Cuticle deposition improves the biosecurity of eggs through the laying cycle and can be measured on hatching eggs without compromising embryonic development

Maureen M. Bain* Jiangxia Zheng[†], Melissa Zigler[§], Natasha Whenham[1§], Fiona Quinlan-Pluck[#], Anita Jones[#], Mark Roberts*, Wiebke Icken[I], Victor Olori[II], Ian C. Dunn[§]

[*] IBAHCM, MVLS, University of Glasgow, Glasgow, Scotland, UK;[†] College of Animal Science and Technology, China Agricultural University, Bejing 100193, China; [‡] The Roslin Institute, University of Edinburgh, Easter Bush Campus, Midlothian, EH25 9RG, Scotland, UK;[#] School of Chemistry, University of Edinburgh, Joseph Black Building, Edinburgh, EH9 3FJ, Scotland, UK; [I] Lohmann Tierzucht, 27472 Cuxhaven, Germany; [II] Aviagen, 11 Lochend Road, Ratho Station, Newbridge, Midlothian, EH28 8SZ, United Kingdom

Scientific Section: Microbiology and Food Safety

Corresponding author: maureen.bain@glasgow.ac.uk

Phone: +44 (0)141 330 5719

[1] Current affiliation: AB Vista, 3 Woodstock Court, Blenheim Road, Marlborough, SN8 4AN

ABSTRACT

The cuticle is part of the egg's natural defence and it can be improved by genetic selection. Prior to adoption of this measurement in breeding programs, questions that need addressed are whether improved cuticle deposition will result in a reduced risk of eggs becoming contaminated and whether selection for this trait will have any unintended consequences on the incubation process.

Bacterial penetration experiments were carried out using eggs from a pedigree line of Broiler Breeders (BB) and Rhode Island Red (RIR) layers. Within the natural variation in cuticle deposition in each line, a good cuticle was shown to significantly reduce an egg's susceptibility to penetration by *E.coli* (BB, $P=0.023$) and *S. Typhimurium* (RIR, $P<0.001$). Deglycosylation of cuticle proteins had little effect on their antimicrobial activity. The effect of bird age on cuticle deposition was also examined. Shell colour decreased with age as anticipated, however, we found no evidence that cuticle deposition decreases with age, up to at least 50wks.

A thicker cuticle could affect the water vapour conductance (WPC) of hatching eggs. The WPC of eggs was therefore measured on eggs selected from the top and tail of the cuticle distribution, this time in a Lohmann selected leghorn (LSL) pedigree line. BB eggs were also tested. No evidence of a relationship between cuticle deposition and WPC was found for LSL or BB eggs.

Cuticle deposition measurements require eggs to be stained. Here we show that this has no adverse effect on embryo development at day 12 of incubation. Thus, we conclude that cuticle deposition is important in preventing bacterial penetration of eggs in genetically divergent breeds of chicken and that the measurement can be practically incorporated into breeding programs. This will contribute to improving the biosecurity of eggs by reducing

vertical and horizontal transmission of potentially zoonotic and pathogenic organisms from parent to offspring.

Key Words: cuticle, biosecurity, bacterial penetration, selection, water vapour conductance

INTRODUCTION

A number of bacterial infections of economic and zoonotic significance to the poultry industry are known to be transmitted vertically from parent to offspring via the egg. These include *Escherichia coli* (Poulsen et al., 2017), a number of salmonellas (Liljebjelke et al., 2005) and several important Mycoplasma spp, (Barrow 1994). Vertical transmission (parent to offspring) occurs when the reproductive tissues of breeding females become colonised and the organism is periodically shed and becomes incorporated into the egg as it is forming . Vertical transmission can also occur when the surface of the newly laid egg becomes soiled with contaminated faeces either at, or just after, oviposition. The risk of cross contamination occurring during egg collection, transport and storage (horizontal transmission) is a major cause for concern for commercial hatcheries, especially if some of the eggs are already heavily contaminated (Bailey et al., 1996). In this context, particular targets where elimination would be of benefit are *E.coli* species and *Enterococcus faecalis* (Fertner et al., 2011; Petersen et al., 2006). Irrespective of the route or site of transfer, entry of pathogenic or zoonotic organisms into the egg contents is undesirable for both animal and public health, and food safety.

Eggs are naturally equipped with a range of both physical and chemical defences to protect the embryo and the contents from bacterial ingress and growth (Baron and Jan 2011; D'Alba and Shawkey 2015). The cuticle, for example, forms the egg's first line of defence (Bain, et al., 2013). This largely organic layer forms on the surface of the calcified shell during the final 1 to 1.5 hr of egg formation (Baker and Balch 1962; Sparks and Board 1985; Wilson et al., 2017) and consists of several proteins, including ovocalyxin-36, kunitz-like protease inhibitor, ovocleidin-116, ovocleidin-17, ovocalyxin 25, clusterin and ovocalyxin-32 (Bain et al., 2013; Anon 2001; Miksik et al., 2003; Wellman-Labadie et al., 2008). Several of these proteins have either known or suspected antimicrobial roles (Gautron et al., 2001; Gautron et

al., 2007). As well as contributing to the eggs chemical defence, the cuticle also creates an effective physical barrier to bacterial ingress by plugging the external openings of the gaseous exchange pores. This prevents both water and solids, including debris and microorganisms, from passing through the shell into the egg contents (Sparks 1994). Wild birds nesting in humid, dirtier habitats, therefore, have a tendency to have evolved a cuticle that is more resistant to water uptake than those nesting in less risky inhabits (D'Alba et al., 2017). In modern poultry breeding programs, with strict on-farm biosecurity and the widespread use of artificial incubation (where hygiene and temperature and humidity are closely monitored), emphasis has not been placed on the artificial selection for this trait. In support of this we previously demonstrated that there is considerable natural variation in the amount of cuticle deposited on eggs from individual birds in a pure line of Rhode Island Red (RIR) laying hens and showed that eggs from hens with a poor cuticle were more often penetrated by a laboratory strain of *E.coli* than eggs from hens with good cuticles (Bain et al., 2013). In this paper we have extended these observations to include more breeds of chicken (layers and broiler breeders) and other strains of bacteria e.g. *Salmonella enterica*, serotype Typhimurium (*S*. Typhimurium). Another aim of the current work was to establish if cuticle deposition changes with bird age. Many common egg shell traits, including eggshell colour (Mills, et al., 1991) and breaking strength (Rodriguez-Navarro et al., 2002), decrease with age, indeed this is what currently determines the end of a flock's productive life (Bain et al., 2016). If cuticle deposition or its chemistry decline with age (Kulshreshtha et al., 2018; Rodriguez-Navarro et al., 2013) then this could have important implications for the risk of vertical and horizontal transmission in eggs from older hens.

Most of the proteins associated with the cuticle are thought to be heavily glycosylated (Hincke et al., 2011). Glycosylation of cuticle proteins could be important to the function of the cuticle, its stability and/or its adhesiveness to the underlying calcified substrate. A further

objective of this study was to test the hypothesis that glycosylation (i.e. the attachment of sugar moieties) is important to the cuticle's antimicrobial activity.

