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

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ABSTRACT The cuticle is part of the egg's natural defense and it can be improved by genetic selection. Prior to adoption of this measurement in breeding programs, questions that need to be 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 reduce an egg's susceptibility to penetration by *Escherichia coli* (BB, $P = 0.023$) and *Salmonella 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 color decreased with age as anticipated; however, we found no evidence that cuticle deposition decreases with

age, at least up to 50 wk. A thicker cuticle could affect the water vapor 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. Broiler breeder 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 d 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 vapor conductance

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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 *Salmonella* spp. (Liljebjelke et al., 2005), and several important *Mycoplasma* spp. (Barrow, 1994). Vertical transmission (from parent to offspring)

can occur when the reproductive tissues of breeding females become colonized 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 feces 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 have benefit are *E. coli* species and *Enterococcus faecalis* (Petersen et al., 2006; Fertner et al., 2011). Irrespective of the route or site

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of transfer, entry of pathogenic or zoonotic organisms into the egg contents is undesirable for both animal and public health.

Eggs are naturally equipped with a range of both physical and chemical defenses 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 defense (Bain et al., 2013). This organic layer largely forms on the surface of the calcified shell during the final 1 to 1.5 h 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 (Miksik et al., 2003; Wellman-Labadie et al., 2008; Bain et al., 2013). Several of these proteins have either known or suspected antimicrobial roles (Gautron et al., 2001, 2007). As well as contributing to the eggs chemical defense, 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 inhabs (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 the present paper, we have extended these observations to include more breeds of chicken (layers and broiler breeders [BB]) and other strains of bacteria, e.g., *Salmonella enterica*, serotype typhimurium (*S. typhimurium*). Another aim of the present work was to establish if cuticle deposition changes with bird age. Many common egg shell traits, including eggshell color (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 (Rodriguez-Navarro et al., 2013; Kulshreshtha et al., 2018), 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 adhe-

siveness to the underlying calcified substrate. A further objective of the present 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 a 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 (Peebles and Brake, 1986; Deeming, 1987; 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 the present paper, therefore, 1) provide further evidence that the cuticle is important in preventing bacterial penetration of eggs in genetically divergent breeds of chicken; 2) demonstrate that glycosylation *per se* has no effect on the antimicrobial properties of the proteins in the cuticle; 3) show that cuticle deposition does not diminish with bird age; and 4) 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 an RIR pure line that contributes to the male used to produce Lohmann Brown commercial layers (Lohmann Tierzucht GmbH, Am Seedeich, Cuxhaven), as previously studied (Dunn et al., 2005, 2009, 2012; Bain et al., 2013). The population from which the eggs were sampled consisted of 1,262 female offspring from three hatches. The birds were housed in individual tiered cages in 3 separate environmentally controlled houses on the same site, and were on 16 h of L/d and fed as per Lohmann management guidelines (https://www.ltz.de/de-wAssets/docs/management-guides/en/PS/LTZ_MG_LB-LSL-PS_EN.pdf).

White eggs were sourced from a White Leghorn pure line, used in the production of Lohmann Selected Leghorn (LSL) commercial layers (Lohmann Tierzucht GmbH, Am Seedeich, Cuxhaven). The population, from which we sampled, in this case, consisted of 948 female offspring derived from 2 hatches, which were housed in

individual tiered cages in 3 separate environmentally controlled houses on the same site. These birds also received 16 h of L/d and were fed as per Lohmann management guidelines.

Broiler breeder eggs were obtained from a fully pedigree female broiler pure line from Aviagen Limited (Newbridge, Scotland, UK) breeding program. The population, from which we sampled, in this case, comprised 1,459 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 h of L/d, and was 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 our 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, UK), and the difference before and after dyeing was used to estimate the cuticle deposition (cuticle Δ Abs@640 nm). The eggs used in our experiments were tested less than 3 d after collection and were transported and maintained at a temperature of between 8°C and 12°C.

In addition, the pre-stain absorbance at 640 nm (Abs@640 nm [pigment]) was used to estimate shell color 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, <https://oceanoptics.com/>) was used to calibrate the instrument between the experiments.

