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

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Abstract

The size and orientation of the calcium carbonate crystals influence the structure and strength of the chickens eggshell. In this study estimates of heritability for crystal size were found to be high (0.6) and moderate for crystal orientation (0.3). There was a strong positive correlation for crystal size and orientation with the thickness of the shell, and in particular the thickness of the mammillary layer (0.65). Correlations with shell breaking strength was positive but with a high error. This was contrary to expectation as in man made materials smaller crystals would be stronger. We believe the results of this study support the hypothesis that the structural organisation of shell, and in particular the mammillary layer, is influenced by crystal size and orientation especially during the initial phase of calcification.

Genetic associations for crystal measurements were observed between haplotype blocks or individual markers for a number of eggshell matrix proteins. *Ovalbumin*, and *ovotransferrin (LTF)* markers for example were associated with crystal size while *ovocleidin-116* and *ovocalyxin 32 (RARRES1)* markers were associated with crystal orientation. The location of these proteins in the eggshell is consistent with different phases of the shell formation process.

In conclusion the variability of crystal size and to a lesser extent orientation appear to have a large genetic component and the formation of calcite crystals are intimately related to the ultrastructure of the eggshell. Moreover this study also provides evidence that proteins in the shell influence the variability of crystal traits and in turn the shells thickness profile. The crystal measurements and/or the associated genetic markers may therefore prove to be useful in selection programs to improve eggshell quality.

Keywords: Eggshell, Egg, $CaCO_3$ Crystal, Matrix protein, markers

1 Introduction

2

3 Cracked and damaged eggs amount to 6 and 8% of total production (Hamilton et al.,
4 1979) and therefore result in substantial economic loss to the egg industry. Improving
5 the quality of the eggshell by genetic selection is therefore of importance as this will
6 help ameliorate these losses (Preisinger and Flock, 2000). However before this is
7 possible it is first necessary to establish and then measure the basic components of the
8 eggshells which contribute to its mechanical strength.
9 The eggshell of the domestic chicken is a bioceramic material comprised of columnar
10 calcite ($CaCO_3$) crystals and a pervading organic proteinaceous matrix. It forms a
11 unique protective barrier that impedes bacterial penetration while allowing the
12 interchange of water and gases needed for the development of the chick embryo (Nys
13 et al., 1999). It is well established that thickness of the shell (typically 300-400 μm)
14 contributes to its breaking strength and its integrity (Tyler and Geake, 1961, Bain,
15 1990). However, the size of the calcite crystals, their shape and crystallographic
16 orientation (collectively referred to in the literature as the microstructural properties of
17 the shell) can also significantly contribute to the shell's mechanical properties
18 (Rodriguez-Navarro et al., 2002). For instance, the microstructure of the guinea fowl
19 eggshell, formed by the intricate interlacing of crystal units, are much tougher than
20 eggs of similar thickness formed by straight columnar units such as that found in
21 chicken eggs (Panheleux et al., 1999). Moreover chicken eggshells consisting of
22 highly oriented crystals of abnormal sizes have been reported to be generally weaker
23 than those consisting of smaller and less oriented crystals (Rodriguez-Navarro et al.,
24 2002, Ahmed et al., 2005). This is not surprising given that in other polycrystalline
25 materials the resistance to fracture or toughness increases as the crystal size decreases

(Hall, 1951) since less external energy (e.g. an external insult) is required for a crack to propagate across brittle large crystals than among smaller crystals. Thus an increased preferential orientation of calcite crystals in the chicken egg should result in a weaker eggshell (Rodriguez-Navarro et al., 2002). Given this argument one would expect that the microstructural organisation of an eggshell would have a strong genetic determination (Rodriguez-Navarro, 2007). This implies that within a species there may be useful genetic variation in the nucleation and growth of calcite crystals during shell formation which are controlled by the eggshell matrix protein precursors present in the uterine fluid (Hernandez-Hernandez et al., 2008). If this is the case then it should be possible to improve eggshell quality by genetic selection of hens with eggshell properties that provide a mechanical advantage. However, until now this has not been possible as quantification of eggshell microstructure has been time consuming and tedious.

In this study a new rapid and efficient method for measuring the size and orientation of eggshell microstructural properties is described . The method described is based on the analysis of two dimensional X-ray diffraction (2D-XRD) patterns formed by intact eggshells and recorded with an area detector (Rodriguez-Navarro, 2007). The intensity of the spots displayed in these patterns is directly related to the size of crystals in egg shells (Rodríguez-Navarro et al., 2006, Rodriguez-Navarro et al., 2007).