In some species, a thick cuticle has been shown to increase the diffusion pathway for respiratory gases and to lower the shell's conductance (Sparks 1994). In commercial Peking duck production for example it is common practice to chemically remove the cuticle before incubation (Anon 2006), although this is not unequivocally the case (Pouvreau and Baudon 2016). For chicken eggs, the cuticle is not thought to be a significant factor determining the shell's conductance (Sparks and Board 1984); however, the evidence for this is confusing (Deeming 1987; Peebles and Brake 1986; Peebles et al., 1987) and is reliant on studies where the cuticle is chemically degraded and unquantified. A further aim was, therefore, to compare the conductance of fertile eggs laid by hens that naturally vary in their cuticle deposition, and to demonstrate that staining eggs to measure cuticle deposition can be carried out without compromising embryo development. The latter is important to know, if the measurement of cuticle deposition is to be applied in practice.

The experiments described in this paper, therefore: a) provide further evidence that the cuticle is important in preventing bacterial penetration of eggs in genetically divergent breeds of chicken; b) demonstrate that glycosylation *per se* has no effect on the antimicrobial properties of the proteins in the cuticle; c) show that cuticle deposition does not diminish with bird age; and d) illustrate that the measurement of cuticle deposition can be incorporated into poultry breeding programs without compromising embryonic development.

MATERIALS AND METHODS

Source of Eggs

Brown eggs were sourced from a Rhode Island Red (RIR) pure line that contributes to the male used to produce Lohmann Brown commercial layers (Lohmann Tierzucht GmbH), as

previously studied (Bain et al., 2013; Dunn et al., 2005, 2009; Dunn et al., 2012). The population from which the eggs were sampled consisted of 1262 female offspring from three hatches. The birds were housed in individual cages in 3 separate environmentally controlled houses on the same site, and were on 16 hours of light per day and fed as per Lohmann pure-line management guidelines.

White eggs were sourced from a white leghorn pure line, used in the production of Lohmann selected leghorn (LSL) commercial layers (Lohmann Tierzucht GmbH). The population, from which we sampled, in this case, consisted of 948 female offspring derived from two hatches which were housed in individual cages in 3 separate environmentally controlled houses on the same site. The birds and received 16 hours of light per day and fed as per Lohmann pure-line management guidelines.

Broiler Breeder (BB) eggs were obtained from a fully pedigree female broiler pure line from Aviagen Limited's breeding program. The population, from which we sampled, in this case, comprised 1459 female offspring spread across 13 flocks, housed in floor pens with trap nesting facilities to facilitate recording of each egg laid by each hen. Each flock received 14 hours of light per day and were managed as per company specific management guidelines.

The advantage of sampling eggs from individual hens from pedigree populations was that this made it possible to identify the eggs from individual birds in all of the experiments.

Measurement of Cuticle Deposition

Cuticle deposition was measured as described by Bain et al. (2013), except that in this instance the absorbance of eggs at 640 nm was measured prior to dyeing with MST cuticle blue stain (MS Technologies, U.K) and the difference before and after dyeing was used to estimate the cuticle deposition (Cuticle ΔAbs @640nm). The eggs used in our experiments

were tested <3 days after collection and were stored and transported at an ambient temperature of between 8- 12^{o}C.

In addition, the pre-stain absorbance at 640nm (Abs@640nm[pigment]) was used to estimate shell colour or brownness, as the peak of protoporphyrin absorbance is around 644 nm.

All initial measurements were carried out using a USB4000-VIS-NIR spectrometer coupled to an ISP-REF integrating sphere, as previously described by Bain et al. (2013). During the study period, however, there was a progressive development of technology to improve the speed of data acquisition. The basic principle used to measure the cuticle deposition however remained the same viz. The measurement of the amount of light absorbed by the cuticle-bound stain, as a proxy of cuticle deposition and in all cases a WS-1 diffuse reflectance PTFE standard tile (Ocean optics) was used to calibrate the instrument between experiments.

Bacterial Penetration Experiments

To increase our power to detect differences in the effect of cuticle deposition on bacterial penetration, two intact eggs from 23 hens at the top and 23 hens from the tail of the cuticle deposition distribution were sampled from the RIR population at 51wks of age. This was possible as we had previously measured the cuticle deposition in the entire population at 30-32wks of age and the genetic correlation within hens at different ages for cuticle coverage was very high (1.00; Dunn et al., in prep). The test organism used on the RIR eggs was a non-pathogenic laboratory strain of *S. Typhimurium* containing plasmid pGlo (*St-pGlo:* SL1344 *htr*A mutant pGLO). Cuticle deposition was also measured on an additional two eggs from each hen.

A different approach was used for the penetration studies carried out on BB eggs, where fewer eggs were available and we had no *a priori* knowledge about quantities of cuticle deposition. Three eggs were collected from 73 individuals from one of the BB flocks at

41wks of age for this study. One egg from each hen was used for bacteriology; the other two were used to measure cuticle deposition. The test organism used for BB eggs was *E. coli* containing plasmid pGLO (*E-pGlo*, BIO-RAD laboratories) as described previously (Bain et al., 2013).

Our penetration experiments followed the method described previously (Bain et al., 2013). Eggs were first warmed and then individually immersed into a zip-lock bag containing a chilled inoculum of the test organism for 15 min. The eggs were then placed into another sterile zip-lock bag and incubated for 24 h at 37℃. After removal of the egg content, the inner surface of each egg was viewed under a long-wave UV light source. Penetration by the test organism was confirmed by the presence or absence of bright luminescent areas on the inner shell membranes.

For RIR eggs, each hen (n=46) was given a score of between 0 and 2 depending on whether zero, one, or two out of two eggs were penetrated by the test organism (*St-pGlo).* These scores were then used as a factor in an unbalanced analysis of variance using Genstat regression (Genstat 13th edition, VSN International Ltd) for cuticle coverage. The hens in this population came from 2 different hatches so this was fitted in the analysis as a nuisance factor.

For BB eggs, the single test eggs were simply categorised as being penetrated or not penetrated by *E-pGlo.* These scores were then used as a factor in an unbalanced analysis of variance using Genstat regression (Genstat 13th edition, VSN International Ltd) for cuticle deposition. Because the penetration sites were more discrete in BB eggs it was also possible to categorise each single test eggs as having no penetration, low penetration (<3 discrete luminescent areas per egg) or high penetration (>3 discrete luminescent areas per egg). This allowed a more refined statistical analysis with 3 rather than 2 possible scores.

Glycosylation and Antibacterial Activity

To test if glycosylation influences the cuticles antimicrobial properties the cuticle from a number of freshly laid RIR eggs was extracted using 5% EDTA. The pooled cuticle extract was divided into three and treated in one of the following ways: 1) untreated (glycosylated); 2) denatured and then deglycosylated using a New England BioLabs Protein Deglycosylation Mix (P6039S); 3) degylosylated using the same P60395 kit but without completing the denaturing step. Gel purification (15%w/v SDS) was subsequently used to remove residual enzyme and separate the cuticle proteins in each sample into fractions of <30 kDa and >30 kDa. Protein concentration was normalized between samples by dilution based on OD600 measurements. A broth-based antimicrobial assay was then used to determine the efficacy of each sample fraction against a gram negative bacterium *E.coli DH5*α, and a gram positive bacterium, *Staphylococcus aureus* RN422. Both strains are commonly used non-pathogenic laboratory strains. In each case the test bacteria were cultured overnight at 37^{o}C in Luria broth (LB) for *E. coli* DH5α, and Tryptone soya broth (TSB) for *S. aureus (RN4220)*; 250 µl of overnight culture was sub-cultured into 20 ml of LB and incubated at 37^{o}C for 3 h. After the second incubation, 20 µl of culture was diluted with 2 ml of PBS, pH 7.4. Glycosylated or deglycosylated cuticle protein fraction (10 µl) was then added to 50 µl of diluted culture. After vortexing, this was incubated at 37^{o}C for 3 h, and then the suspensions were serially diluted to $1x10^{-4}$ with 225 µl of PBS; all dilutions were then plated on LB agar or Tryptone Soya agar plates in duplicate and incubated overnight at 37^{o}C. The colonies were then counted and the results expressed as a reduction in colony-forming units per millilitre (CFU/ml) compared to a PBS control.

