,** 986-990.

Parsons AH (1982) Structure of the eggshell. *Poultry Science* **61,** 2013-2021.

Pavlovski Z, Skrbic Z, Lukic M (2004) Influence of husbandry system on the internal egg quality traits in small flocks hen layers. *Zivinarstvo* **39,** 19-23.

Petek M, Fazli A, Sule Gezen S, Cibikn R (2009) Effects of housing system and age on early stage egg production and quality in commercial laying hens. *The Journal of the Faculty of Veterinary Medicine, University of Kafkas* **15,** 57-62.

Poppe C, Irwin RJ, Forsberg CM, Clarke RC, Oggel J (1991) The prevalence of Salmonella enteritidis and other Salmonella spp. among Canadian registered commercial layer flocks. *Epidemiology & Infection* **106,** 259-270.

Protais J, Queguiner S, Boscher E, Piquet JC, Nagard B, Salvat G (2003) Effect of housing system on the bacterial flora in the air and on egg shells, In: Proceedings of the X European symposium on the quality of eggs and egg products, St. Brieuc-Ploufragan, pp. 142-149.

Quarles CL, Gentry RF, Bressler GO (1970) Bacterial contamination in poultry houses and its relationship to egg hatchability. *Poultry Science* **49,** 60-66.

R Core Team (2012). R: A language and environment for

statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.
Radkowski M (2002a) Survival of *Salmonella* Enteritidis on the eggshells. *Polish Journal of Food and Nutrition Sciences* **11,** 61-63.
Radkowski M (2002b) Effect of moisture and temperature on survival of *Salmonella* Enteritidis on shell eggs. *Archiv fur Geflugelkunde* **66,** 119-123.
Raevuori M, Seuna E, Nurmi E (1978) An epidemic of *Salmonella infantis* infection in Finnish broiler chickens in 1975--76. *Acta Veterinara Scandinavia.* **19,** 317-330.
Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galán JE, Ginocchio C, Curtiss R 3rd, Gyles CL (1992) Amplification of an invA gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and Cellular Probes* **6,** 271-279.
Reid J (1983). The use of the plasma chemistry unit as an aid to the scanning electron microscope study of avian egg-shell structure. *British Poultry Science* **24,** 233-235
Ricke SC, Birkhold SG, Gast RK (2001) Eggs and egg products. In: Downes FP, Ito K. editors. Compendium of methods for the microbiological examination of foods. American Public Health Assoc. Washington, D.C. pp. 473-479.
Roberts JR (2004) Factors affecting egg internal quality and egg shell quality in laying hens. *International Journal of Poultry Science* **41,** 166-177.
Roberts J, Ball W (2003) 'Egg quality guidelines for the Australian egg industry'. (University of New England and Australian Egg Corporation Limited, Australia).
Roberts JR, Ball W (2006) Egg and eggshell quality guidelines for the Australian egg industry. In 'The Amazing Egg: Nature's Perfect Functional Food for Health Promotion'. (Eds By JS Sim, HH Sunwoo) pp. 499-508. (Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada).
Roberts JR, Brackpool CE (1994) The ultrastructure of avian egg shells. *Poultry Science Reviews* **5,** 245-272.
Rodriguez-Navarro A, Kalin O, Nys Y, Garcia-Ruiz JM (2002) Influence of the microstructure on the shell strength of eggs laid by hens of different ages. *British Poultry Science* **43,** 395–403.
Roland DA (1979) Factors influencing shell quality of aging hens. *Poultry Science* **58,** 774-777.
Roland DA, Sloan DR, Harms RH (1975) The ability of hens to maintain calcium deposition in the eggshell and egg yolk as the hen ages. *Poultry Science* **54,** 1720-1723.
Samiullah, Roberts JR (2013) Location of pigment in brown shelled eggs. *Poultry Science* **92,** 2783-2788
Sauter EA, Petersen CF (1974) The effect of eggshell quality on penetration by various *Salmonella*. *Poultry Science* **53,** 2159–2162.
Schoeni J, Glass KA, McDernott JL, Wong ACL (1995) Growth and penetration of *Salmonella* Enteritidis, *Salmonella* Heidelberg and *Salmonella* Typhimurium in eggs. *International Journal of Food Microbiology* **24,** 385-396.
Sekeroglu A, Sarica M, Demir E, Ulutas Z, Tilki M, Saatci M, Omed H (2010) Effects of different housing systems on some performance traits and egg qualities of laying hens. *Journal of Animal and Veterinary Advances* **9,** 1739-1744.
Sencic D, Antunovic Z, Domacinovic M, Speranda M, Steiner Z (2006) Egg quality from free range and cage system of keeping layers. *Stocarstvo* **60,** 173-179.
Sergeant ESG, Grimes TM, Jackson C, Baldock FC, Whan IF (2003) *Salmonella enteritidis* surveillance and response options for the Australian egg industry. A report for the Rural Industries Research and Development Corporation. Retrieved from: http://www.salmonella360.com/content/files/323/Sci.%20Report_EN_RIRDC-Australia_2003_S.Enteritidis%20Surveillance.pdf.
Shea JE, Hensel M, Gleeson C, Holden DW (1996) Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. *Proceedings of the National Academy of Sciences* **93,** 2593-2597.