The main aim of our study was to use this 2D-XRD method to estimate how much of the variance observed in the size and orientation of crystals in eggshells can be attributed to genetics by quantifying the heritability of these microstructural parameters in a line of Rhode Island Red hens which we have previously characterised for other egg quality traits (Dunn et al., 2005, Dunn et al., 2009). In

addition we wanted to investigate which genes determine the variability of an eggshells microstructural properties so we also looked for association between molecular markers in genes involved in egg shell formation, including eggshell matrix proteins, (Dunn et al., 2009). These organic components are known from biochemical and crystallization *in-vitro* tests, to control the nucleation and growth of calcite crystals (Arias and Fernandez, 2001, Fernandez et al., 2004, Hernandez-Hernandez et al., 2008) but the precise role of these components during the different stages of eggshell formation are not yet fully understood.

Methods

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. The population has previously been described in other publications (Dunn et al., 2005, Dunn et al., 2009). Briefly the study population comprised 32 sires and 237 dams with samples of 2 eggs from 898 of the female offspring for crystal microstructure. The offspring resulted from 5 hatches that were housed in individual cages on 16 hours of light per day in two separate houses at the same location. The eggs were sampled in separate batches between 38 and 42 weeks of age in such a way that each batch of eggs came from a quarter of the population on each occasion. In this study our objective was to extend our existing data set of phenotypic eggshell quality traits for this population with the newly available crystal measurements which were carried out on the same eggs. This allowed comparison with the existing measurements.

Phenotypic measurements

Weight and mechanical traits

Eggs were weighed (g) and, the dynamic stiffness ($K_{dyn,}$ N/m), breaking strength (N) , and stiffness (N/mm) (defined as breaking strength/deformation at fracture), were measured as described previously (Dunn et al., 2005).

Thickness traits

The thickness of the mammillary layer (mm) , the effective thickness (combined palisade, vertical crystal layer and cuticle (mm)) and the total shell thickness (mm) were measured by scanning electron microscopy (SEM) (Panheleux et al., 1999) on 3 sections of eggshell derived from each egg.

Assessment of Eggshell microstructure

Pieces (about 1x1 cm) of eggshell were removed from the equator of each egg using a dental drill fitted with a diamond tipped circular saw. These were then mounted on a sample holder of a single-crystal diffractometer equipped with a CCD area detector (D8 Smart APEX, Bruker, Germany). In the 2D-XRD measurements, the working conditions were: Mo Kα (λ = 0.7093 Å), 50 kV and 30 mA, a pin-hole collimator of 0.5 mm in diameter, and an exposure time of 20 s per frame. Samples were mounted so that the outer shell surface faced the area detector and the inner surface faced the incident X-ray beam. Using this set up the X-ray beam passes through the sample and a 2D diffraction pattern or frame is recorded on the area detector. The registered diffraction patterns from eggshells consists of concentric spotty rings (Debye-Scherrer rings). Each spot within a ring corresponds to a *hkl* reflection of a calcite crystal whose (*hkl*) planes are oriented in diffraction conditions.

XRD2DScan software (Rodríguez-Navarro et al., 2006) was used to automatically analyze the 2D diffraction patterns by measuring the intensity of the reflection spots

in five Debye-Scherrer rings, associated with the strongest calcite reflections: 104, 110, 113, 108, 202. The data from the five rings were added to give the total peak area (TA) to minimise the influence of any preferential orientation of crystals and to lower data variability. Each eggshell sample was measured in three different locations to further improve the estimate. To convert the TA value to crystal size, the TA values were calibrated against the average crystal size determined by analyzing thin sections of 10 eggshells using optical microscopy (Rodriguez-Navarro et al., 2006; 2007). The selected eggshells used for this calibration covered a wide range of TA values (figure 2). As crystal size and TA were linearly related, we have chosen to use crystal size in this paper to facilitate understanding.

A quantitative estimation of the degree of crystal orientation was obtained using the ratio between the integrated intensities of the calcite reflections from the intact eggshell and that of a random sample. The integrated intensities of the strongest calcite reflections were normalized by their values in a calcite powder standard representing a randomly oriented sample. Using the slope of the regression line between the normalized intensity ratios and the interfacial angle between crystal planes, a measure of the orientation of crystals was determined (OI Lineal) (Rodriguez-Navarro et al., 2002). The value of OI Lineal describes the degree of preferential orientation of crystals, a zero value would be for a sample of randomly oriented crystals and highly negative value for a sample constituted by highly oriented crystals. This parameter can be converted to the parameter FWHM which represents the angular scattering in the orientation of the calcite ***c***-axis (Rodriguez-Navarro et al., 2002).