Bacterial Penetration Experiments

To increase our power to detect differences in the effect of cuticle deposition on bacterial penetration, 2 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 51 wk of age. This was possible as we had previously measured the cuticle deposition in the entire population at 30 to 32 wk of age, and the genetic correlation within hens at different ages for cuticle coverage was very high (1.00; I.C.Dunn,

unpublished data). The test organism used on the RIR eggs was a non-pathogenic laboratory strain of *S. typhimurium* containing plasmid pGlo (*St-pGlo*: SL1344 *htrA* mutant pGLO). Cuticle deposition was also measured on 2 additional 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 1 of the BB flocks at 41 wk of age for this study. One egg from each hen was used for bacteriology; the other 2 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°C. 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 between 0 and 2 depending on whether zero, 1, or 2 out of 10 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 categorized 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 further categorize each single test egg 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 to be carried out 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 3 and treated in 1 of the following ways: 1) untreated (glycosylated); 2) denatured and then deglycosylated

using a New England BioLabs Protein Deglycosylation Mix (P6039S); and 3) deglycosylated using the same P6039S 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 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 as non-pathogenic laboratory strains. In each case, the test bacteria were cultured overnight at 37°C in Luria broth (LB) for *E. coli* DH5 α , and Tryptone Soya Broth for *S. aureus* (RN4220); 250 μ l of overnight culture was sub-cultured into 20 ml of LB and incubated at 37°C for 3 h. After the second incubation, 20 μ l of culture was diluted with 2 ml of PBS, pH 7.4. Glycosylated or deglycosylated cuticle protein fraction (10 μ l) was then added to 50 μ l of diluted culture. After vortexing, this was incubated at 37°C for 3 h, and then the suspensions were serially diluted to 1×10^{-4} with 225 μ l of PBS; all dilutions were then plated on LB agar or Tryptone Soya Agar plates in duplicate and incubated overnight at 37°C. The colonies were then counted, and the results were expressed as a reduction in colony-forming units per milliliter compared to a PBS control.

Cuticle Deposition and Shell Color (Brownness) with Bird Age

Eggs from 4 consecutive d of production from 32 individual birds in our RIR population were collected every 5 wk from 25 to 45 wk of age. Shell color or brownness (Abs 640 nm [pigment]) and cuticle deposition (cuticle Δ Abs@640 nm) measurements were carried out on the 1st 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 to 4 wk from 27 to 50 wk 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 color or brownness.

Cuticle Deposition and Water Vapor Conductance

Four eggs from 24 LSL laying hens at the top and 24 from the tail of the cuticle distribution at 51 wk of age, and 2 eggs from 85 individual BB hens from the same flock at 41 wk of age were used in this study. For the LSL eggs, cuticle deposition measurements were carried out on 2 of the eggs per individual, and 2 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 (De Ketelaere et al., 2000; Bain et al., 2006) to ensure that only intact eggs were used.

Water vapor conductance (WPC) measurements were carried out using the method described by Peebles and McDaniel (2004). In brief, all eggs were held under standard storage conditions for 24 h. Fresh egg weight was then measured to an accuracy of 0.1 mg, prior to the eggs being placed randomly into 1 of 2 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°C for 4 d. Every 24 h the desiccant in each cabinet was replenished and the average local temperature (°C) and barometric pressure (mm Hg: Torr) were recorded. Egg weight was recorded at 24 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 2 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 1 of 4 groups so that there was an equal weight distribution in each group. Groups 1 and 2 eggs were stained for cuticle deposition, and 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 1 of 2 tabletop incubators (OvaEasy 380 Advance EX Series II, Brinsea, North Somerset, UK) and incubated at 37.5°C and RH of 60%. On d 12, the eggs were removed from the incubator, weighed, and then placed in a fridge (4°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 1st photographing and then measuring using ImageJ software (<https://imagej.nih.gov/ij/>). 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 analyzed using a General Linear Model with treatment (stained or wetted) and incubator (A or B) as the main

