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# **Enhancing the egg's natural defence against bacterial penetration by increasing cuticle deposition**

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## Summary

The cuticle is a proteinaceous layer covering the avian egg and is believed to form a defence to microorganism ingress. In birds that lay eggs in challenging environments the cuticle is thicker suggesting evolutionary pressure, however in poultry selection pressure for this trait has been removed because of artificial incubation. This study aimed to quantify cuticle deposition, to estimate its genetic parameters and its role on trans-shell penetration of bacteria. Additionally cuticle proteins were characterised to establish if alleles for these genes explained variation in deposition.

A novel and reliable quantification was achieved using the difference in reflectance of the egg at 650nm before and after staining with a specific dye. The heritability of this novel measurement was moderate (0.27) and bacteria penetration was dependent on the natural variation in cuticle deposition. Eggs with the best cuticle were never penetrated by bacteria ( $P < 0.001$ ). The cuticle proteome consisted of six major proteins. A significant association was found between alleles of one of these protein genes, ovocalyxin-116, and cuticle deposition ( $P = 0.015$ ) and also between alleles of oestrogen receptor and cuticle deposition ( $P = 0.008$ ).

With the heritability observed, genetic selection should be possible to increase cuticle deposition in commercial poultry, so reducing trans-generational transmission of micro-organisms and reversing the lack of selection pressure for this trait during recent domestication.

**Keywords:** eggshell cuticle deposition, avian eggs, egg natural defence, trans-generational transmission of microorganisms

## **Introduction**

The cuticle is the proteinaceous layer which is deposited onto the surface of eggs during the final 1-1.5 hours prior to oviposition (Baker & Balch 1962). At oviposition the cuticle takes about 3 minutes to dry (Sparks & Board 1985) creating an effective barrier to the movement of water across the shell by plugging the gaseous exchange pores (Ruiz & Lunam 2000). The movement of water across the eggshell through the pores provides a pathway for bacterial contamination of the egg contents (Sparks & Board 1984). This is corroborated by the evidence that the removal of the cuticle or egg washing can enhance bacterial penetration of the shell (Board & Halls 1973; Wang & Slavik 1998). The cuticle, therefore, is the eggs first line of defence against bacterial invasion. Normally the cuticle is likely to be under strong natural selection in birds such as pelicans or flamingos that live in damp and presumably more microbiologically challenging environments have much thicker cuticles than chickens or quail (Kusuda *et al.* 2011). In modern egg production to a large extent the use of artificial incubation will have lessened the selection pressure for this trait.

The belief that the cuticle forms a physical barrier to bacterial penetration has been tested by several authors and a range of methods e.g.(Board & Halls 1973; Messens *et al.* 2005) but these accounts focus on the presence or absence of the cuticle rather than the natural variation in cuticle deposition. Moreover, the use of bacteria to assess eggshell penetration has been hampered by the requirement for asepsis and complicated procedures requiring sectioning of eggs or filling eggs with agar (Messens *et al.* 2005).

The fact that the cuticle of the chicken's egg reacts with a large number of histochemical stains specific for protein moieties confirms that protein is a major

component (Sharp 1932). Whilst the use of proteomics has led to the discovery of a cornucopia of proteins in the eggshell, albumen and yolk (Mann *et al.* 2006; Mann 2007; D'Ambrosio *et al.* 2008; Mann & Mann 2008; Farinazzo *et al.* 2009), the cuticle has proven to be much more difficult to analyse because it is not easy to solubilise. Despite this, results are available from both proteomic studies (Miksik *et al.* 2010; Rose-Martel *et al.* 2012) and studies that have identified individual cuticle associated proteins either by western blotting or immunohistochemistry (Gautron *et al.* 2001; Wellman-Labadie *et al.* 2008). The antimicrobial activity of some of these cuticle associated proteins has been suggested (Gautron *et al.* 2001; Gautron *et al.* 2007) and in some cases demonstrated (Wellman-Labadie *et al.* 2008). This is not surprising given that the cuticle is strategically located for antimicrobial defence.