Cuticle Deposition and Shell Colour (Brownness) with Bird Age

Eggs from 4 consecutive days of production from 32 individual birds in our RIR population were collected every 5 wks from 25 to 45wks of age. Shell colour or brownness

(Abs@640nm[pigment]) and cuticle deposition (Cuticle ΔAbs@640nm) measurements were carried out on the first two intact eggs from each hen at each time point. A similar approach was used to establish how the cuticle changes with age in our BB population, but in this case we assessed 2 eggs from 100 individuals from the same flock every 2-4 wks from 27 to 50 wks of age. A repeated measurement analysis was applied using Minitab® Statistical Software, V18, to examine the effect of bird age on cuticle deposition and shell colour or brownness.

Cuticle Deposition and Water Vapour Conductance

Four eggs from 24 LSL laying hens at the top and 24 from the tail of the cuticle distribution at 51 wks of age, and two eggs from 85 individual BB hens from the same flock at 41 wks of age were used in this study. For the LSL eggs, cuticle deposition measurements were carried out on two of the eggs per individual, and two for conductance measurements. For BB eggs cuticle deposition was carried out on the same eggs subsequently used in the conductance experiment. All eggs used in the conductance experiments were tested using an acoustic crack detector (Bain et al., 2006; De Ketelaere et al., 2000) to ensure that only intact eggs were used.

Water vapour conductance measurements were carried out using the method described by Peebles and McDaniel (2004). In brief, all eggs were held under standard storage conditions for 24h. Fresh egg weight was then measured to an accuracy of 0.1 mg, prior to the eggs being placed randomly into one of two large glass desiccator cabinets each fitted with 4 shelves containing deep trays filled with dry desiccant. Each cabinet had the capacity to hold 100 eggs. The cabinets were then placed in an oven and held at a constant temperature of 26^{o}C for 4 days. Every 24 h the desiccant in each cabinet was replenished and the average local temperature (^{0}C) and barometric pressure (mmHg: Torr) were recorded. Egg weight was recorded at 24 h and 96 h. These data were used to calculate the eggshell conductance (mg

H2O / d / Torr) and then the relative eggshell conductance (mg H2O / d /Torr / 100 g) of each egg, as described by (Ar, et al., 1974).

Regression analysis was used to investigate the relationship between the relative eggshell conductance and cuticle deposition using the mean of the two eggs for each individual sampled in both LSL and BB pure lines (Genstat 13th edition, VSN International Ltd).

Staining Eggs for Cuticle Deposition Measurement and Embryonic Development

Two eggs from BB hens (n=84) were ranked by weight and then randomly placed into one of four groups so that there was an equal weight distribution in each group. Groups 1 and 2 eggs were stained for cuticle deposition, groups 3 and 4 eggs were wetted with tap water for the same amount of time (1 min). The stained and unstained eggs were then randomly placed on setter trays in one of two table top incubators (OvaEasy 380 Advance EX Series II , Brinsea, North Somerset, UK) and incubated at 37.5^{o}C and RH of 60%. On day 12 the eggs were removed from the incubator, weighed and then placed in a fridge (4^{o}C) overnight. The chick embryos were then staged using the HH system (Hamburger and Hamilton, 1951) and weighed (embryo wet weight minus the yolk sac). The length of the 3rd toe and each lower mandible were also determined by first photographing and then measuring using ImageJ. Any early deaths and any eggs which had failed to develop were also recorded.

The effect of staining for cuticle deposition on % egg weight loss during incubation, stage of development, wet chick weight (g), length of 3rd toe (mm) and length of lower mandible (mm) were analysed using a GLM with treatment (stained for cuticle versus wetted) and incubator (A and B) as the main effects. A chi-squared test was used to determine whether there was any association of incubator and treatment on the number of early deaths using Minitab® Statistical Software, V18.

Ethical approval to carry out this experiment was granted by the University of Glasgow, School of Veterinary Medicine Ethical Committee.

RESULTS

Bacterial Penetration Experiments

A significant relationship (P<0.001) was found between cuticle deposition and the *St-pGlo* penetration score for eggs sampled from the top and tail of the cuticle distribution in our RIR pedigree population (Figure 1). Hens whose eggs were never penetrated by *St-pGlo* (0/2, n=31) had good cuticle deposition, hens where one out of two egg were penetrated (1/2, n=10) had intermediate cuticle deposition and hens where both eggs were penetrated (2/2, n=5) had the poorest cuticle deposition.

For BB eggs tested with *E. coli* (*E-pGlo)*, the mean (+/- St.dev) score for cuticle deposition was significantly (P=0.011) lower in penetrated eggs (0.231+/- 0.101; n=33) than in non-penetrated eggs (n=40; 0.288+/- 0.090). There was also a significant difference (P=0.023) when penetrated BB eggs were further categorised as having Low penetration (<3 discrete luminescent areas, per egg) or High penetration (>3 discrete luminescent areas). Eggs from hens that had low penetration (n=14) had moderate cuticle deposition, and eggs from hens with high penetration (n=19) had poorer cuticle deposition. Eggs from hens that were never penetrated (n=40) had good cuticle deposition (Figure 2).

Glycosylation and Antibacterial Activity

Glycosylated cuticle extract fractions possessed antibacterial activity against both gram negative and gram positive organisms (Figure 3). The larger cuticle extract proteins (>30kDa) were the most effective, achieving a 95% reduction in *E. coli* and a 97% reduction in *S. aureus*. This activity was not reduced when the >30kDa cuticle protein extract was deglycosylated; however, when this protein fraction was denatured before being

deglycosylated there was a significant reduction in the ability to kill *E. coli* (Figure 3A). The smaller cuticle extract proteins (<30kDa) showed no activity against *E. coli* (Figure A), and only moderate activity (46%) against *S. aureus*. For *S. aureus* the antimicrobial activity increased to 75% when this fraction was deglycosylated, but only when the proteins were not denatured prior to deglycosylation (Figure 3B).

Cuticle Deposition and Shell Colour (Brownness) with Bird Age

Shell colour or brownness significantly decreased ($P<0.001$) in the RIR population of laying hens between 25 and 45 wks of age (Abs@640nm[pigment], Figure 4A). However, we found no clear evidence to support an age-related decline in cuticle deposition in this population (Cuticle ΔAbs@640nm, $P<0.077$, Figure 4A).

BB eggs contain much less brown pigment than RIR layer and, as for layers, the amount of pigment significantly decreased with bird age (Abs@640nm [pigment], $P<0.001$; Figure 4B). The outcome of the repeated measures analysis for cuticle deposition in this case, however, was also significant (Cuticle ΔAbs @640nm, $P<0.001$), Figure 4B) although, as was the case in the RIR, this was not associated with an overall decline in cuticle deposition, but due to random changes over the time course of the study. Indeed cuticle deposition at 50 wks was similar to that observed at 27 wks.