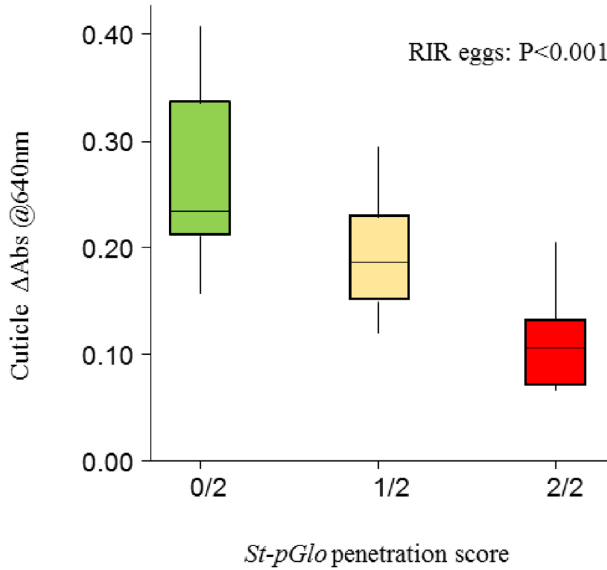


Figure 1. Cuticle deposition and *S. typhimurium* penetration of RIR eggs. Cuticle deposition (cuticle $\Delta\text{Abs}@640\text{nm}$) and *S. typhimurium* (*St-pGlo*) penetration scores for $n = 2$ eggs tested from 46 pure line RIR laying hens (0/2 = no eggs out of 2 eggs penetrated; 1/2 = 1 out of 2 egg penetrated; and 2/2 = 2 out of 2 eggs penetrated). Data presented as box and whisker plots with median in the box, with 25 to 75 percentile range as the box and the whisker as 10 to 90 percentiles.

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 (cuticle $\Delta\text{Abs}@640\text{nm}$) and *S. typhimurium* (*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 1 out of 2 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 cuticle deposition (cuticle $\Delta\text{Abs}@640\text{nm}$) was lower ($P = 0.011$) in penetrated eggs (0.231 ± 0.101 ; $n = 33$) than in non-penetrated eggs (0.288 ± 0.090 ; $n = 40$). There was also a significant difference ($P = 0.023$) when penetrated BB eggs were further categorized as having no, low (<3 discrete luminescent areas per egg), or high penetration (>3 discrete luminescent areas per egg). Eggs from hens that had low penetration ($n = 14$) had moderate cuticle deposition, and eggs from hens with

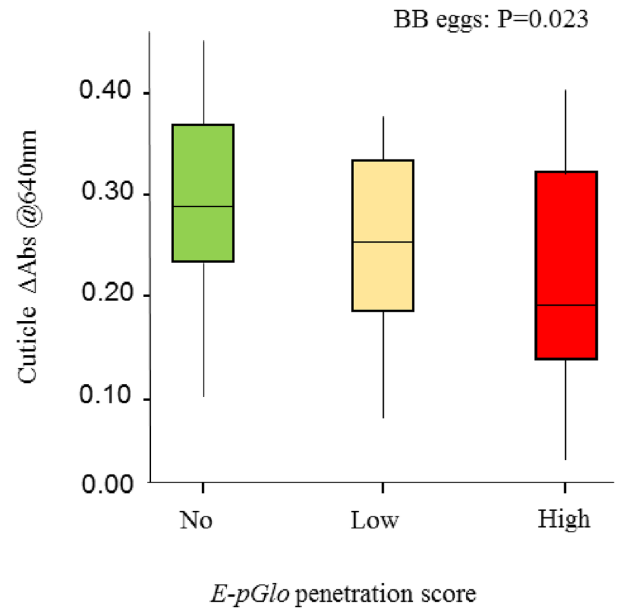


Figure 2. Cuticle deposition and *Escherichia coli* penetration of broiler breeder (BB) eggs. Cuticle deposition (cuticle $\Delta\text{Abs}@640\text{nm}$) and *E. coli* (*E-pGlo*) penetration scores for BB eggs sampled from $n = 73$ birds. (No = no penetration; low = <3 translucent areas per egg; and high = >3 translucent areas per egg). Data are presented as box and whisker plots with median in the box, with 25 to 75 percentile range as the box and the whisker as 10 to 90 percentiles.