A simple qualitative staining technique to assess the cuticle coverage of eggs which involves immersing eggs in 1% (w/v) aqueous solution of Edicol Supra Pea Green H dye was described in the 1970's (Board & Halls 1973). Using a subjective assessment of the eggshells staining characteristics it was observed that there is a great deal of variation in cuticle deposition on eggs laid by individual hens and between different breeds (Ball *et al.* 1975; Sparks 1994) Further evidence for breed differences are observations that the cuticle is thicker in brown versus white eggs (Simons 1971; Board & Halls 1973). Taken together this suggested that genetics may be responsible for a significant component of this traits variation and there may be a genetic link between pigment and cuticle deposition.

The aims of this study were to establish a method to reliably quantify the cuticle deposition in brown eggs and to establish the cuticle's functional significance in the context of its natural variation. Specifically we have established the role of the cuticle in preventing bacterial penetration within the normal biological range of variation and

have quantified the heritability and genetic correlations of our measurement. This has allowed its potential for genetic selection to be assessed to counter the effects of the lack of natural selection. We have also determined the composition of the cuticle using proteomics and examined whether alleles of genes encoding for these proteins, as well as a gene involved with oviduct function at the time of cuticle deposition, have association with cuticle deposition.

## **Methods**

### **Assessment of egg colour and cuticle deposition**

#### *Animals and egg collection*

A Rhode Island Red pedigree line that contributes to the male line used to produce Lohmann Brown commercial layer hens was used in this study. Specifically we used a population of 684 female offspring derived from 38 sires and 142 dams. This population was a later generation from the same genetic line previously described (Dunn *et al.* 2005; Dunn *et al.* 2009; Dunn *et al.* 2012) . The hens were housed in individual cages on 16 hours of light per day in four separate huts at the same location. Two eggs per bird were collected between 50 -52 weeks of age for cuticle deposition determination. The total egg production over the lifespan of each bird (20-48, and 62-68 weeks of age) and breaking strength at 43 weeks of age as described in (Dunn *et al.* 2005) was used to quantify genetic correlation with the cuticle deposition.

#### *Method for measuring cuticle deposition*

The % reflectance spectrum of each egg was determined at three equidistant points around the equator of each egg using an ISP-REF integrated sphere

spectrophotometer (Ocean Optics, Inc) calibrated against a reference white and black tile. Each egg was then immersed in cuticle stain (MST cuticle blue, M.S. Technologies Ltd, Telford Way, Kettering, Northants) for 15 minutes rinsed in clean water to remove excess stain then dried for 24 hours. The % reflectance spectra measurements were subsequently repeated on each egg post staining. The pre versus post spectra for each egg was compared to determine the optimal wavelength at which the greatest difference in % reflectance occurred (Figure 1). The pre-stain % reflectance at this optimum wavelength was used as our measurement of shell colour intensity. Our cuticle deposition measurement was taken as the difference in pre versus post stain % reflectance at the optimum wavelength (average of  $n = 3$  measurements per egg).

### **Calculations of heritability and genetic correlation for cuticle deposition**

The methods used to calculate heritability and genetic correlations have been described previously (Dunn *et al.* 2005). All calculations were based on data from the average of two eggs per bird laid between 50 and 52 weeks of age. Heritabilities were estimated from the following model,

$$Y_{ijk} = \mu + h_i + s_j + d_{jk} + e_{ijk}. \quad (1)$$

where  $Y_{ijk}$  is the trait,  $\mu$  is the overall mean,  $h_i$  is the fixed effect of the house,  $s_j$  and  $d_{jk}$  are the random effects of sires and dams within sires and  $e_{ijk}$  is the residual. Model parameters were estimated by residual maximum likelihood (REML, (Patterson & Thompson 1971)). Genetic correlations were estimated from a bivariate mixed model with the same linear terms as model (1) above. All calculations were performed in Genstat version 12.1 (VSN International Ltd, Oxford, UK).

