



Dunn, I.C., Rodriguez-Navarro, A.B., Mcdade, K., Schmutz, M., Preisinger, R., Waddington, D., Wilson, P.W., and Bain, M.(2012) Genetic variation in eggshell crystal size and orientation is large and these traits are correlated with shell thickness and are associated with eggshell matrix protein markers. *Animal Genetics*, 43 (4). pp. 410-418. ISSN 0268-9146(doi:10.1111/j.1365-2052.2011.02280.x)

<http://eprints.gla.ac.uk/62555/>

Deposited on: 25th July 2012

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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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1 Abstract

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

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

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

25 Keywords: Eggshell, Egg, CaCO₃ Crystal, Matrix protein, markers

26

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

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

14 In this study a new rapid and efficient method for measuring the size and orientation
15 of eggshell microstructural properties is described . The method described is based
16 on the analysis of two dimensional X-ray diffraction (2D-XRD) patterns formed by
17 intact eggshells and recorded with an area detector (Rodriguez-Navarro, 2007). The
18 intensity of the spots displayed in these patterns is directly related to the size of
19 crystals in egg shells (Rodríguez-Navarro et al., 2006, Rodriguez-Navarro et al.,
20 2007).

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

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

9

10 Methods

11 **Animals and egg collection**

12 A Rhode Island Red pedigree line that contributes to the male line used to produce
13 Lohmann Brown commercial layer hens was used in this study. The population has
14 previously been described in other publications (Dunn et al., 2005, Dunn et al., 2009).
15 Briefly the study population comprised 32 sires and 237 dams with samples of 2 eggs
16 from 898 of the female offspring for crystal microstructure. The offspring resulted
17 from 5 hatches that were housed in individual cages on 16 hours of light per day in
18 two separate houses at the same location. The eggs were sampled in separate batches
19 between 38 and 42 weeks of age in such a way that each batch of eggs came from a
20 quarter of the population on each occasion. In this study our objective was to extend
21 our existing data set of phenotypic eggshell quality traits for this population with the
22 newly available crystal measurements which were carried out on the same eggs. This
23 allowed comparison with the existing measurements.

24

25

1 **Phenotypic measurements**

2 *Weight and mechanical traits*

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

6 *Thickness traits*

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

11 *Assessment of Eggshell microstructure*

12 Pieces (about 1x1 cm) of eggshell were removed from the equator of each egg using a
13 dental drill fitted with a diamond tipped circular saw. These were then mounted on a
14 sample holder of a single-crystal diffractometer equipped with a CCD area detector
15 (D8 Smart APEX, Bruker, Germany). In the 2D-XRD measurements, the working
16 conditions were: Mo $K\alpha$ ($\lambda = 0.7093 \text{ \AA}$), 50 kV and 30 mA, a pin-hole collimator of
17 0.5 mm in diameter, and an exposure time of 20 s per frame. Samples were mounted
18 so that the outer shell surface faced the area detector and the inner surface faced the
19 incident X-ray beam. Using this set up the X-ray beam passes through the sample and
20 a 2D diffraction pattern or frame is recorded on the area detector. The registered
21 diffraction patterns from eggshells consists of concentric spotty rings (Debye-Scherrer
22 rings). Each spot within a ring corresponds to a hkl reflection of a calcite crystal
23 whose (hkl) planes are oriented in diffraction conditions.

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

1 in five Debye-Scherrer rings, associated with the strongest calcite reflections: 104,
2 110, 113, 108, 202. The data from the five rings were added to give the total peak
3 area (TA) to minimise the influence of any preferential orientation of crystals and to
4 lower data variability. Each eggshell sample was measured in three different locations
5 to further improve the estimate. To convert the TA value to crystal size, the TA
6 values were calibrated against the average crystal size determined by analyzing thin
7 sections of 10 eggshells using optical microscopy (Rodriguez-Navarro et al., 2006;
8 2007). The selected eggshells used for this calibration covered a wide range of TA
9 values (figure 2). As crystal size and TA were linearly related, we have chosen to use
10 crystal size in this paper to facilitate understanding.