To give approximate normality and consistency of variances the log of crystal size -80 ($\log_{10}$(crystal size-80)) was taken and used in calculations although the non

transformed values are presented for the association analysis to allow ease of interpretation of the size of effects.

Single nucleotide polymorphisms and association analysis.

The SNPs markers used for association analysis were from organic eggshell matrix candidate genes; *ovocleidin-116*, *osteopontin (SPP1*), *ovocalyxin-32* (*RARRES1*), *ovotransferrin (LTF)*, *ovalbumin* and *ovocalyxin-36* and key genes involved in the maintenance and function of the shell gland region of the hens oviduct; *estrogen receptor (ESR1)* and *carbonic anhydrase II* (*CAII*). The position and genotyping were as described previously (Dunn et al., 2009). A further 34 SNP markers were added from re-sequencing the genome of the population around the *ovocleidin-116* region, (12 SNPs; Chr4: 47.118-47.123,GGoc116nnnnnNN where nnnnn is a code number and NN represents the 2 possible bases), *ovocalyxin-32(RARRES1)* gene (10 SNPs; Chr9: 23.995-24.045, GGovc32nnnnnNN) and *ovalbumin* gene (12 SNPs; chr2 68.905-68.915Mb, GGovalbnnnnnNN). These markers were at gene loci that had previously shown an association with egg quality traits (Dunn et al., 2009) and the SNP information and allele frequencies have been submitted to dbSNP with submitter SNP (ss) accession numbers running from ss410759452-ss410759486 . Genotyping was performed by KBiosciences (Hoddesdon, Herts). Association with crystal size and orientation was determined by fitting as fixed effects, hatch (h), house (w), and their interaction and the marker genotypes (g), together with sires (s) and dams within sires (d) and error (e) as random effects to the responses (y), as

$$y_{ijklmn} = s_i + d_{ij} + h.w_{kl} + g_m + e_{ijklmn}$$

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.

Calculations of heritability and genetic correlation

All calculations are based on data from the average of two eggs per bird between 38 and 42 weeks of age. Heritabilities were estimated from the following model,

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

where Y_{ijkl} is the trait, μ is the overall mean, h_i is the fixed effect of the hatch date, s_j and d_{jk} are the random effects of sires and dams within sires and e_{ijkl} is the residual, with variance components σ_s^2, σ_d^2 and σ_e^2, respectively. Model parameters were estimated by residual maximum likelihood (REML, (Patterson and Thompson, 1971)), and heritabilities from the formulae below. Estimates of standard errors were obtained by the delta method, which approximates the variance of a function using the first term of its Taylor series expansion about the mean.

$$h_s^2 = 4.\frac{\sigma_s^2}{(\sigma_s^2 + \sigma_e^2)}$$

$$h_d^2 = 4.\frac{\sigma_d^2}{(\sigma_d^2 + \sigma_e^2)}$$

$$h_{s+d}^2 = 2.\frac{\sigma_s^2 + \sigma_d^2}{(\sigma_s^2 + \sigma_d^2 + \sigma_e^2)}$$

$h^2_{s,}$ h^2_d and h^2_{s+d} are heritabilities based on sire, dam and sire + dam components of variance. Genetic correlations were estimated from a bivariate mixed model with the same linear terms as model (1) above.

$$\begin{bmatrix} y_1 \\ y_2 \end{bmatrix} = \begin{bmatrix} \mu_1 \\ \mu_2 \end{bmatrix} + \begin{bmatrix} h_1 \\ h_2 \end{bmatrix} + \begin{bmatrix} s_1 \\ s_2 \end{bmatrix} + \begin{bmatrix} d_1 \\ d_2 \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \end{bmatrix}$$