## **Investigating the cuticle proteome**

### *Source of eggs*

The eggs used in this experiment were obtained from birds which had been transferred to sterile single bird plastic cages just prior to the collection period. Each egg was collected from the cage front using an inverted sterile re-sealable plastic bag (Transpack Ltd, Hants Southampton, UK). All eggs were subsequently washed under running distilled water for 15 minutes and candled before being chosen for the extraction experiments. Every attempt was made to avoid handling the eggs throughout these procedures. The eggs were then placed in new sterile re-sealable plastic bags and treated as follows.

### *Liberation of the cuticle using 5% EDTA*

Six eggs were washed with 70% ethanol before being individually placed into sterile re-sealable plastic bags containing a 10ml solution of 5% (0.13mol/l) EDTA (PH 7.6) with 10 mmol/l 2-mercaptoethanol. The insoluble organic layer on the surface of each egg was liberated by gently rubbing the solution over the eggs surface for 1 hour at room temperature. The eggs were removed and the solution plus liberated cuticle centrifuged at 1000g for 15 minutes. The pellets from each egg were pooled, re-suspended in water, centrifuged again (x3) then lyophilised and stored at -20°C.

### *Liberation of the cuticle using 1N HCl*

A 10ml solution of 1N HCl was added to a sterile re-sealable plastic bag containing a single egg. The solution was massaged over the eggs surface for 1 minute. The egg was then removed and replaced by another egg. This procedure was repeated using a



total of 6 eggs. The accumulated cuticle extract was then dialyzed against Milli-Q water, lyophilized and stored at -20°C.

#### *Trypsin enzymatic cleavage, separation of peptides*

The lyophilized cuticle extracts were reconstituted in ammonium hydrogen carbonate buffer (pH 7.8). A 100µL aliquot of Trypsin dissolved in 0.01mol/L HCL (pH 2) was added; the samples were sonicated for 5 minutes and then incubated at 37°C for 36hrs. Each sample was then centrifuged for 10 mins at 2000g and the supernatants removed and stored at -20°C. Any remaining solid material was also stored for subsequent investigation.

#### *Electron spray Ionisation (ESI) Mass Spectrometry*

Tryptic peptide samples were separated on an LC system (Famos / Switchos / Ultimate, LC Packings) before being analysed by electrospray ionisation (ESI) mass spectrometry on a Q-STAR<sup>®</sup> Pulsar i hybrid LC/MS/MS System. Peptide separation was performed on a Pepmap C18 reversed phase column (LC Packings), using a 5 - 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45 min. The flow rate was maintained at 0.2 µl / min / min. Mass spectrometric analysis was performed using a 3 second survey MS scan followed by up to four MS/MS analyses of the most abundant peptides (3 seconds per peak) in information dependent acquisition' (IDA) mode, choosing 2+ to 4+ ions above threshold of 30 counts, with dynamic exclusion for 180 seconds.

#### *Data analysis and criteria for protein identification*

Data generated from the Q-STAR<sup>®</sup> Pulsar i hybrid mass spectrometer was analysed using Applied Biosystems Analyst QS (v1.1) software and the automated Matrix Science Mascot Daemon server (v2.1.06). Protein identifications were assigned using the Mascot search engine, which gives each protein a probability based MOWSE score. Searches were performed against all of the vertebrate sequences in the NCBI database. In all cases variable methionine oxidation was allowed in searches. An MS tolerance of 1.2 Daltons (Da) for MS and 0.4 Da for MS/MS analysis was used. The relative quantification of the identified proteins was calculated using the exponentially modified protein abundance index (emPAI) (Ishihama *et al.* 2005).

## **Genetic Association Studies**

Blood and DNA were obtained as described previously (Dunn *et al.* 2004). SNP markers known to segregate in the population for ovocleidin 116, ovocalyxin 36, ovoclayxin 32, ovalbumin and oestrogen receptor (Marker names: Oc116\_310, Oc116\_1336, Ovocal36, Ovocal32\_626, OVAL, Oer\_2571), were tested for association with our measure of cuticle deposition as described previously (Dunn *et al.* 2009). These genes are either present in the shell or have evidence of affecting egg formation. Briefly, linear models were fitted by REML, followed by approximate Student's t-tests to assess marker effects. The additive effect of each marker was estimated as half the difference between homozygote means.