Silversides FG (1994) The Haugh unit correction for egg weight is not adequate for comparing eggs from chickens of different lines and ages. *Journal of Applied Poultry Research* **3,** 120-126.

Silversides FG, Budgell K (2004) The relationships among measures of egg albumen height, pH, and whipping volume. *Poultry Science* **83,** 1619-1623.

Silversides FG, Scott TA (2001) Effect of storage and layer age on quality of eggs from two lines of hens. *Poultry Science* **80,** 1240-1245.

Singh R, Cheng KM, Silversides FG (2009) Production performance and egg quality of four strains of laying hens kept in conventional cages and floor pens. *Poultry Science* **88,** 256–264.

Skyberg JA, Logue CM, Nolan LK (2006) Virulence genotyping of Salmonella spp. with multiplex PCR. *Avian Diseases* **50,** 77-81.

Slinko VG, McCall BJ, Stafford RJ, Bell RJ, Hiley LA, Sandberg SM, White SA, Bell KM (2009) Outbreaks of Salmonella Typhimurium phage type 197 of multiple genotypes linked to an egg producer. *Communicable Diseases Intelligence* **33,** 419-425.

Smeltzer TI, Orange K, Peel B, Runge GI (1979) Bacterial penetration in floor and nest box eggs from meat and layer birds. *Australian Veterinary Journal* **55,** 592-593.

Smith A, Rose SP, Wells RG, Pirgozliev V (2000) The effect of changing the excreta moisture of caged laying hens on the excreta and microbial contamination of their eggshells. *British Poultry Science* **41,** 168-173.

Solomon S (1986) Translucency - some causative factors, *Poultry International* **25,** 24-26.

Solomon SE (1991) 'Egg and eggshell quality', Wolfe Publishing Limited, London.

Solomon SE (1992a) Can eggshell structure measure quality? *Poultry International,* **31,** 24-27.

Solomon SE (1992b) Eggshell quality and microbial penetration. *Poultry International,* **31,** 20-22.

Solomon, S.E. (2009). Foundation is key for eggshell quality. *World Poultry* **25,** 16-18.

Sparks NH, Burgess AD (1993) Effect of spray sanitising on hatching egg cuticle efficacy and hatchability. *British Poultry Science* **34,** 655-662.

Sparks NH, Board RG (1984) Cuticle, shell porosity and water uptake through hen's eggshells. *British Poultry Science* **25,** 267-276.

Stępień-Pyśniak D (2010) Occurrence of Gram-negative bacteria in hens' eggs depending on their source and storage conditions. *Polish Journal of Veterinary Sciences* **13,** 507-513.

Stipkovits L, Kempf I (1996) Mycoplasmosis in poultry. *Revue Scientifique et Technique* (International Office of Epizootics) **15,** 1495-1525.