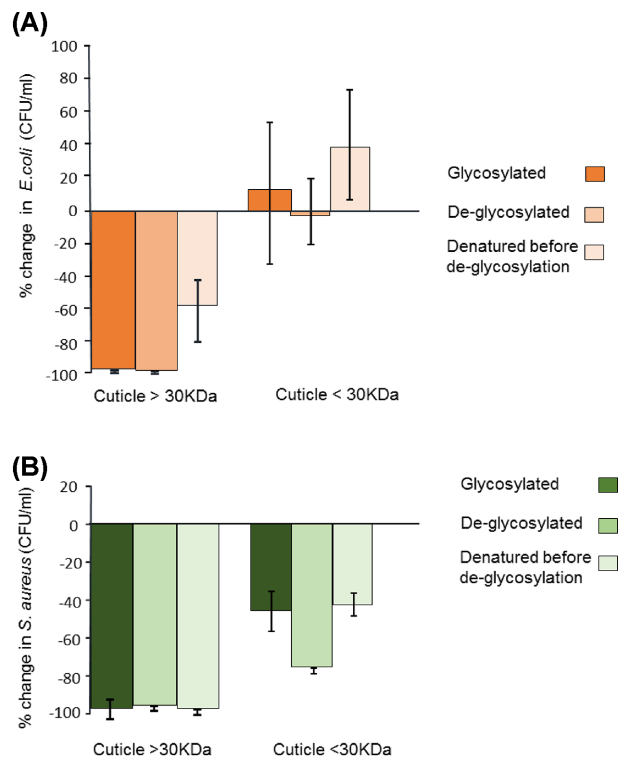


Figure 3. Deglycosylation and antimicrobial properties. The effect of deglycosylation on the antimicrobial properties of fractions of cuticle extracts > 30 and < 30 kDa. Each fraction was incubated for 3 h at 37°C with (A) *Escherichia coli* or (B) *Staphylococcus aureus* in PBS, and the number of surviving bacteria was counted. Results are expressed as a % change in colony-forming units per milliliter when compared to a PBS control.

high penetration ($n = 19$) had poorer cuticle deposition. Eggs from hens that were not 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 (>30 kDa) 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 >30 kDa cuticle protein extract was deglycosylated; however, when this protein fraction was denatured prior to being deglycosylated, there was a significant reduction in the ability to kill *E. coli* (Figure 3A). The smaller cuticle extract proteins (<30 kDa) showed no activity against *E. coli* (Figure 3A), and only moderate activity (46%) against *S. aureus*. For *S. aureus*, the antimicrobial activity of this fraction 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 Color (Brownness) with Bird Age

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

Broiler breeder eggs contain much less brown pigment than RIR layer and, as for layers, the amount of pigment significantly decreased with bird age (BB: Abs@640 nm [pigment], $P < 0.001$; Figure 4C). The outcome of the repeated measures analysis for cuticle deposition in this case, however, was also significant (BB: cuticle Δ Abs@640 nm, $P < 0.001$), Figure 4D), 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 wk was similar to that observed at 27 wk.

Cuticle Deposition and Water Vapor Conductance

The results of the regression analysis for cuticle deposition and the relative eggshell conductance measurements are shown 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.

Staining Eggs for Cuticle Deposition Measurement and Embryonic Development

Staining eggs for cuticle assessment prior to setting had no effect on % egg weight loss, embryo wet weight, length of the 3rd toe, length of the lower mandible, or the HH stage of embryonic development after 12 d of incubation, when compared to the wetted controls (Table 1). An incubator effect ($P < 0.001$) was observed for embryo wet weight (minus yolk) and the HH stage of development ($P < 0.01$). The length of the lower mandible also tended to be greater in incubator A. No interaction between treatment and incubator was observed for any of the measurements.

DISCUSSION

Previously, we reported that RIR hens that laid eggs with good cuticle deposition were never penetrated by *E. coli*, whilst those with poor cuticle deposition were often penetrated (Bain et al., 2013). Now, by including a laboratory strain of *Salmonella* and also BB eggs in our penetration studies, we have demonstrated that these observations are likely to be ubiquitous. For BB eggs, we found that there was a lot of variation in cuticle deposition and so even within the higher penetration category the distribution appears skewed (Figure 2). Eggshell quality traits in general have received limited attention in BB breeding programs compared to layers and so it could be that factors other than cuticle deposition had an effect on the BB results. Nevertheless the overall impression is that cuticle deposition is also of importance in meat types of chicken.

Cuticle deposition has previously been shown to have a moderate heritability in the same pure line of RIR sample as was sampled from here (Bain et al., 2013). Recent evidence shows that this is also the case across independent and generically divergent lines (Dunn et al., unpublished data). Collectively, these results provide evidence that incorporating our cuticle deposition measurement into breeding programs of egg and meat types of chicken will lead to improvement in cuticle coverage and hence a reduction in the transmission of potentially pathogenic organisms via the egg. This will help to improve biosecurity in the poultry industry.