## **Cuticle deposition and susceptibility to bacterial ingress**

### *Source of eggs*

Using the offspring from the population used to calculate the heritability and genetic correlation we were able to identify dams that were likely to produce offspring that

laid eggs with poor or good cuticle deposition which we confirmed by measurement. This increased our statistical power to detect the effect of cuticle deposition on bacterial penetration. Four unstained eggs from the 27 individuals displaying the extremes in terms of cuticle coverage were collected at 40 weeks of age and used in the bacterial penetration assay described below.

#### *Bacterial Penetration Assay*

*E. coli* containing plasmid pGLO (BIO-RAD Laboratories), were grown overnight with shaking at 37°C in L-broth containing 100 µg/ml carbenicillin. Cultures were inoculated at a dilution of 1:50 in fresh L-broth containing 100 µg/ml carbenicillin and either 5 mM or 1 mM L-arabinose. These were grown for a further 2-3 h with shaking at 37°C to an OD<sub>600</sub> of 0.3-0.4 (this corresponds to approximately 5 x 10<sup>8</sup> cfu/ml). 10ml aliquots of this broth were then decanted into sterile re-sealable plastic bags and placed into an ice bath.

All eggs was candled, swabbed with ethanol, then individually placed in a clean re-sealable plastic bag and incubated for 1 hour at 37°C. Each egg was then transferred into one of the zip lock bags containing 10 mls of the chilled inoculum and immersed into an ice bath for 15 minutes. The egg was subsequently transferred back into its original locker-sealable plastic bag and incubated for a further 24hours at 37°C.

Penetration of the shell by *E. coli* pGLO was determined by observing the inner surface of each egg after removal of the egg contents under a long wave UV light source. Penetration by *E. coli* pGLO was confirmed by the presence or absence of luminescence spots (Figure 2). Normally no light is emitted from the inner surface of an eggshell in response to the UV part of the spectrum. If any one of the four eggs

laid by an individual hen were penetrated, then the bird was assigned a score of 1. If no eggs from an individual hen were penetrated the bird was assigned a score of 0.

The average cuticle deposition measurement for each bird was calculated using an additional four eggs from each individual.

### *Data analysis*

A Mann-Whitney U (Wilcoxon rank-sum) test was used to analyse the data from the penetration experiments because the data was not normally distributed due to the selection of hens from families predicted to be at the top and tail of the cuticle deposition distribution.

## **Results**

### **Cuticle Deposition Measurement**

To determine the optimal wavelength to measure the cuticle deposition, the spectrum of each unstained egg was compared with the reflectance spectrum of the same egg after staining (Figure 1). The greatest difference in reflectance pre and post staining occurred between ~ 590nm and 700nm. This corresponds to the orange-red part of the visible spectrum.

The difference in % reflectance at 650nm ( $\Delta 650\text{nm}$ ) had the highest repeatability score, the between bird component accounted for 66.3% of the variance whilst between egg within bird variance was 0.2% and the between sample within egg 1.4%. This wavelength was therefore used in subsequent experiments to determine the heritability and genetic correlation of cuticle deposition and in the subsequent association studies.

In Table 1 the % reflectance pre-staining provides an estimate of the intensity of shell colour whilst the difference between the pre and post staining measurements ( $\Delta 650\text{nm}$ ) provides an estimate of the cuticle deposition with a mean decrease in the reflectance of 26.9%.

## **Heritability and Genetic Correlations**

The sire estimate of heritability for our measurement of cuticle deposition ( $\Delta 650\text{nm}$ ) was moderate whilst as expected the heritability for our measure of intensity of shell colour (% reflectance pre staining) was high (Table 2). The genetic correlation between cuticle deposition and intensity of shell colour was  $0.31 \pm 0.29$  while the phenotypic correlation was 0.08. This suggests that there was no significant genetic correlation between cuticle deposition and intensity of shell colour. Similarly there was no evidence of a genetic correlation between cuticle deposition and total egg production or breaking strength at 43 weeks of age ( $0.19 \pm 0.52$  and  $0.29 \pm 0.43$  respectively).