Suzuki K, Origlia J, Alvarez F, Faccioli M, Silva M, Caballero J, Nunrez L, Castro L (2009) Relative risk estimation for *Mycoplasma synoviae* in backyard chickens in Paraguay. *International Journal of Poultry Science* **8,** 842-847.

Synnott M, Brindley M, Gray J, Dawson J (1998) An Outbreak of *Salmonella* Agona infection associated with precooked turkey meat, *Communicable Disease and Public Health*, **15,** 176-180.

Talbot CJ, Tyler C (1974) A study of the fundamental cause of natural translucent areas in egg shells. *British Poultry Science* **15,** 205-215.

Tanaka T, Hurnik JF (199). Comparison of behavior and performance of laying hens housed in battery cages and an aviary. *Poultry Science* **71,** 235-243.

Travel, A, Nys Y, Bain M (2011) Effect of hen age, moult, laying environment and egg storage on egg quality. In 'Improving the safety and quality of eggs and egg products' Vol 1. (Eds Y. Nys, M. Bain, F. Van Immerseel) pp. 300-329. (Woodhead Publishing Limited: Cambridge, U.K.).

Tullett S (1978) Pore Size verses Pore Number in Avian Eggshells. In *Respiratory Function in Birds, Adult and Embryonic.* (ed Piiper, J). 219-226. (Springer-Verlag, Berlin, Heidelberg, New York).

Tumova E, Ebeid T (2005) Effect of time of oviposition on egg quality characteristics in cages and in a litter housing system. *Czech Journal of Animal Science* **50,** 129–134.

Tumova E, Englmaierova M, Ledvinka Z, Charvatova V (2011) Interaction between housing system and genotype in relation to internal and external egg quality parameters. *Czech Journal of Animal Science* **56,** 490-498.

Tumova E, Ledvinka Z (2009) The effect of time of oviposition and age on egg weight, egg components weight and eggshell quality. *Archive fur Geflugelkunde* **73,** 110-115.

Tyler C (1956) Studies of eggshells. VII. Some aspects of structure as shown by plastic models. *Journal of Science, Food and Agriculture* **7,** 483-493.

Van Den Brand H, Parmentier HK, Kemp B (2004) Effects of housing system (outdoor vs cages) and age of laying hens on egg characteristics. *British Poultry Science* **45,** 745-752.

Varguez-Montero G, Sarmiento-Franco L, Santos-Ricalde R, Segura-Correa J (2012) Egg production and quality under three housings in the tropics. *Tropical Animal Health and Production* **44,** 201-204.

Wall H, Tauson R, Sorgjerd S (2008) Bacterial contamination of eggshells in furnished and conventional cages. *Journal of Applied Poultry Research* **17,** 11-16.

Wang H, Slavik MF (1998) Bacterial penetration into eggs washed with various chemicals and stored at different temperatures and times. *Journal of Food Protection* **61,** 276-279.

Wang X L, Zheng JX, Ning ZH, Qu LJ, Xu GY, Yang N (2009) Laying hen performance and egg quality of blue-shelled layers as affected by different housing systems. *Poultry Science* **88,** 1485-1492.

Washburn KW (1982) Incidence, cause, and prevention of egg shell breakage in commercial production. *Poultry Science* **61,** 2005-2012.

Williams JE, Dillard LH, Hall GO (1968) The penetration patterns of *Salmonella* Typhimurium through the outer structures of chicken eggs. *Avian Diseases* **12,** 445–466.

Williams KC (1992) Some factors affecting albumen quality with particular reference to Haugh unit Score. *World's Poultry Science Journal* **48,** 5-16.

Zita L, Tumova E, Stolc L (2009) Effects of genotype, age and their interaction on egg quality in brown-egg laying hens. *Acta Veterinaria Brno* **78,** 85-91.

Publications Arising from Project

Journal Articles

Gole VC, Chousalkar KK, Roberts JR (2012) Prevalence of antibodies to *Mycoplasma synoviae* in laying hens and possible effects on egg shell quality. *Preventative Veterinary Medicine* **106,** 75-78.