11 A quantitative estimation of the degree of crystal orientation was obtained using the
12 ratio between the integrated intensities of the calcite reflections from the intact
13 eggshell and that of a random sample. The integrated intensities of the strongest
14 calcite reflections were normalized by their values in a calcite powder standard
15 representing a randomly oriented sample. Using the slope of the regression line
16 between the normalized intensity ratios and the interfacial angle between crystal
17 planes, a measure of the orientation of crystals was determined (OI Lineal)
18 (Rodriguez-Navarro et al., 2002). The value of OI Lineal describes the degree of
19 preferential orientation of crystals, a zero value would be for a sample of randomly
20 oriented crystals and highly negative value for a sample constituted by highly oriented
21 crystals. This parameter can be converted to the parameter FWHM which represents
22 the angular scattering in the orientation of the calcite *c*-axis (Rodriguez-Navarro et al.,
23 2002).

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

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

3 **Single nucleotide polymorphisms and association analysis.**

4 The SNPs markers used for association analysis were from organic eggshell matrix
5 candidate genes; *ovocleidin-116*, *osteopontin (SPP1)*, *ovocalyxin-32 (RARRES1)*,
6 *ovotransferrin (LTF)*, *ovalbumin* and *ovocalyxin-36* and key genes involved in the
7 maintenance and function of the shell gland region of the hens oviduct; *estrogen*
8 *receptor (ESR1)* and *carbonic anhydrase II (CAII)*. The position and genotyping were
9 as described previously (Dunn et al., 2009). A further 34 SNP markers were added
10 from re-sequencing the genome of the population around the *ovocleidin-116* region,
11 (12 SNPs; Chr4: 47.118-47.123, GGoc116nnnnnNN where nnnnn is a code number
12 and NN represents the 2 possible bases), *ovocalyxin-32(RARRES1)* gene (10 SNPs;
13 Chr9: 23.995-24.045, GGovc32nnnnnNN) and *ovalbumin* gene (12 SNPs; chr2
14 68.905-68.915Mb, GGovalbnnnnnNN). These markers were at gene loci that had
15 previously shown an association with egg quality traits (Dunn et al., 2009) and the
16 SNP information and allele frequencies have been submitted to dbSNP with submitter
17 SNP (ss) accession numbers running from ss410759452-ss410759486 . Genotyping
18 was performed by KBiosciences (Hoddesdon, Herts). Association with crystal size
19 and orientation was determined by fitting as fixed effects, hatch (h), house (w), and
20 their interaction and the marker genotypes (g), together with sires (s) and dams within
21 sires (d) and error (e) as random effects to the responses (y), as

$$22 \quad y_{ijklmn} = s_i + d_{ij} + h.w_{kl} + g_m + e_{ijklmn}$$

23 Linear models were fitted by REML, followed by approximate Student's t-tests to
24 assess marker effects. The additive effect of each marker was estimated as half the
25 difference between homozygote means.

1

2 **Calculations of heritability and genetic correlation**

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

$$5 Y_{ijkl} = \mu + h_i + s_j + d_{jk} + e_{ijkl}. \quad (1)$$

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

$$13 h_s^2 = 4 \cdot \frac{\sigma_s^2}{(\sigma_s^2 + \sigma_e^2)}$$

$$14 h_d^2 = 4 \cdot \frac{\sigma_d^2}{(\sigma_d^2 + \sigma_e^2)}$$

$$15 h_{s+d}^2 = 2 \cdot \frac{\sigma_s^2 + \sigma_d^2}{(\sigma_s^2 + \sigma_d^2 + \sigma_e^2)}$$

16 h_s^2 , h_d^2 and h_{s+d}^2 are heritabilities based on sire, dam and sire + dam components of
17 variance. Genetic correlations were estimated from a bivariate mixed model with the
18 same linear terms as model (1) above.

19

$$20 \begin{bmatrix} y_1 \\ y_2 \end{bmatrix} = \begin{bmatrix} \mu_1 \\ \mu_2 \end{bmatrix} + \begin{bmatrix} h_1 \\ h_2 \end{bmatrix} + \begin{bmatrix} s_1 \\ s_2 \end{bmatrix} + \begin{bmatrix} d_1 \\ d_2 \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \end{bmatrix}$$

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

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

6

$$7 \text{ cov} \begin{bmatrix} s_1 \\ s_2 \end{bmatrix} = \begin{bmatrix} \sigma_1^2 & \rho\sigma_1\sigma_2 \\ \rho\sigma_1\sigma_2 & \sigma_2^2 \end{bmatrix}$$

8

9 where σ_1^2 and σ_2^2 are the additive sire genetic variances for traits 1 and 2, and ρ is