Where y_1 is either crystal size or crystal orientation and y_2 is an egg trait, and the omitted additional subscripts ijkl, are the same as in model (1) above. The terms also

correspond to model (1): $\mu_{1/2}$ is the mean of $y_{1/2}$, $h_{1/2}$ is the fixed effect of the hatch date, $s_{1/2}$ and $d_{1/2}$ are the random effects of sires and dams within sires and $e_{1/2}$ is the residual. In addition to the components of random variation for each of the traits, the three final terms also have covariance terms to model the sire, dam and residual correlations between the 2 traits. For example, for the sire effects on traits y_1 and y_2:

$$\text{cov}\begin{bmatrix} s_1 \\ s_2 \end{bmatrix} = \begin{bmatrix} \sigma_1^2 & \rho\sigma_1\sigma_2 \\ \rho\sigma_1\sigma_2 & \sigma_2^2 \end{bmatrix}$$

where σ_1^2 and σ_2^2 are the additive sire genetic variances for traits 1 and 2, and ρ is the (additive) genetic correlation between the traits. Phenotypic correlations were calculated according to the following equation;

$$\frac{\sigma_{s1/2}^2 + \sigma_{d1/2}^2 + \sigma_{e1/2}^2}{\sqrt{(\sigma_{s1}^2 + \sigma_{d1}^2 + \sigma_{e1}^2)(\sigma_{s2}^2 + \sigma_{d2}^2 + \sigma_{e2}^2)}}$$

The terms are the same as used in the heritability equations except for $\sigma_{s1/2}^2$, $\sigma_{d1/2}^2$ and $\sigma_{e1/2}^2$ which are the sire dam and error covariances of the two traits. Model parameters were estimated by REML and standard errors for the sire-based genetic correlation were approximated by the delta method. All calculations were performed in Genstat version 6.1 (VSN International Ltd, Oxford, UK).

Results

The results of our calibration experiment (figure 2) demonstrates that there is a good correlation (r^2=0.84) between the estimate of total peak area as determined from the 2D-X-ray diffraction patterns of eggshells (TA) and the estimate of crystal size obtained using polarised light microscopy of polished sections of the same eggshells.

Thus the total peak area measured from 2D-XRD analysis of the intact shell provides a good estimate of the average size of crystals comprising the eggshell.

Using the data from two eggs from 898 hens and the calibration from figure 2 the mean crystal size for eggs from hens in the population was calculated to be 100.8±0.2 µm (Table 1) but the data is positively skewed with an Anderson Darling (AD) value of <0.005. The intensities of the peaks were largest in the 104 diffraction ring (A_104) (Table 1) since the 104 reflection is the strongest for calcite (Rodriguez-Navarro et al., 2007). The estimate of crystal orientation, OI lineal, is normally distributed (AD = 0.89).

The heritability estimate for crystal size (Table 2) was high, whilst the estimate for crystal orientation, OI lineal, was moderate (Table 2).

The genetic correlation of crystal size with egg weight, mammillary thickness and total thickness (Table 3) was at least twice its error, with the correlation with mammillary thickness being of the largest magnitude (0.65). This was larger than the correlation with total thickness or effective thickness (combined palisade, and vertical crystal layers). The genetic correlation of crystal orientation with mammillary thickness was also larger (0.66) than that observed for the total and effective thickness measurement (Table 3). There was also evidence that crystal size was genetically correlated with crystal orientation (Table 3) with animals laying eggs with larger crystals having a more randomly orientated crystal structure.

For the 2 microstructural traits measured there were 27 SNP markers out of 69 which had test statistics of additive effects that gave p-values < 0.05. However, since the markers in the regions around the genes with dense genotyping are close together many were in haplotype blocks as determined by haploview (Barrett et al., 2005). Using this approach we identified 2 markers or marker blocks for crystal size and 3

for OI lineal out of 24 independent markers or marker blocks that are above the nominal 0.05 p value. Applying a Bonferroni correction conservatively within this experiment, assuming independent markers & traits, the probability required would be ≤0.001. Table 4 shows the SNP with the most significant association in each haplotype block. The following haplotype blocks were represented by a marker and contains the markers indicated in brackets; GGovalb1927GC (GGovalb1927GC, GGovalb1936GA) GGovalb3173CT (GGovalb3173CT, GGovalb4511GA) and are associated with crystal size. For association with OI lineal the haplotype blocks are represented by Oc116_310 (Oc116_310 , GGov1161991GA, GGov1162073CT, GGov1162344CA, GGov1162611GA, GGov1162644CA GGov1162799CT, GGov1163981CT) and GGovc321992GA (GGovc321992GA, GGovc32834CT, GGovc321205GA, GGovc323915CT, GGovc324760GA, GGovc326132GT GGovc3210051CT).The associations with crystal orientation had relatively high p values, in particular the markers linked to Oc116_310 on chromosome 4 and GGovc321992GA on chromosome 9 (Table 4). When the effect of substituting the beneficial allele in the population on the population mean was calculated the effects are quite small, typically around 1% or less for crystal size although somewhat larger up to 9.4% for the crystal orientation measurement (Table 4).