Chousalkar KK, Roberts JR (2012) Recovery of *Salmonella* from egg shell wash, egg shell crush and egg internal contents of unwashed commercial shell eggs in Australia. *Poultry Science* **91,** 1739-1741.

Samiullah, Chousalkar KK, Roberts JR, Sexton M, May D, Kiermeier A. (2013) Effects of egg shell quality and washing on *Salmonella* Infantis penetration. *International Journal of Food Microbiology* **165,** 77-83.

Gole V, Chousalkar KK, Roberts JR (2013) Survey of *Enterobacteriaceae* contamination of table eggs collected from layer flocks in Australia. *International Journal of Food Microbiology* **164,** 161-165.

Roberts, J.R., Chousalkar, K.K. and Samiullah. 2013, in press. Egg quality and age of laying hens: implications for product safety. *Animal Production Science* **53,** 1291-1297.

Samiullah, Roberts JR, Chousalkar KK (2013, in press) Effect of production system and flock age on egg quality and total bacterial load in commercial laying hens. *Journal of Applied Poultry Research*.

Gole VC, Chousalkar KK, Roberts JR, Sexton M, May D, Tan J, Kiermeier A. Effect of egg washing and correlation between eggshell characteristics and egg penetration by various *Salmonella* Typhimurium phage types. In preparation for submission to *PLOS one*.

Refereed Conference Papers

Already Presented

Gole VC, Chousalkar KK, Lievart J, Roberts JR (2012) Prevalence of *Mycoplasma synoviae* in eggs from laying hens using ELISA. *Proceedings of the Australian Poultry Science Symposium (J. Roberts Ed.)* **23,** 205-208.

Chousalkar KK, Roberts JR (2012) Recovery of *Enterobacteriaceae* from shell surface and shell in early mid and late lay. *Proceedings of the Australian Poultry Science Symposium (J. Roberts Ed.)* **23,** 233-236.

Roberts JR, Chousalkar KK (2012) Egg quality and food safety of table eggs: egg quality and age of flock – a horizontal study. *Proceedings of the Australian Poultry Science Symposium (J. Roberts Ed.)* **23,** 241-244.

Samiullah, S, Roberts, JR, Chousalkar KK (2012) Egg quality and food safety of table eggs: egg quality and age of flock – a longitudinal study. *Proceedings of the Australian Poultry Science Symposium (J. Roberts Ed.)* **23,** 245-248.

Ray A, Roberts JR (2013) The structural basis of egg shell translucency and its role in food safety of table eggs. *Proceedings of the Australian Poultry Science Symposium (J.R. Roberts, Ed)*. **24,** 162.

Gole VG, Chousalkar KK, Roberts JR, Sexton M, May D, Kiermeier A (2013) Egg penetration by *Salmonella* Typhimurium in washed and unwashed eggs. *Proceedings of the Australian Poultry Science Symposium (J.R. Roberts, Ed*). **24,** 252-255.

Samiullah S, Roberts JR (2013) Protoporphyrin IX in shell and cuticle of brown shelled eggs. *Proceedings of the Australian Poultry Science Symposium (J.R. Roberts, Ed*). **24,** 260-263.

Roberts JR, Chousalkar KK (2013) Egg quality and food safety of table eggs. *Proceedings of the Australian Poultry Science Symposium (J.R. Roberts, Ed).* **24,** 242.

Submitted for 2014

Ray A, Roberts JR, Chousalkar KK, Haling R (2014) Eggshell uniformity and the relationship between shell structures and shell translucency, examined by computated tomography

(CT) and scanning electron microscopy (SEM). Submitted for Australian Poultry Science Symposium 2014.

Non-refereed Conference Papers

Roberts, J.R. 2010. Egg quality and food safety. *Proceedings of the 50th Anniversary New Zealand Poultry Industry Conference*, Palmerston North, New Zealand, October 2010, *10,* 85-95.

Roberts, J.R. and Chousalkar K.K. 2012. Egg shell quality and the risks of foodborne pathogens. *Australian Veterinary Poultry Association Conference*, Gold Coast, Australia, May 23-24, 2012.