Cuticle Deposition and Water Vapour Conductance

The results of the regression analysis for cuticle deposition and the relative eggshell conductance measurements are presented in Figure 5. There was no strong relationship between these two traits in eggs from LSL hens, which we predicted would have good and poor cuticles, or in eggs from individual hens in our BB population.

294 ***Staining Eggs for Cuticle Deposition Measurement and Embryonic Development***

295 Staining eggs for cuticle assessment prior to setting had no effect on % egg weight loss,
296 embryo wet weight, length of the 3rd toe, length of the lower mandible or the HH stage of
297 embryonic development after 12 days incubation, when compared to the wetted controls
298 (Table 1). A significant incubator effect was observed for embryo wet weight and the HH
299 stage of development. The length of the lower mandible also tended to be greater in Incubator
300 1. No interaction between treatment and incubator was observed for any of the parameters
301 assessed.

302

303 **DISCUSSION**

304 Previously we reported that RIR hens that laid eggs with good cuticle deposition were never
305 penetrated by *E.coli* whilst those with poor cuticle deposition were often penetrated (Bain et
306 al., 2013). Now, by including a laboratory strain of *Salmonella* and also broiler breeder eggs
307 in our penetration studies, we have demonstrated that these observations are likely to be
308 ubiquitous. Cuticle deposition has previously been shown to have a moderate heritability in
309 the same pure line of RIR sampled as was sampled from here (Bain et al., 2013). Recent
310 evidence shows that this is also the case across independent and generically divergent lines
311 (Dunn et al., in prep). Collectively, these results provide evidence that incorporating our
312 cuticle deposition measurement into breeding programmes of egg- and meat-types of chicken
313 will lead to improvement in cuticle coverage and hence a reduction in the transmission of
314 potentially pathogenic organisms via the egg. This will help to improve biosecurity in the
315 poultry industry.

316 Rodriguez-Navarro et al. (2013) proposed that glycosylation of proteins in the cuticle was
317 critical to their protective functional role. Our *in vitro* experiments showed that proteins >30

kDa were the most potent against both gram negative (*E.coli*) and gram positive bacteria (*S.aureus)* and that deglycosylation *per se* had no effect on their antimicrobial activity. Denaturation of these high molecular weight proteins, however, did reduce potency but only against *E. coli. S.aureus,* unlike *E. coli,* lacks an outer membrane (OM). It might be that correct folding of the antimicrobial protein(s) is required to penetrate the OM. This could explain the differential effect denaturation had on the antimicrobial activity of the >30 kDa proteins.

Cuticle extract containing small molecular weight proteins (<30kDa) had no activity against *E.coli* and only moderate potency against *S. aureus.* Glycosylation of small molecular weight proteins could therefore be of greater importance to the cuticle's protective function *in situ* against gram positive bacteria. Potency against *S.aureus* however was further enhanced by deglycosylation. Glycosylation may therefore be more important to the adhesive properties of the cuticle to the underlying calcified shell substrate. This warrants further investigation, as the widespread use of sanitisers in commercial hatcheries (Buhr et al., 2013) may alter these adhesive properties and significantly affect the integrity and protective role of the cuticle during incubation.

A number of important eggshell quality traits, including shell breaking strength, shell colour and albumen quality, are known to decrease with bird age (Bain et al., 2016; Bozkurt and Tekerli 2009; Kemps et al., 2006; Samiullah et al., 2015; Sirri et al., 2018). In our study, we measured the brownness of eggs as the absorbance at 640nm and found this to decrease in both pedigree lines of RIR and BB's. However, an age-related decline in cuticle deposition was not observed. This is consistent with the findings of Ball et al. (1975), but might be unexpected if the reports that significant amounts of pigment are found in the cuticle were correct (Lang and Wells 1987; Samiullah and Roberts 2013), as we might expect that reduction in both to be correlated. Although there was variance in our cuticle deposition measurement from sample to sample, the cuticle deposition values at the end of the study

period were similar to those observed at the beginning of the study period in both populations. Looking more closely at the data, it was possible to see a statistical difference when some of the different age samples were directly compared. However, when all the data were examined there was no cumulative decline with age. If we had sampled at only two time points, say 27 and 39 wks in the case of our BB population, we might have concluded that there was an age-related difference and that hens reduced cuticle deposition with age. Had the current study been extended beyond 50 wks (the oldest age in this study), it is also possible that we would have observed a decline in cuticle deposition. Indeed a recent study, where staining was also used to measure the cuticle deposition, showed that there was less cuticle on eggs laid by hens which were 60 wks of age compared to 25 wk old hens (Dominguez-Gasca et al., 2017). However, a limitation of the latter study, and indeed of other similar studies, is that often cuticle measurements are not carried out on eggs from the same hens. Further but limited evidence for an age-related decline in cuticle quality comes from two studies where the cuticle was measured by both staining and infrared spectroscopy (Kulshreshtha et al., 2018; Rodriguez-Navarro et al., 2013). Infrared spectroscopy provides information about the chemical composition of the cuticle (Rodriguez-Navarro et al., 2013). In the latter study, samples again came from different birds and different flocks at different ages, potentially providing conflicting evidence, depending on the measurement and whether the eggs were from brown or white layers. Further studies where individual hens are followed for a longer duration are, therefore, warranted, to confirm if cuticle deposition, or its chemical composition and quality decline with age, and if this is linked to oviposition time. If our results can be validated, then cuticle deposition is protected over other egg quality traits from an age-related decline, which substantiates its role as the egg's first line of defence. Confirmation would also support the view that the cuticle deposition is distinct from pigment deposition, as observed in previously reported physiological studies (Wilson et al., 2017).

The cuticle has inconsistently been reported to impede or enhance water vapour diffusion in broiler breeder eggs, depending on the age of the bird which laid the egg (Deeming 1987; Peebles and Brake 1986; Peebles et al., 1987). Unlike these earlier studies, which relied on chemical methods to degrade the cuticle, we tested eggs from the top and tail of the cuticle distribution in an LSL pedigree line which should have maximized our chances of finding a significant effect. For BB eggs, we focused on testing eggs representing the natural variability in cuticle deposition. In both cases we found no evidence of a relationship between our measurement of cuticle deposition and the water vapour conductance of the hen's eggshell. This is reassuring and supports the contention that selection for improved cuticle deposition will not have any unintended consequences on such process that are essential for normal development.

The recent development of customised equipment that can rapidly measure cuticle deposition and process the data (Ecutimeter 3, Lomond Instruments, UK) means that the implementation of our cuticle deposition measurement into breeding programs should be relatively straight forward. This is further supported by the consistent estimates of heritability that we have obtained, across genetically diverse lines of commercial chickens and with age (Bain et al., 2013). However, as the measurement still requires the egg to be immersed in stain for 30 sec, it was important to test whether this had any effect on normal embryonic development. In our experiment, we compared the development of embryos in wetted versus stained eggs, at 12 days of incubation, and found no evidence that chick development had been compromised by either treatment. We considered it reasonable to use wetted eggs rather than dry eggs as controls, as it has been common practice to measure the specific gravity on eggs prior to setting, in broiler breeder selection programs, for decades (Wolc et al., 2010).