Rodriguez-Navarro et al. (2013) proposed that glycosylation of proteins in the cuticle was 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*. *Staphylococcus aureus*, unlike *E. coli*, lacks an outer membrane. It might be that correct folding of the antimicrobial protein(s) is required to penetrate the outer membrane. This could explain the differential effect denaturation

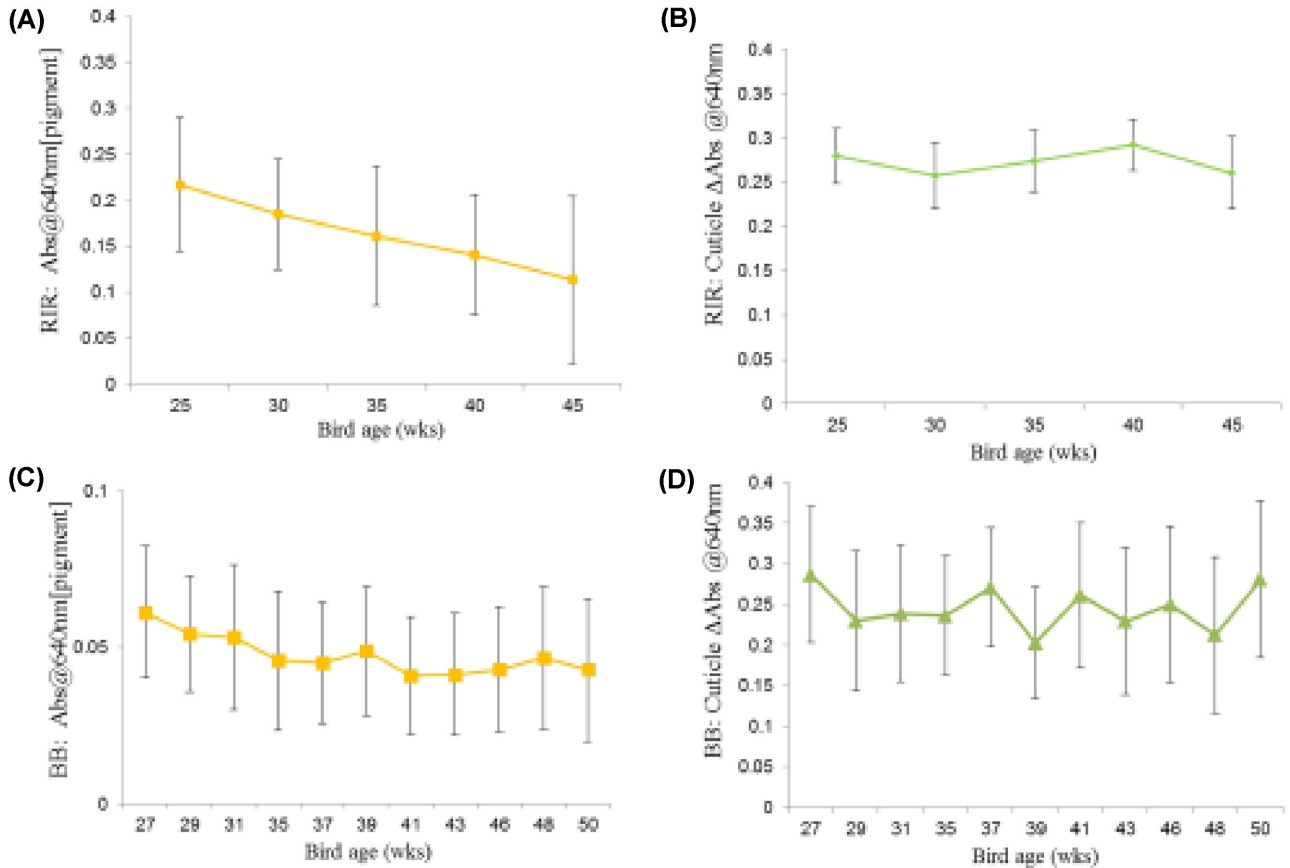


Figure 4. Bird age effect on shell color and cuticle deposition. **A** and **B**: Shell color or brownness (RIR: Abs 640nm [pigment]) and cuticle deposition (RIR: cuticle Δ Abs@640 nm) measurements on $n = 2$ eggs sampled from the same individual RIR laying hens ($n = 32$) between 25 and 45 wk of age. **C** and **D**: Measurements of shell color or brownness (broiler breeder [BB]: Abs@640 nm [pigment]) and cuticle deposition (BB: cuticle Δ Abs@640 nm) on $n = 2$ eggs sampled from the same BB hens ($n = 100$) between 27 and 50 wk of age.

had on the antimicrobial activity of the >30 kDa proteins.