Discussion

It seems self evident that the microstructural characteristics (i.e., size and orientation of the calcite crystals) of an eggshell are important in terms of its mechanical strength and indeed this has been alluded to before. (Ahmed et al., 2005, Rodriguez-Navarro, 2007, Rodríguez-Navarro et al., 2006, Rodríguez-Navarro et al., 2000). However the software applied in this study has only now made it possible to rapidly measure these

traits in eggshells from sufficient numbers of animals to estimate their genetic basis and to look for correlations with existing measurements of egg shell quality. This new development also means that it is now possible for these measurements to be carried out in an egg testing laboratory on large numbers of eggs given the correct equipment. In our study population, the average crystal size was estimated to be 100 µm (Table 1) which is larger than the published estimates of 80µm previously reported for commercial hybrids (Rodriguez-Navarro et al., 2002). The estimate for crystal orientation (OI lineal = -0.014; Table 1) which corresponds to a FWHM value of 90 degrees is in the range previously observed in chicken eggshells (FWHM 50 -120 deg; (Rodriguez-Navarro, 2007, Rodriguez-Navarro et al., 2002). For comparison the nearly parallel calcite crystals of an ostrich eggshell have a OI_lineal value of -0.606 and a FWHM value of 18 deg (Rodriguez-Navarro, 2007).

A striking aspect of this study has been the observation of a very high value for the estimate of heritability for crystal size (Table 2). At around 0.6, this is higher than the measurement for egg colour in brown eggs (Francesch et al., 1997) or egg weight (0.52) previously reported for this population (Dunn et al., 2005). Both egg colour and egg weight are traditionally considered to be the traits with the highest heritability estimates in egg layers. The estimate of heritability for the degree of preferred crystal orientation was somewhat lower at 0.35 although still more than twice the error estimate.

The Rhode Island Red line used in this study already had a large quantity of detailed phenotypic data available which allowed us to make genetic correlations with appropriate traits (Dunn et al., 2009, Dunn et al., 2005). The largest genetic correlation for both of our microstructural traits was with the mammillary layer thickness, followed by the total eggshell thickness which includes the mammillary

layer. In contrast, the effective thickness which has the largest contribution to eggshell strength (Vantoledo et al., 1982, Bain, 1990) was not highly genetically correlated with either crystal size or orientation. The genetic correlation of crystal size and breaking strength was low 0.32±0.28 and positive which is contrary to that expected from studies of man made polycrystalline materials (Hall, 1951) but in order to determine if this estimate can be relied upon it will be necessary to repeat our experiments with a larger sample because of the size of the error. Lastly there is positive correlation between crystal size and orientation indicating that bigger crystals are less regular. This is consistent with stronger shells having a more random crystal orientation (Rodriguez-Navarro et al., 2002) but interestingly we did not observe a direct genetic correlation between orientation and breaking strength (Table 3) in our study. The genetic correlation of crystal size and orientation with egg production between month 1 and 6 of production was always negative (-0.4 and –0.65 respectively) but in all cases this was not significant.

Our results therefore provide strong evidence for a relationship between crystal size or orientation and the thickness of the shell with particular emphasis on the mammillary layer. This suggests that the relationship between microstructure and shell strength is also important as has been hypothesised but this could not be proven. The strong relationship between microstructure and the thickness of the mammillary layer nevertheless may have an extremely important consequence if in fact what we are indirectly measuring is the relationship between the mammillary density, (which is a result of the number of nucleation sites on the outer shell membrane during the early stages of shell formation), and the dimensions of the crystal columns which make up the palisade layer of the shell. If, for example, the individual mammillae are close together during shell formation then it seems logical that this would have a limiting

effect on the width of the crystals in the palisade layer since the latter forms at the point at which the individual mammillae fuse (Solomon, 1991)). The potential thickness of the mammillary layer would as a consequence also be reduced. If on the other hand the mammillae are more widely spaced then the width of the crystal in the palisade layer would be comparatively larger and the mammillary layer thicker. This concept is represented in figure 3 and is supported by the fact that the columnar microstructure and the preferential orientation of calcite crystals in eggshells are the result of a competitive crystal growth process in which crystals emerging from the mammillary cores compete for the available space such that only those favourably oriented continue to grow outward forming the columnar units of the palisade layer. The outcome of this process and resulting material microstructure is thus mainly defined by the spacing between adjacent crystals units (or the density of the mammillae) and the relative growth rate of different crystallographic directions within a calcite crystal (Rodriguez-Navarro and Garcia-Ruiz, 2000). These parameters are thought to be modulated by specific organic matrix components.