In conclusion, new evidence is presented that clearly demonstrates that selecting hens that lay eggs with better cuticles will reduce the risk of potential pathogenic organisms from gaining

393 entry to the egg contents. We have also demonstrated for the first time that cuticle deposition
394 does not naturally decrease in genetically diverse lines of egg- and meat-types of chicken, at
395 least up to 50 wks. of age. We also found no evidence that selection for improved cuticle
396 deposition will have an adverse effect on water vapour conductance of the shell. This is
397 important as a controlled loss of water through incubation is critical for normal embryo
398 development. In the broiler industry, where eggs are especially precious (Hocking, 2014),
399 there is no evidence, at least with the power available in this study, that those eggs cannot be
400 successfully incubated after the staining and measurement has been carried out.

401

402 **ACKNOWLEDGEMENT**

403 The work was funded by the BBSRC, Lohman Tierzucht and Aviagen through the BBSRC
404 LINK grants BB/K006096/1 and BB/K0070921/1 'Cute-Egg'. The Roslin Institute is funded
405 with a BBSRC institute strategic programme grant. The authors would also like to thank
406 Christine Whitelaw, Aynsley Sharp and George Loaning for technical assistance provided
407 throughout the project.

408

409 **REFERENCES**

410 Anon. 2006. Pekin duck egg incubation International Hatchery Practice 20:19.
411 Ar, A., C. V. Paganelli, R. B. Reeves, D. G. Greene, and H. Rahn. 1974. The avian egg:
412 water vapor conductance, shell thickness, and functional pore area. The Condor 76:153-158.
413 Bailey, J. S., R. J. Buhr, N. A. Cox, and M. E. Berrang. 1996. Effect of hatching cabinet
414 sanitation treatments on Salmonella cross-contamination and hatchability of broiler eggs.
415 Poult Sci 75:191-196.

Bain, M. M., I. C. Dunn, P. W. Wilson, N. Joseph, B. De Ketelaere, J. De Baerdemaeker, and D. Waddington. 2006. Probability of an egg cracking during packing can be predicted using a simple non-destructive acoustic test. Br Poult Sci 47:462-469.

Bain, M. M., K. McDade, R. Burchmore, A. Law, P. W. Wilson, M. Schmutz, R. Preisinger, and I. C. Dunn. 2013. Enhancing the egg's natural defence against bacterial penetration by increasing cuticle deposition. Anim. Genet. 44:661-668.

Bain, M. M., Y. Nys, and I. C. Dunn. 2016. Increasing persistency in lay and stabilising egg quality in longer laying cycles. What are the challenges? Br Poult Sci. 57: 330–338.

Baker, J. R., and D. A. Balch. 1962. A study of organic material of hens-egg shell. Biochem. J. 82:352-361.

Ball, R. F., V. Logan, and J. F. Hill. 1975. Factors affecting the cuticle of the egg as measured by intensity of staining. Poultry Sci 54:1479-1484.

Baron, F., and S. Jan. 2011. Egg and egg product microbiology. Pages 330-350 in Woodhead Publ Food S. Y. Nys, M. Bain, and F. VanImmerseel eds. Woodhead Publ Ltd, Cambridge.

Barrow, P. A. 1994 The microflora of the alimentary tract and avian pathogens: translocation and vertical transmission. In: Board R.G., Fuller R. (eds) Microbiology of the Avian Egg. Springer, Boston, MA.

Bozkurt, Z., and M. Tekerli. 2009. The effects of hen age, genotype, period and temperature of storage on egg quality. Kafkas Univ Vet Fak 15:517-524.

Buhr, R. J., J. L. Spickler, A. R. Ritter, D. V. Bourassa, N. A. Cox, L. J. Richardson, and J. L. Wilson. 2013. Efficacy of combination chemicals as sanitizers of Salmonella-inoculated broiler hatching eggshells. J Appl Poultry Res 22:27-35.

D'Alba, L., and M. D. Shawkey. 2015. Mechanisms of antimicrobial defense in avian eggs. Journal of Ornithology 156:S399-S408.

D'Alba, L., R. Torres, G. I. N. Waterhouse, C. Eliason, M. E. Hauber, and M. D. Shawkey. 2017. What does the eggshell cuticle do? A functional comparison of avian eggshell cuticles. Physiological and Biochemical Zoology 90:588-599.

De Ketelaere, B., P. Coucke, and J. De Baerdemaeker. 2000. Eggshell crack detection based on acoustic resonance frequency analysis. J. Agr. Eng. Res. 76:157-163.

Deeming, D. C. 1987. Effect of cuticle removal on the water vapour conductance of egg shells of several species of domestic bird. Brit Poultry Sci 28:231-237.

Dominguez-Gasca, N., A. Munoz, and A. B. Rodriguez-Navarro. 2017. Quality assessment of chicken eggshell cuticle by infrared spectroscopy and staining techniques: a comparative study. Brit Poultry Sci 58:517-522.

Dunn, I. C., M. Bain, A. Edmond, P. W. Wilson, N. Joseph, S. Solomon, B. De Ketelaere, J. De Baerdemaeker, M. Schmutz, R. Preisinger, and D. Waddington. 2005. Heritability and genetic correlation of measurements derived from acoustic resonance frequency analysis; a novel method of determining eggshell quality in domestic hens. Br. Poult. Sci. 46:280-286.

Dunn, I. C., N. T. Joseph, M. Bain, A. Edmond, P. W. Wilson, P. Milona, Y. Nys, J. Gautron, M. Schmutz, R. Preisinger, and D. Waddington. 2009. Polymorphisms in eggshell organic matrix genes are associated with eggshell quality measurements in pedigree Rhode Island Red hens. Anim. Genet. 40:110-114.

Dunn, I. C., A. B. Rodriguez-Navarro, K. McDade, M. Schmutz, R. Preisinger, D. Waddington, P. W. Wilson, and M. M. Bain. 2012. 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. Anim. Genet. 43:410-418.

Fertner, M. E., R. H. Olsen, M. Bisgaard, and H. Christensen. 2011. Transmission and genetic diversity of Enterococcus faecalis among layer chickens during hatch. Acta Veterinaria Scandinavica 53.

Gautron, J., M. T. Hincke, K. Mann, M. Panheleux, M. Bain, M. D. McKee, S. E. Solomon, and Y. Nys. 2001. Ovocalyxin-32, a novel chicken eggshell matrix protein - Isolation, amino acid sequencing, cloning, and immunocytochemical localization. Journal of Biological Chemistry 276:39243-39252.

Gautron, J., E. Murayama, A. Vignal, M. Morisson, M. D. McKee, S. Rehault, V. Labas, M. Belghazi, M.-L. Vidal, Y. Nys, and M. T. Hincke. 2007. Cloning of ovocalyxin-36, a novel chicken eggshell protein related to lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins, and plunc family proteins. J. Biol. Chem. 282:5273-5286.

Hincke, M., J. Gautron, Y. Nys, A. B. Rodriguez-Navarro, and M. D. McKee. 2011. The eggshell: structure and protective function. Woodhead Publ Food S:151-182.

Hocking, P. M. 2014. Unexpected consequences of genetic selection in broilers and turkeys: problems and solutions. Br. Poult. Sci. 55:1-12.

Kemps, B. J., T. Govaerts, B. De Ketelaere, K. Mertens, F. R. Bamelis, M. M. Bain, E. M. Decuypere, and J. G. De Baerdemaeker. 2006. The influence of line and laying period on the relationship between different eggshell and membrane strength parameters. Poult Sci 85:1309-1317.