10 the (additive) genetic correlation between the traits. Phenotypic correlations were

11 calculated according to the following equation;

$$12 \frac{\sigma_{s1/2}^2 + \sigma_{d1/2}^2 + \sigma_{e1/2}^2}{\sqrt{(\sigma_{s1}^2 + \sigma_{d1}^2 + \sigma_{e1}^2)(\sigma_{s2}^2 + \sigma_{d2}^2 + \sigma_{e2}^2)}}$$

13 The terms are the same as used in the heritability equations except for $\sigma_{s1/2}^2$, $\sigma_{d1/2}^2$

14 and $\sigma_{e1/2}^2$ which are the sire dam and error covariances of the two traits. Model

15 parameters were estimated by REML and standard errors for the sire-based genetic

16 correlation were approximated by the delta method. All calculations were performed

17 in Genstat version 6.1 (VSN International Ltd, Oxford, UK).

18

19 **Results**

20 The results of our calibration experiment (figure 2) demonstrates that there is a good

21 correlation ($r^2=0.84$) between the estimate of total peak area as determined from the

22 2D-X-ray diffraction patterns of eggshells (TA) and the estimate of crystal size

23 obtained using polarised light microscopy of polished sections of the same eggshells.

1 Thus the total peak area measured from 2D-XRD analysis of the intact shell provides
2 a good estimate of the average size of crystals comprising the eggshell.
3 Using the data from two eggs from 898 hens and the calibration from figure 2 the
4 mean crystal size for eggs from hens in the population was calculated to be 100.8 ± 0.2
5 μm (Table 1) but the data is positively skewed with an Anderson Darling (AD) value
6 of < 0.005 . The intensities of the peaks were largest in the 104 diffraction ring
7 (A_104) (Table 1) since the 104 reflection is the strongest for calcite (Rodriguez-
8 Navarro et al., 2007). The estimate of crystal orientation, OI lineal, is normally
9 distributed (AD = 0.89).

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

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

21 For the 2 microstructural traits measured there were 27 SNP markers out of 69 which
22 had test statistics of additive effects that gave p-values < 0.05 . However, since the
23 markers in the regions around the genes with dense genotyping are close together
24 many were in haplotype blocks as determined by haploview (Barrett et al., 2005).

25 Using this approach we identified 2 markers or marker blocks for crystal size and 3

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

19

20 **Discussion**

21 It seems self evident that the microstructural characteristics (i.e., size and orientation
22 of the calcite crystals) of an eggshell are important in terms of its mechanical strength
23 and indeed this has been alluded to before. (Ahmed et al., 2005, Rodriguez-Navarro,
24 2007, Rodríguez-Navarro et al., 2006, Rodríguez-Navarro et al., 2000). However the
25 software applied in this study has only now made it possible to rapidly measure these

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

13 A striking aspect of this study has been the observation of a very high value for the
14 estimate of heritability for crystal size (Table 2). At around 0.6, this is higher than the
15 measurement for egg colour in brown eggs (Francesch et al., 1997) or egg weight
16 (0.52) previously reported for this population (Dunn et al., 2005). Both egg colour
17 and egg weight are traditionally considered to be the traits with the highest heritability
18 estimates in egg layers. The estimate of heritability for the degree of preferred crystal
19 orientation was somewhat lower at 0.35 although still more than twice the error
20 estimate.

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

1 layer. In contrast, the effective thickness which has the largest contribution to
2 eggshell strength (Vantoleto et al., 1982, Bain, 1990) was not highly genetically
3 correlated with either crystal size or orientation. The genetic correlation of crystal
4 size and breaking strength was low 0.32 ± 0.28 and positive which is contrary to that
5 expected from studies of man made polycrystalline materials (Hall, 1951) but in order
6 to determine if this estimate can be relied upon it will be necessary to repeat our
7 experiments with a larger sample because of the size of the error. Lastly there is
8 positive correlation between crystal size and orientation indicating that bigger
9 crystals are less regular. This is consistent with stronger shells having a more random
10 crystal orientation (Rodriguez-Navarro et al., 2002) but interestingly we did not
11 observe a direct genetic correlation between orientation and breaking strength (Table
12 3) in our study. The genetic correlation of crystal size and orientation with egg
13 production between month 1 and 6 of production was always negative (-0.4 and -
14 0.65 respectively) but in all cases this was not significant.