It has previously been noted that there is a large phenotypic correlation between mammillary density and the number of gas exchange pores in the hatching egg (Tullett, 1975). It would be interesting to establish if the density of gaseous exchange pores is also correlated with crystal size. The number and area of apposition between the mammillae and the contact made between mammillae and the shell membranes also has potential to influence the absorption of calcium by the developing embryo, since these structures represent the main source of calcium for the developing chick's skeleton (Chien et al., 2009). It could therefore be postulated that crystal size, perhaps influenced by matrix proteins in the shell, is also critical to this process. Thus, the importance of both of these factors to the development of the chick embryo and

the magnitude of the genetic component for crystal size presents a potential route to improving embryo fitness. In this respect a genetic correlation was found between crystal size and egg weight (0.44±0.22) but not between crystal orientation and egg weight (0.09±0.26). It is not obvious how egg weight would be related to crystal size, although one possibility is that egg size is related in some way to the spacing of nucleation sites which would be consistent with our model (figure 3). This assumes that nucleation sites are finite in each bird, determined by a genetically derived pattern, and if egg size increases these would be further apart, in the manner that an elastic net expands with the increasing volume of contents but the number of intersections in the net fabric stays constant. This means that as egg weight and size increases the spacing between nucleation sites increases and the crystal size increases. It also implies that the genes responsible for variation in egg size may well underlie some of the variation in crystal size and shell thickness.

Given these arguments our measurement of crystal size and orientation seems to be getting close to the basic components of the construction of the eggshell. But it is not clear what the fundamental biological units are that determine the variability of all these components, although the proteins involved in crystal nucleation and in the organic matrix of the shell seem good candidates. The mammillae are formed during the slow phase of mineralisation which is associated with the presence of different organic matrix proteins than the rapid phase which forms the palisade layer (Nys et al., 2004). With this in mind we attempted to associate crystal size and crystal orientation with a number of alleles of eggshell matrix proteins and genes known to be involved in eggshell formation. We found a number of associations which are highly significant (Table 4) which included many of the markers in haplotype blocks because of the density in which genotyping was performed. Markers in the densely

genotyped loci tested had previously shown association with egg shell quality traits, in particular *ovocleidin-116* with the total thickness of the shell and *RARRES1* with relative thickness of the mammillary layer (Dunn et al., 2009). The significance of both markers in the current association study is therefore particularly noteworthy. Of the proteins previously localised within the mammillary layer of the shell those seen in the list of markers for crystal size are ovotransferrin (Gautron et al., 2001) and *ovalbumin* (Hincke, 1995). *Ovocleidin-116* and *ovocalyxin-32(RARRES1)* are localised more with the palisade layer and vertical crystal layer (Hincke et al., 1999) and both are associated with the measurement of crystal orientation. Ironically the protein which has been studied most as a catalyst for calcite crystal nucleation in relation to egg shell formation, *ovocleidin 17* (Freeman et al., 2010), has not been isolated as an EST nor is it represented in the chicken genome so it has not been possible to examine the relationship between alleles for this gene and crystal formation. This is despite its appearance in proteomic studies (Mann et al., 2006) Although the effects of each marker on the trait mean values are relatively small, each is of sufficient size to merit further validation as tools for selection of sires and possibly dams to improve eggshell quality in pedigree poultry breeding programmes. Small increases in shell quality traits can have large effects on the product quality and further work may lead us to understand the importance of these markers on mammillary layer formation and what effect this may have for the developing embryo.

In conclusion, we believe that these measurements bring us closer to reducing eggshell quality to its component parts which will improve our understanding of eggshell quality and safety and the precision of how we define it. Ultimately this contributes to our goal of improvement of egg shell quality through genetic selection.