Kulshreshtha, G., A. Rodriguez-Navarro, E. Sanchez-Rodriguez, T. Diep, and M. T. Hincke. 2018. Cuticle and pore plug properties in the table egg. Poult. Sci. 97:1382-1390.

Lang, M. R., and J. W. Wells. 1987. A review of eggshell pigmentation. Worlds Poult. Sci. J. 43:238-246.

Liljebjelke, K. A., C. L. Hofacre, T. Liu, D. G. White, S. Ayers, S. Young, and J. J. Maurer. 2005. Vertical and horizontal transmission of salmonella within integrated broiler production system. Foodborne pathogens and disease 2:90-102.

Miksik, I., J. Charvatova, A. Eckhardt, and Z. Deyl. 2003. Insoluble eggshell matrix proteins - their peptide mapping and partial characterization by capillary electrophoresis and high-performance liquid chromatography. Electrophoresis 24:843-852.

Mills, A. D., Y. Nys, J. Gautron, and J. Zawadski. 1991. Whitening of brown shelled eggs - individual variation and relationships with age, fearfulness, oviposition interval and stress. Br. Poult. Sci. 32:117-129.

Peebles, E., and C. Mcdaniel. 2004. A practical manual for understanding the shell structure of broiler hatching eggs and measurements of their quality [bulletin 1130] Mississippi Agricutural and Forestry Experiment Station.

Peebles, E. D., and J. Brake. 1986. The role of the cuticle in water-vapor conductance by the eggshell of broiler breeders. Poult. Sci. 65:1034-1039.

Peebles, E. D., J. Brake, and R. P. Gildersleeve. 1987. Effects of eggshell cuticle removal and incubation humidity on embryonic-development and hatchability of broilers. Poultry Sci 66:834-840.

Petersen, A., J. P. Christensen, P. Kuhnert, M. Bisgaard, and J. E. Olsen. 2006. Vertical transmission of a fluoroquinolone-resistant Escherichia coli within an integrated broiler operation. Vet. Microbiol. 116:120-128.

Poulsen, L. L., I. Thofner, M. Bisgaard, J. P. Christensen, R. H. Olsen, and H. Christensen. 2017. Longitudinal study of transmission of Escherichia coli from broiler breeders to broilers. Vet Microbiol 207:13-18.

Pouvreau, P., and S. Baudon. 2016. Incubation of Pekin ducks by single loading with the cuticle on. International Hatchery Practice 30.

Rodriguez-Navarro, A., O. Kalin, Y. Nys, and J. M. Garcia-Ruiz. 2002. Influence of the microstructure on the shell strength of eggs laid by hens of different ages. Br. Poult. Sci. 43:395-403.

513 Rodriguez-Navarro, A. B., N. Dominguez-Gasca, A. Munoz, and M. Ortega-Huertas. 2013.
514 Change in the chicken eggshell cuticle with hen age and egg freshness. Poult. Sci. 92:3026-
515 3035.
516 Samiullah, S., and J. R. Roberts. 2013. The location of protoporphyrin in the eggshell of
517 brown-shelled eggs. Poult. Sci. 92:2783-2788.
518 Samiullah, S., J. R. Roberts, and K. Chousalkar. 2015. Eggshell color in brown-egg laying
519 hens - a review. Poult. Sci. 94:2566-2575.
520 Sirri, F., M. Zampiga, A. Berardinelli, and A. Meluzzi. 2018. Variability and interaction of
521 some egg physical and eggshell quality attributes during the entire laying hen cycle. Poultry
522 Sci 97:1818-1823.
523 Sparks, N. H. C. 1994. Shell accessory materials: structure and function. In: Board R.G.,
524 Fuller R. (eds) Microbiology of the Avian Egg. Springer, Boston, MA:25-40.
525 Sparks, N. H. C., and R. G. Board. 1984. Cuticle, shell porosity and water-uptake through
526 hens eggshells. Br. Poult. Sci. 25:267-276.
527 Sparks, N. H. C., and R. G. Board. 1985. Bacterial penetration of the recently oviposited shell
528 of hens eggs. Aust. Vet. J. 62:169-170.
529 Wellman-Labadie, O., J. Picman, and M. T. Hincke. 2008. Antimicrobial activity of cuticle
530 and outer eggshell protein extracts from three species of domestic birds. Br. Poult. Sci.
531 49:133-143.
532 Wilson, P. W., C. S. Suther, M. M. Bain, W. Icken, A. Jones, F. Quinlan-Pluck, V. Olori, J.
533 Gautron, and I. C. Dunn. 2017. Understanding avian egg cuticle formation in the oviduct: a
534 study of its origin and deposition. Biol. Reprod. 97:39-49.
535 Wolc, A., I. M. S. White, W. G. Hill, and V. E. Olori. 2010. Inheritance of hatchability in
536 broiler chickens and its relationship to egg quality traits. Poultry Sci 89:2334-2340.

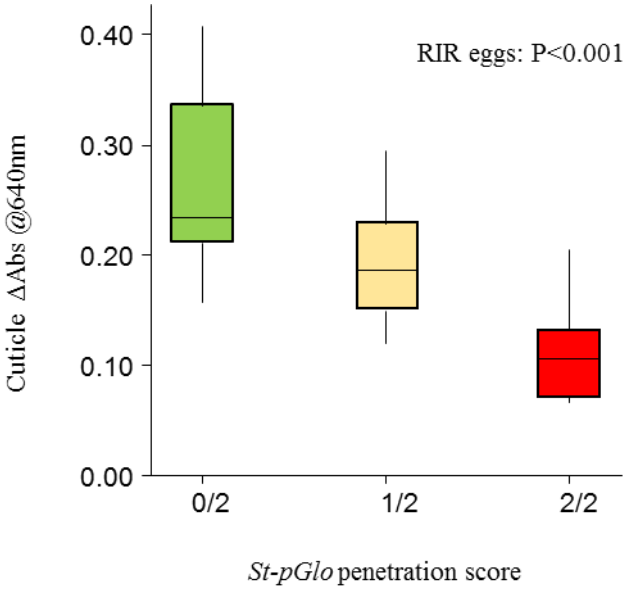
537

538 **Figure 1. Cuticle deposition and *S. Typhimurium* penetration of RIR eggs**

539 Cuticle deposition (Cuticle ΔAbs @640nm) and *S. Typhimurium* (*St-pGlo)* penetration scores
540 for n=2 eggs tested from 46 pure line RIR laying hens (0/2 = no eggs out of two eggs
541 penetrated; 1/2 =one out of two egg penetrated; 2/2 = two out of two eggs penetrated). Data
542 presented as box and whisker plots with median in the box, with 25-75 percentile range as the
543 box and the whisker as 10-90 percentiles.