Roberts, J.R. and Chousalkar, K.K. 2012. Egg quality and food safety of table eggs: egg quality and age of flock – a horizontal study. *World's Poultry Congress*, Salvador, Brazil, August 5-9, 2012 (Oral Presentation)

Samiullah, Roberts, J.R. and Chousalkar, K.K. 2012. Egg quality and food safety of table eggs. *World's Poultry Congress*, Salvador, Brazil, August 5-9, 2012 (Poster Presentation)

Gole, V.C., Chousalkar, K.K., Lievaart, J. and Roberts, J.R. 2012. *Mycoplasma synoviae* in laying hens and effects on egg shell quality. *World's Poultry Congress*, Salvador, Brazil, August 5-9, 2012 (Poster Presentation)

Samiullah, Roberts, J.R. and Chousalkar, K. Whole egg penetration of table eggs with *Salmonella enterica* serovar Infantis. *Proceedings of the XXI European Symposium on the Quality of Poultry Meat and the XV European Symposium on the Quality of Eggs and Egg Products*, Bergamo, Italy September 15-19 (Poster Presentation).

Gole, V.C., Chousalkar, K.K., Roberts, J.R., Sexton, M., May, D. and Kiermeier, A. Egg penetration ability of various *Salmonella* serovars in washed and unwashed eggs. *Proceedings of the XXI European Symposium on the Quality of Poultry Meat and the XV European Symposium on the Quality of Eggs and Egg Products*, Bergamo, Italy September 15-19 (Oral Presentation).

Research Students associated with the project

Samiullah, Master of Rural Science, University of New England (completed)

Vaihbah Gole, PhD student, Charles Sturt University then University of Adelaide (current)

Aaron Ray, Bachelor of Science (Animal Science) Honours student, University of New England (completed)

Aaron Ray, PhD student, University of New England (current)

Plain English Compendium Summary

Sub-Project Title:	Eggshell quality and the risks of food borne pathogens
Poultry CRC Sub-Project No.:	3.2.2
Researcher:	Juliet R Roberts
Organisation:	University of New England, Armidale, NSW 2351
Phone:	(02) 6773 1995
Fax:	(02) 6773 3922
Email:	jrobert2@une.edu.au
Sub-Project Overview	This sub-project investigated the incidence of bacterial contamination of commercial eggs and the relationship between egg quality and the ability of bacteria to penetrate the egg shell and enter the egg contents. Shell quality varies with flock age and shell quality was also found to be related to the ease with which bacteria are able to pass across the egg shell. Certain shell quality aspects were found to increase the risk of bacteria moving across egg shells.
Background	Although the eggs produced by the Australian commercial egg industry are generally regarded as being of very high quality, eggs are commonly implicated in cases of food borne salmonellosis. An intact, good quality egg shell provides a significant barrier to bacterial entry into the egg contents. However, defects in the egg shell may potentiate bacterial entry into the egg. The main source of bacteria is from the outside of the egg and bacteria may enter the egg via pores or defects in the egg shell or they may contaminate the egg contents during food preparation. Therefore, it is important to know what bacteria are found on commercial eggs in Australia and to understand the risks of these bacteria entering the egg contents.
Research	A horizontal study (different flocks at different ages) was conducted involving 34 commercial flocks of laying hens. Egg quality and bacterial contamination of the eggs were evaluated. A vertical study of one cage flock and one free range flock was also conducted. Many aspects of egg quality were found to decline with increasing flock age. The bacteria found on the outside of the egg shell and in the pores were identified. The most common serovar of *Salmonella* found was *S.* Infantis. A number of serovars and phage types was used to determine how easily the bacteria could move from the outside of the egg shell into the egg contents and it was found that there were differences among the types of Salmonella. The incidence of the phenomenon of translucency and the extent of cuticle cover, along with the ultrastructural features of the mammillary layer were all found to be associated with the risk of bacterial ingress.
Implications	The incidence of organisms capable of causing food borne illness needs to be monitored regularly in the Australian egg industry.
Publications	Journal Articles – 5 published, 2 in press, 1 submitted Refereed Conference Papers – 8 published Non-refereed Conference Papers – 7 published