Cuticle extract containing small molecular weight proteins (<30 kDa) 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 sanitizers 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 color, and albumen quality, are known to decrease with bird age (Kemps et al., 2006; Bozkurt and Tekerli, 2009; Samiullah et al., 2015; Bain et al., 2016; Sirri et al., 2018). In the present study, we measured the brownness of eggs as the absorbance at 640 nm and found this to decrease in both pedigree lines of RIR and BB's. However, an age-related decline in cuticle deposition was not ob-

served. This is consistent with the findings of Ball et al.'s (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 2 time points, say 27 and 39 wk 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 present study been extended beyond 50 wk (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 wk of age compared to 25-wk-old hens (Dominguez-Gasca et al., 2017).

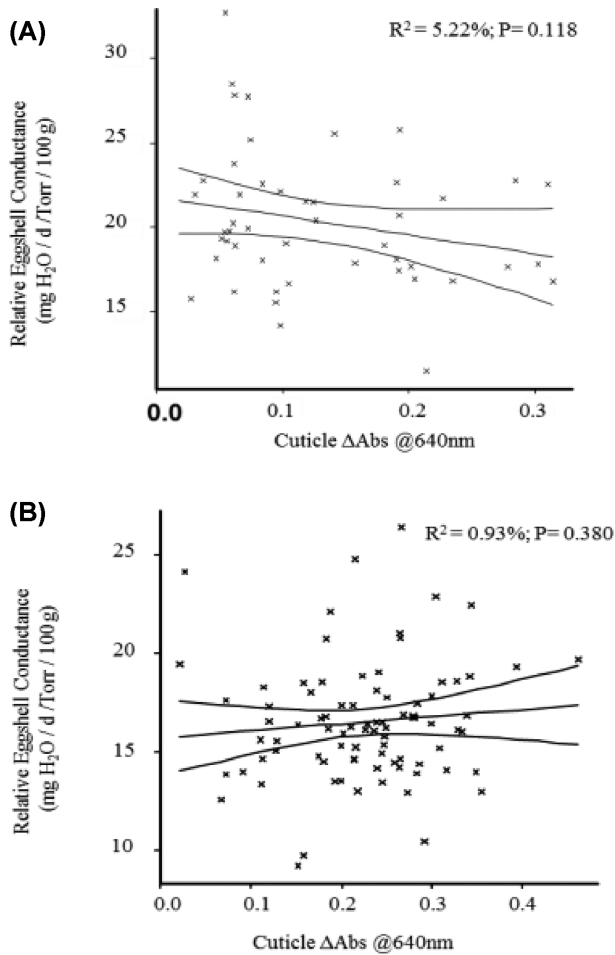


Figure 5. Cuticle deposition and relative eggshell conductance. Relationship between cuticle deposition (cuticle $\Delta\text{Abs}@640\text{ nm}$) and relative eggshell conductance in eggs sampled from **A**) Lohmann Selected Leghorn (LSL) layers and **B**) broiler breeders (BB). The correlation coefficient (R^2) and significance value for each regression line are indicated.

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 limited evidence for an age-related decline in cuticle quality comes from 2 studies where the cuticle was measured by both staining and infrared spectroscopy (Rodriguez-Navarro et al., 2013; Kulshreshtha et al., 2018). 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 defense. 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 vapor diffusion in BB eggs, depending on the age of the bird which laid the egg (Peebles and Brake 1986; Deeming, 1987; 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 WPC 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 customized 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 s, it was important to test whether this had any effect on normal embryonic development. In

Table 1. Staining and embryo development at 12 d of incubation. Embryo development in stained and wetted eggs after 12 d of incubation. Two eggs from 84 broiler breeder (BB) hens were ranked by weight and then randomized into 1 of 4 groups, treated (wetted or stained), and then incubated in 1 of 2 incubators (A or B) such that each incubator contained equal numbers of stained or wetted eggs.

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

our experiment, we compared the development of embryos in wetted vs. stained eggs, at 12 d 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 a common practice to measure the specific gravity on eggs prior to setting, in BB 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 entry to the egg contents. We have also demonstrated for the first time that cuticle deposition does not naturally decrease in genetically diverse lines of egg and meat types of chicken, at least up to 50 wk of age. We also found no evidence that selection for improved cuticle deposition will have an adverse effect on WPC of the shell. This is important as a controlled loss of water through incubation is critical for normal embryo development. In the broiler industry, where eggs are especially precious (Hocking, 2014), there is no evidence, at least with the power available in this study, that those eggs cannot be successfully incubated after the staining and measurement has been carried out.

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