544

545

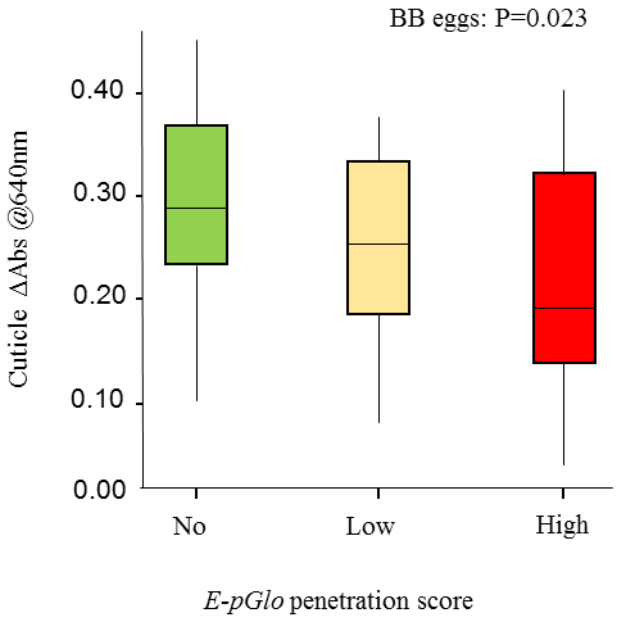

RIR eggs: P<0.001
Cuticle ΔAbs @640nm
0.40
0.30
0.20
0.10
0.00
0/2
1/2
2/2
St-pGlo penetration score

546

Figure 2. Cuticle deposition and *E. coli* penetration of BB eggs

Cuticle deposition (Cuticle ΔAbs @640nm) and *E. coli* (*E-pGlo)* penetration scores for BB eggs sampled from n=73 birds. (No= No penetration; Low <3 translucent areas per egg; High >3 translucent areas per egg). Data is presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles.

553

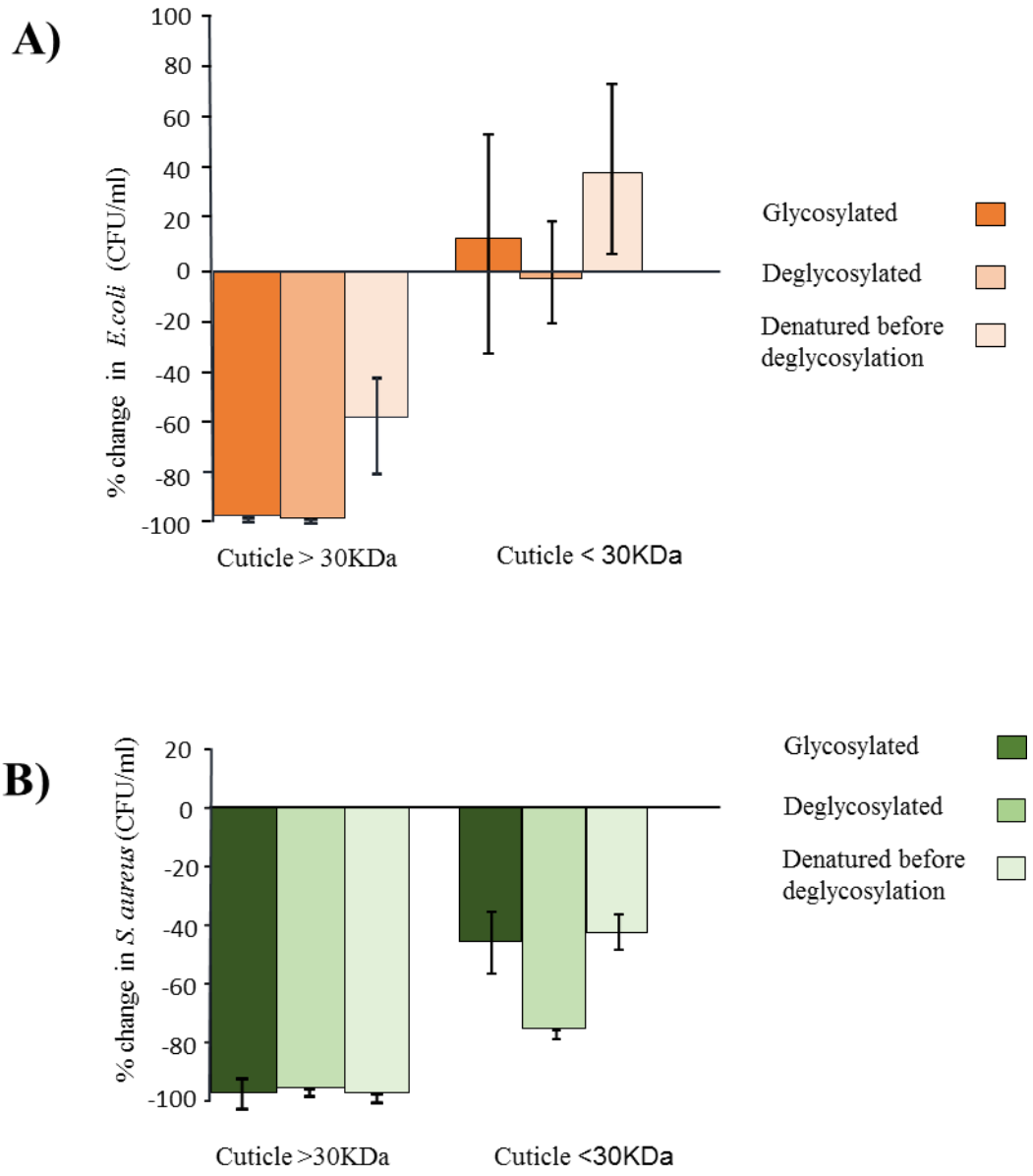

BB eggs: P=0.023
Cuticle ΔAbs @640nm
0.40
0.30
0.20
0.10
0.00
No
Low
High
E-pGlo penetration score

554

555 **Figure 3: Deglycosylation and antimicrobial properties**

556 The effect of deglycosylation on the antimicrobial properties of fractions of cuticle extract >
557 30kDa and < 30kDa. Each fraction was incubated for 3 hr at 37°C with (**A**) *E. coli* or (**B**) *S.*
558 *aureus* in PBS, and the number of surviving bacteria was counted. Results are expressed as a
559 % change in CFU/ml when compared to a PBS control.

560

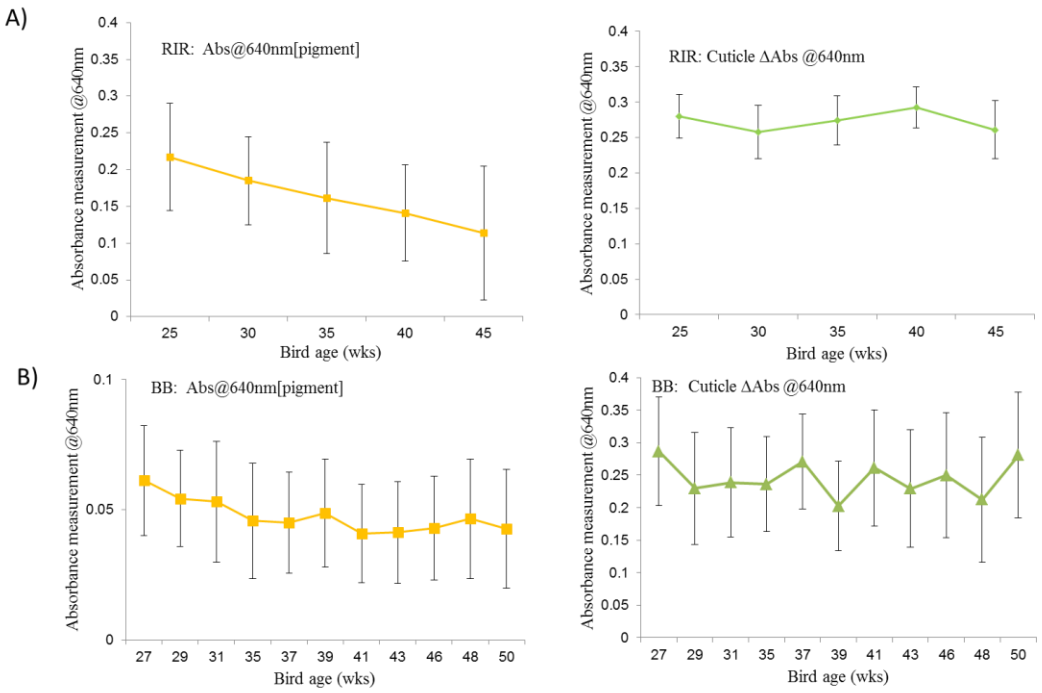

A)
100
80
60
40
20
0
-20
-40
-60
-80
-100
% change in E.coli (CFU/ml)
Cuticle > 30KDa
Cuticle < 30KDa
Glycosylated
Deglycosylated
Denatured before deglycosylation
B)
20
0
-20
-40
-60
-80
-100
% change in S. aureus (CFU/ml)
Cuticle >30KDa
Cuticle <30KDa
Glycosylated
Deglycosylated
Denatured before deglycosylation