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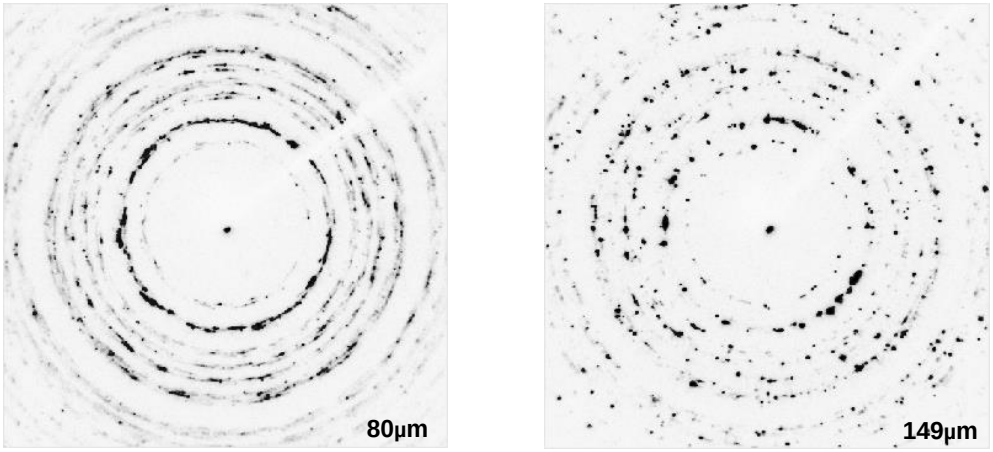


Figure 1. The effect of crystal size on the X-ray diffraction pattern of egg shell samples with average crystal sizes of 80 and 149um estimated from by cross polarised light microscopy (figure 2) . The number of spots decreases and their intensity and size increases as crystal size increases.

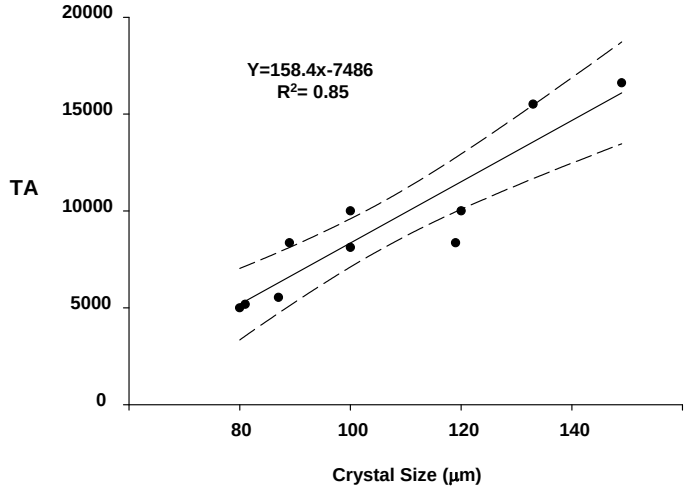


Figure 2. Graph of the relationship between average crystal size determined by cross polarised light microscopy and the total peak area (TA) estimate by X-ray diffraction of 10 individual egg shells from a pedigree Rhode Island Red population. The line is fitted using linear regression and the dashed lines represent 95% confidence intervals.

Trait	Mean ± SD
Values for mean intensity of individual crystal orientations	
*A*_104	4371±782
*A*_108	833±116
*A*_110	1000±154
*A*_113	916±127
*A*_202	1215±185
Overall mean intensity	
TA	8336±1231
Crystal size calculated from calibration of TA	
Crystal size (um)	100.8±6.5
Crystal orientation	
OI lineal	-0.014±0.003

Table 1. Summary statistics for the estimates of the average intensities of peaks along the Debye-Scherrer ring associated with the most important hkl calcite reflections (A_104, 110, 113, 202, 108) their sum, the total peak area (TA) and the average crystal size determined by calibration from cross polarised light microscopy using the equation in figure 1. The estimate of preferred crystal orientation is represented by OI lineal where a value of 0 represents a completely random orientation with increasingly negative values representing a more orientated crystal structure. The shells of two eggs laid by 898 pedigree Rhode Island Red hens aged between 38 and 42 weeks were used for the estimates.

Trait	Sire estimate	Dam estimate	Sire+Dam estimate
A_104	*0.51±0.15*	*0.55±0.13*	*0.53±0.09*
A_110	*0.45±0.19*	*0.55±0.13*	*0.50±0.08*
A_113	*0.57±0.17*	*0.44±0.12*	*0.51±0.09*
A_202	*0.43±0.14*	*0.43±0.13*	*0.43±0.08*
A_108	*0.54±0.17*	*0.55±0.13*	*0.55±0.09*
crystal size	0.60±0.18	0.62±0.13	0.61±0.09
OI lineal= crystal orientation	0.35±0.13	0.39±0.13	0.37±0.08

Table 2. Estimates of heritability ± standard error for the average intensities of peaks along the Debye-Scherrer ring associated with the most important hkl calcite reflections (104, 110, 113, 202, 108). These were then summed to provide the total average intensity (TA), which was converted to calcite crystal size using the equation in figure 1. The heritability estimates associated with that value are presented and the estimate of OI lineal which is a measure of crystal orientation.