## **The cuticle proteome**

The merged results of our proteomic studies on the EDTA and 1N HCL cuticle extracts are presented in Table 3. The two methods of extraction gave similar results in that six major proteins were identified. The most abundant cuticle protein detected in the EDTA cuticle extract was ovocalyxin-36 then ovocalyxin-32 with emPAI values of 0.34 and 0.26, respectively. For the 1NHCl cuticle extract the most abundant proteins detected were ovocleidin-116 and ovocleidin-17 (emPAI values of 0.67 and 0.56).

## **Genetic Association Studies**

Significant association was seen with alleles for the marker Oc116\_310 (ovocalyxin-116) accounting for 15% of trait SD and Oer\_2571(oestrogen receptor) representing 17% of the trait SD (Table 4). There was no significant association with any of the other candidate gene markers tested. If the beneficial alleles were fixed in the flock the improvement to the flock mean for cuticle deposition would be 1 and 8% for the Oc116\_310 and the Oer\_2571 marker respectively.

## **Cuticle Deposition and Bacterial Penetration**

The presence of luminescent spots on the inner surface of the eggshell confirmed that some of our eggs had been penetrated by our test bacteria (Figure 2).

There was a significant difference in cuticle deposition between the eggs which had been penetrated and those which had not been penetrated (18.2 $\pm$ 2.6 versus 31.5 $\pm$ 2.2;  $P < 0.001$ ). The difference between penetrated and non-penetrated eggs in terms of their cuticle deposition can be visualised in Figure 3. The lower panel shows that there were no eggs penetrated when the cuticle deposition was at its best. Conversely the upper panel demonstrates that all eggs were penetrated if they had the poorest cuticle quality.

## **Discussion**

In this study we have successfully quantified the natural variation of cuticle deposition in a line of laying hens using a combination of staining and reflectometry. The innovation in this study is the development of a repeatable quantifiable measurement of cuticle deposition as previous attempts were either qualitative or had poor repeatability (Ball *et al.* 1975; Drysdale 1985; Leleu *et al.* 2011). Using single

wavelength reflectometry at 650nm before and after staining with a cuticle dye (Figure 1), we found good repeatability for our  $\Delta 650\text{nm}$  measurement of cuticle deposition between eggs from the same individual (0.66) compared to taking the same measurements at other single wavelengths. Values obtained using the difference between the Lab colour measurement value before and after staining had low repeatability (0.14) possibly due to the multiple factors which this system measures and the way it represents colour although it is very successful for improving the colour of eggs (Forster *et al.* 1996).

Using our  $\Delta 650\text{nm}$  measurement we then demonstrated that this trait has moderate heritability (0.27, Table 1) and can be used in selection programs to improve cuticle deposition. This may help reverse any reduction in cuticle deposition and functionality which has occurred due to the high use of artificial incubation in modern commercial stock. We have also proven that variation in cuticle deposition determines variation in bacterial penetration of the eggshell (Figure 3). This result strongly suggests that both vertical and horizontal transmission (Sparks & Burgess 1993; Stanley *et al.* 2003) would be reduced, and the health of poultry and the safety of table eggs improved, by selecting for cuticle deposition. While the moderate heritability observed is adequate to allow for selection we believe that further improvements in the methodology to reduce its complexity would improve the repeatability and increase the estimate of the genetic component of variance.

The lack of significant genetic correlation between shell colour and cuticle deposition was not predicted (Table 2). The dogma suggests that the cuticle contains most of shell pigment (Lang & Wells 1987) which would suggest that greater cuticle deposition would have a positive correlation with pigment quantity. However a correlation of 0.31 and an error of 0.29 would suggest otherwise although it may also

be that we require larger numbers to get a better estimate. This discrepancy may also be due to over enthusiastic removal of the cuticle by some researchers which is typically removed using chelating agents or acidic solutions (as in our proteomic studies) which erode the underlying vertical crystal layer where most of the pigment may in reality be located. From a positive point of view there was no evidence of any significant negative genetic correlation with key production traits.