561

562

Figure 4: Bird age effect on shell colour and cuticle deposition

Shell colour or brownness (Abs@640nm[pigment]), and cuticle deposition (Cuticle ΔAbs @640nm) measurements on n=2 eggs from **A)** the same individual RIR laying hens (n=32) between 25 and 45wks of age and, **B)** broiler breeder (BB) hens (n=100) between 27 and 50wks of age.


A)
RIR: Abs@640nm[pigment]
Absorbance measurement @640nm
Bird age (wks)
RIR: Cuticle ΔAbs @640nm
Absorbance measurement @640nm
Bird age (wks)
B)
BB: Abs@640nm[pigment]
Absorbance measurement @640nm
Bird age (wks)
BB: Cuticle ΔAbs @640nm
Absorbance measurement @640nm
Bird age (wks)

569

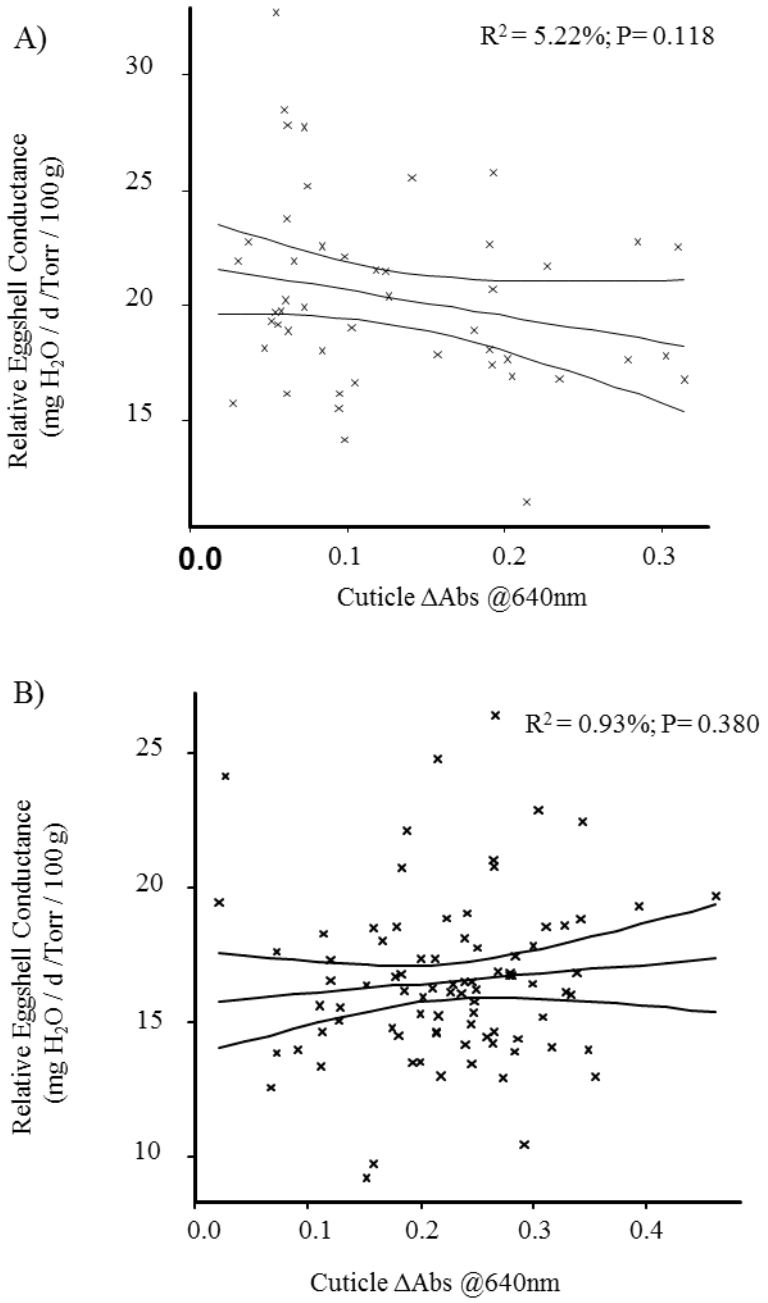
570

571 **Figure 5: Cuticle deposition and relative eggshell conductance**

572

573 Relationship between cuticle deposition (Cuticle ΔAbs @640nm) and relative eggshell
574 conductance in eggs sampled from **A)** LSL layers and **B)** broiler breeders. The correlation
575 coefficient (R^2) and significance value for each regression line is indicated.

576


A)
R^2 = 5.22%; P= 0.118
Relative Eggshell Conductance
(mg H_2O / d /Torr / 100 g)
30
25
20
15
0.0
0.1
0.2
0.3
Cuticle ΔAbs @640nm
B)
R^2 = 0.93%; P= 0.380
Relative Eggshell Conductance
(mg H_2O / d /Torr / 100 g)
25
20
15
10
0.0
0.1
0.2
0.3
0.4
Cuticle ΔAbs @640nm

577

578 **Table 1: Staining and embryo development at 12days incubation**

579 Embryo development in stained and wetted eggs after * 12 days of incubation. Two eggs
580 from 84 broiler breeder hens were weighed, ranked and then randomised into one of four
581 groups, treated (wetted or stained) then incubated in one of two incubators such that each
582 incubator contained equal numbers of stained or wetted eggs.

583

584

	Incubator 1		Incubator 2		P value		
Parameter assessed	Stained (n=47)	Wetted (n= 46)	Stained (n=36)	Wetted (n=46)	Treatment	Incubator	T*I
Egg weight loss (%)	7.37 ± 1.00	7.36 ± 1.28	6.96 ± 0.87	7.49 ± 2.88	0.357	0.682	0.315
Embryo wet weight (minus yolk)(g)	5.75 ± 0.63	5.64 ± 0.60	4.88 ± 0.58	4.86 ± 0.54	0.430	**<.001**	0.641
HH embryonic stage of development	37.32 ± 0.50	37.20 ± 0.40	36.97 ± 0.29	36.96 ± 0.21	0.183	**<.001**	0.328
Length 3rd toe (mm)	6.73 ± 0.60	6.77 ± 0.72	6.65 ± 0.88	6.58 ± 0.79	0.948	0.237	0.627
Length lower mandible (mm)	14.67 ± 1.20	14.93 ± 1.20	14.54 ± 1.44	14.32 ± 1.25	0.554	0.396	0.051

585

586

587