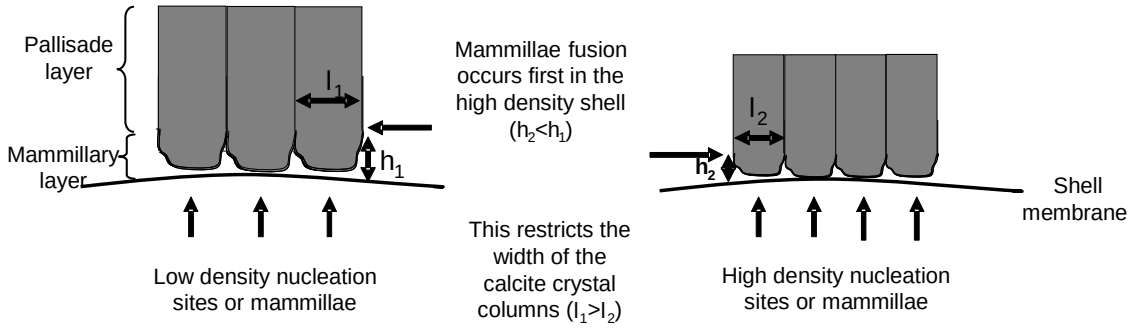
Correlation of:	**Crystal size**		**Crystal orientation**	
with	**Genetic**	**Phenotypic**	**Genetic**	**Phenotypic**
Egg weight and shape				
Egg Weight	0.45±0.21	0.13	0.09±0.26	0.10
Quasi-static measurements				
Breaking strength (equatorial)	0.32±0.28	0.08	-0.08±0.33	-0.07
SEM thickness measurements				
Mean thickness	0.42±0.22	0.20	0.51±0.25	-0.02
Mean effective thickness	0.32±0.24	0.15	0.40±0.27	-0.05
Mean mammillary thickness	0.61±0.20	0.24	0.66±0.24	-0.10
Crystal size				
Crystal Size			0.45±0.21	0.23

Table 3. Estimates of genetic correlation (± error) and phenotypic correlation between crystal size or crystal orientation and measurements of egg weight, egg quality and shape, breaking strength, static and dynamic stiffness and egg shell thickness measurements derived by scanning electron microscopy (SEM) .

Marker	Geno[1] type	Trait[2] mean	Size of[3] additive effect±SE	p[4]	Effect[5] as a % of SD	MAF[6]	Selection[7] effect (% change)
Trait Crystal size							
GGovalb1927GC	1	101.7	0.87±0.37	0.018	13	42	0.7
	2	100.0					
	3	101.5					
Ovotrans	1	97.6	-1.70±0.75	0.026	-26	31	0.8
	2	100.97					
	3	99.8					
Trait OI lineal							
Oc116310poly	1	-0.0132	0.0006±0.0002	0.001	31	46	5.6
	2	-0.0144					
	3	-0.0135					
GGovc321992GA	1	-0.0133	0.0007±0.0003	0.004	40	23	9.4
	2	-0.0148					
	3	-0.0137					

Table 4. The most significant representatives of the marker haplotypes associated with crystal size and crystal orientation are listed with the estimated size of the additive effect and its error and its size relative to the trait standard deviation. [1]Genotypes represented by the SNP where 1 and 2 are homozygotes and 3 is the heterozygote; [2]Trait means from the full-sib model given in the methods section. [3]Size of the additive effect, (AA-aa)/2. [4]Probability from full-sib model, [5]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 and hatch. [6]Minimum allele frequency. [7]An estimate is given of the expected increase in the trait mean if the beneficial allele was selected for in the population

Model for the interaction between crystal growth and egg shell structure



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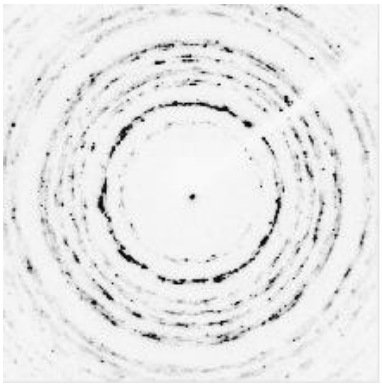
2 *Figure 3 Model of how crystal size may be related to the thickness of the mammillary*
3 *layer and in turn the thickness of the shell.*
4



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