The role of the cuticle in terms of the eggs natural defence has been previously observed in terms of the presence or absence of the cuticle (Sparks & Board 1984; Messens *et al.* 2005). In this study the penetration of eggs by micro-organisms has been shown for the first time to be directly dependent on variation in cuticle deposition within the natural range observed within a flock of laying hens. Eggs with the poorest cuticle deposition were most frequently penetrated whilst eggs with the best cuticle deposition were never penetrated (Figure 3). Evidence suggests that the cuticle does affect the water vapour conductance of the hens' eggshell (Peebles & Brake 1986) so selection for improvements in cuticle deposition may affect hatchability, an important criterion for fertilised eggs, but this would have to be empirically determined.

Our proteomic categorisation of the cuticle proteins (Table 3) agrees with previously published reports (Gautron *et al.* 2001; Miksik *et al.* 2003; Wellman-Labadie *et al.* 2008; Rose-Martel *et al.* 2012) in that ovocalyxin-32 ovocalyxin-36, similar to kunitz like protease inhibitor, ovocleidin-116, ovocleidin-17 and clusterin were all observed. The antimicrobial activity of at least 2 of these cuticle protein components, ovocalyxin -32 and -36, has been hypothesised (Gautron *et al.* 2001; Gautron *et al.* 2007). It is therefore likely that the cuticle acts both as a physical barrier and as a chemically active antimicrobial layer.



Whilst the number of proteins identified in the current study are conservative in comparison to recently published studies (Rose-Martel *et al.* 2012), we think this may reflect the care we took in minimising the risk of contamination of our eggs by using clean cages to house the birds, no human handling of the eggs and the removal of any eggs suspected of being cracked or having egg white contamination prior to extraction of the cuticle which was identified as a problem in some of these studies. An alternative possibility is the method used by Rose-Martel (Rose-Martel *et al.* 2012) may have better protein in solubilisation than the method employed in this study.

The relative abundance of ovocalyxin-116 and -17 in the 1N HCl cuticle extract sample is consistent with the more caustic action of 1N HCl on the underlying vertical crystal layer of the shell during the cuticle extraction process. Both of these proteins are regarded as major eggshell specific matrix proteins (Rose & Hincke 2009). The presence of ovocalyxin 17 was however not reported in the study performed by Rose-Martel (Rose-Martel *et al.* 2012) and this is therefore a novel finding.

The identification of cuticle proteins in our proteomic study allowed us to carry out association analysis with a range of candidate genes expressing cuticle proteins and genes involved in the maintenance of oviduct function (Table 4). This revealed that the alleles of ovocalyxin-116 (Oc116\_310) had association with cuticle deposition. This gene is also one of the most abundant eggshell specific matrix proteins (Rose & Hincke 2009; Rose-Martel *et al.* 2012)) and has previously been associated with the crystal orientation and thickness of the eggshell (Dunn *et al.* 2009; Dunn *et al.* 2012). A very strong statistical association was also observed between alleles of the oestrogen receptor (Oer\_2571) and cuticle deposition. Clearly the oestrogen receptor is important for the maintenance of a fully developed oviduct (Munro & Kosin 1943), so this result suggest that oviduct function and cuticle deposition are likely to be

affected by the degree of the development of the oviduct. The importance of oestrogen receptor for cuticle deposition suggests that any perturbation to the oviduct is likely to be detrimental to cuticle deposition.

In conclusion within the normal variation observed in a commercial layer population we can demonstrate that better cuticle deposition will improve the eggs natural defence to bacterial ingress. This has clear importance in terms of egg safety and reducing the vertical and horizontal transmission of diseases which has been identified by the European food safety agency as a probable route of trans generational infection ((BIOHAZ) 2011).