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

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

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

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

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

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

21 In conclusion, we believe that these measurements bring us closer to reducing
22 eggshell quality to its component parts which will improve our understanding of
23 eggshell quality and safety and the precision of how we define it. Ultimately this
24 contributes to our goal of improvement of egg shell quality through genetic selection.
25

1 **Acknowledgement**

2 This study was funded by EC FP6 Project “SABRE” FOOD-CT-2006-016250, The
3 Roslin Institute is supported by the UK Biotechnology and Biological Sciences
4 Research Council (BBSRC). ARN thanks for funding from research group RNM179
5 and *P08-RNM-4169* and Julia Romero Pastor and Jose Romero Garzón for help with
6 sample preparation and measurements.

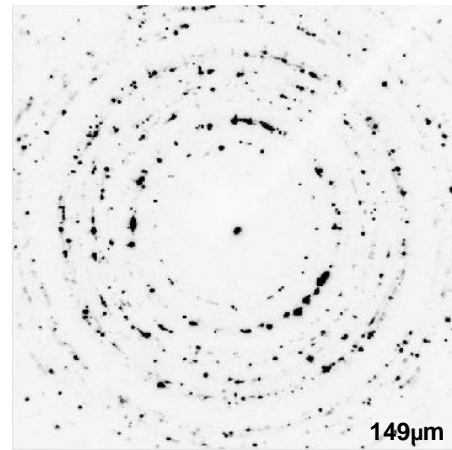
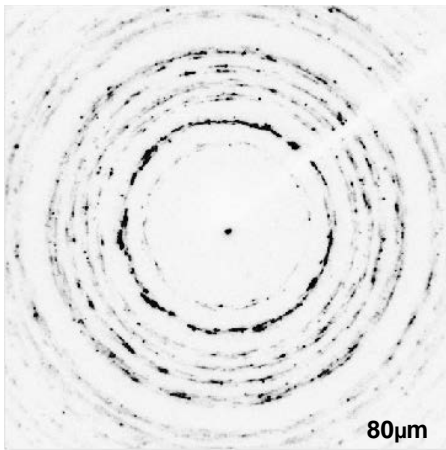
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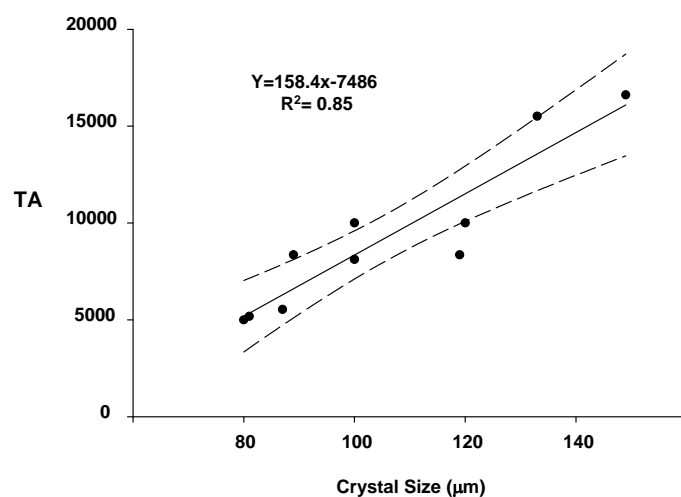
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8



1

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



1
2 *Figure 2. Graph of the relationship between average crystal size determined by cross*
3 *polarised light microscopy and the total peak area (TA) estimate by X-ray diffraction*
4 *of 10 individual egg shells from a pedigree Rhode Island Red population. The line is*
5 *fitted using linear regression and the dashed lines represent 95% confidence*
6 *intervals.*
7

1	Trait	Mean ± SD
2	<i>Values for mean intensity of individual crystal orientations</i>	
3	A_104	4371±782
4	A_108	833±116
5	A_110	1000±154
6	A_113	916±127
7	A_202	1215±185
8	<i>Overall mean intensity</i>	
9	TA	8336±1231
10	<i>Crystal size calculated from calibration of TA</i>	
11	Crystal size (um)	100.8±6.5
12	<i>Crystal orientation</i>	
13	OI lineal	-0.014±0.003