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## Tables

trait	mean $\pm$ s.d
% reflectance at 650nm pre- staining <sup>1</sup>	66.4 $\pm$ 5.5
% reflectance at 650nm post staining <sup>2</sup>	39.5 $\pm$ 9.5
% $\Delta$ 650nm <sup>3</sup>	26.9 $\pm$ 8.2

**Table 1.** The % reflectance pre and post staining and the difference between the two measurements at 650nm are presented. The data is from the mean of the values from two eggs laid by 684 pedigree Rhode Island Red hens aged between 50 and 52 weeks of age. <sup>1</sup>Equates to intensity of shell colour. <sup>2</sup>Equates to intensity of shell colour and cuticle staining. <sup>3</sup>Equates to cuticle deposition.

trait	sire estimate	dam estimate	sire+dam estimate
% reflectance at 650nm pre-staining <sup>1</sup>	0.45 $\pm$ 0.19	0.55 $\pm$ 0.13	0.50 $\pm$ 0.08
% $\Delta$ 650nm <sup>2</sup>	0.27 $\pm$ 0.13	0.54 $\pm$ 0.14	0.40 $\pm$ 0.08

**Table 2.** The estimates of heritability  $\pm$  standard error for % reflectance at 650nm pre staining and for  $\Delta$ 650nm derived after staining eggs with MST cuticle blue are shown. The data is from the mean of the values from two eggs laid by 684 pedigree Rhode Island Red hens aged between 50 and 52 weeks of age. <sup>1</sup>Equates to intensity of shell colour, <sup>2</sup>Equates to cuticle deposition.

protein name	mass (Da)	MS <sub>1</sub>	no's of peptides	emPAI <sub>2</sub>
ovocalyxin-36	48760 [49330]	224 [103]	6 [4]	0.34
ovocalyxin-32	30615 [30900]	144 [78]	8 [6]	0.26
similar to Kunitz-like protease inhibitor	36010 [37606]	90 [92]	2 [2]	0.22
ovocleidin-17	15314 [15657]	81 [48]	2 [3]	0.25
ovocleidin-116	76924 [77266]	177 [333]	5 [15]	0.15
clusterin	51943 [51298]	77 [95]	3 [3]	N/A

**Table 3.** Merged results for cuticle proteins extracted using EDTA and 1N HCl (parenthesis)

<sup>1</sup>Mascot score, <sup>2</sup> exponentially modified protein abundance index



gene marker	maf <sup>1</sup>	geno <sup>2</sup> type	trait <sup>3</sup> mean	effect <sup>4</sup> ±SE	effect as % of the SD <sup>5</sup>	probability <sup>6</sup>
Oer_2571	39	GG	26.5	-1.43±0.53	17%	0.008
		GA	27.1			
		AA	29.4			
Oc116_310	47	TT	28.7	-1.29±0.53	15%	0.015
		CT	26.8			
		CC	26.1			

**Table 4.** Genetic associations for cuticle deposition. Cuticle deposition ( $\Delta 650\text{nm}$ ) trait means of marker genotypes with the estimated size of the additive effects  $\pm$  SE and their sizes relative to the trait standard deviation. Data presented have probabilities less than 0.05. <sup>1</sup>minimum allele frequencies; <sup>2</sup>Genotypes represented by the SNP; <sup>3</sup>Trait means from the full-sib model. <sup>4</sup>Size of the additive effect,  $(AA-aa)/2$ . <sup>5</sup>Effect as % of the SD calculated from the sum of the sire and dam genetic and the environmental variances after fitting the nuisance effects of house. <sup>6</sup>Probability from full-sib model.

**Figure 1.** Average pre and post staining % reflectance spectra for cuticle deposition. The greatest difference in reflectance pre and post staining occurred between ~ 590nm and 700nm (n=50, mean +/- S.E.M).

**Figure 2.** Penetration of the eggshell by *E.coli* pGLO. Penetration by *E.coli* pGLO was confirmed by the presence or absence of luminescence spots on the inner surface of the eggshell after removal of the egg contents under a long wave UV light source. Normally no light is emitted from the inner surface of an eggshell in response to the UV part of the spectrum.

**Figure 3.** The total number of eggs penetrated (lower panel) or not penetrated (upper panel) by *E. coli* versus  $\Delta 650\text{nm}$ . As indicated, a lower  $\Delta 650\text{nm}$  signifies poorer cuticle deposition (n=27).