14

15 *Table 1. Summary statistics for the estimates of the average intensities of peaks*
16 *along the Debye-Scherrer ring associated with the most important hkl calcite*
17 *reflections (A_104, 110, 113, 202, 108) their sum, the total peak area (TA) and the*
18 *average crystal size determined by calibration from cross polarised light*
19 *microscopy using the equation in figure 1. The estimate of preferred crystal*
20 *orientation is represented by OI lineal where a value of 0 represents a completely*
21 *random orientation with increasingly negative values representing a more*
22 *orientated crystal structure. The shells of two eggs laid by 898 pedigree Rhode*
23 *Island Red hens aged between 38 and 42 weeks were used for the estimates.*
24

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

10

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

17

18

	Correlation of:	Crystal size		Crystal orientation	
	with	Genetic	Phenotypic	Genetic	Phenotypic
1	<i>Egg weight and shape</i>				
2	Egg Weight	0.45±0.21	0.13	0.09±0.26	0.10
3	<i>Quasi-static measurements</i>				
4	Breaking strength (equatorial)	0.32±0.28	0.08	-0.08±0.33	-0.07
5	<i>SEM thickness measurements</i>				
6	Mean thickness	0.42±0.22	0.20	0.51±0.25	-0.02
7	Mean effective thickness	0.32±0.24	0.15	0.40±0.27	-0.05
8	Mean mammillary thickness	0.61±0.20	0.24	0.66±0.24	-0.10
9	<i>Crystal size</i>				
10	Crystal Size			0.45±0.21	0.23

14 *Table 3. Estimates of genetic correlation (\pm error) and phenotypic correlation*
15 *between crystal size or crystal orientation and measurements of egg weight, egg*
16 *quality and shape, breaking strength, static and dynamic stiffness and egg shell*
17 *thickness measurements derived by scanning electron microscopy (SEM) .*
18

1

2	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)	
5	Trait Crystal size								
6	GGovalb1927GC	1	101.7	0.87±0.37	0.018	13	42	0.7	
7		2	100.0						
8		3	101.5						
9	Ovotrans	1	97.6	-1.70±0.75	0.026	-26	31	0.8	
10		2	100.97						
11		3	99.8						
12	Trait OI lineal								
13	Oc116310poly	1	-0.0132	0.0006±0.0002	0.001	31	46	5.6	
14		2	-0.0144						
15		3	-0.0135						
16	GGovc321992GA	1	-0.0133	0.0007±0.0003	0.004	40	23	9.4	
17		2	-0.0148						
18		3	-0.0137						

19

20 *Table 4. The most significant representatives of the marker haplotypes associated*
 21 *with crystal size and crystal orientation are listed with the estimated size of the*
 22 *additive effect and its error and its size relative to the trait standard deviation.*

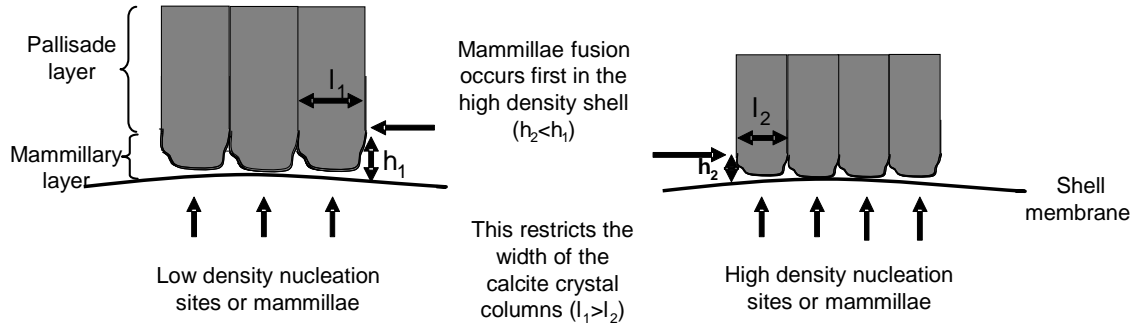
23 ¹*Genotypes represented by the SNP where 1 and 2 are homozygotes and 3 is the*
 24 *heterozygote; ²Trait means from the full-sib model given in the methods section. ³Size*
 25 *of the additive effect, (AA-aa)/2. ⁴Probability from full-sib model, ⁵Effect as % of the*
 26 *SD calculated from the sum of the sire and dam genetic and the environmental*
 27 *variances after fitting the nuisance effects of house and hatch. ⁶Minimum allele*
 28 *frequency. ⁷An estimate is given of the expected increase in the trait mean if the*
 29 *beneficial allele was selected for in the population*

30

31

32

Model for the interaction between crystal growth and egg shell structure



1

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