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Keywords:, eggshell quality, Single nucleotide polymorphisms.

Short title: Candidate genes for eggshell quality

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Summary

Novel and traditional eggshell quality measurements were made from up to 2000 commercial pedigree hens for a candidate gene association analysis with organic eggshell matrix genes; *ovocleidin-116, osteopontin (SPP1*), *ovocalyxin-32* (*RARRES1*), *ovotransferrin (LTF), ovalbumin* and *ovocalyxin-36* and key genes in the maintenance and function of the shell gland; *estrogen receptor (ESR1)* and *carbonic anhydrase II* (*CAII*). Associations were found for i) *ovalbumin* with breaking strength and shell thickness ii) *ovocleidin-116* with elastic modulus, shell thickness and egg shape iii) *RARRES1* with mammillary layer thickness iv) *ESR1* with dynamic stiffness v) *SPP1* with fracture toughness and v) *CAII* with egg shape. The marker effects are as large as 17% of trait standard deviations and could be used to improve eggshell quality.

Damaged eggshells result in losses of 8-11% and provide a route for contamination of egg contents. Genetic selection has been applied to reducing breakage (Preisinger and Flock, 2000) and new measurements of quality are promising (Dunn *et al.*, 2005), however, alternative approaches should be considered. Marker assisted selection would allow the selection of sires for their contribution to female traits and would allow implementation of measurements integrating a number of shell parameters which are difficult to measure. These may predict the eggshell's structural integrity better than a single measurement of thickness or breaking strength (Bain, 1990).

Organic matrix proteins involved in eggshell formation have been identified (Nys, 2004). Guided by these studies we have tested whether single nucleotide polymorphisms (SNP) in *ovocleidin-116*, *SPP1*, *RARRES1*, *LTF*, *ovalbumin* and *ovocalyxin-36* show association with established and novel measurements of eggshell quality. We have also tested association with genes known to be involved in shell gland function; *ESR1* (oviduct maintenance) and *CAII* (bicarbonate secretion).

Blood and DNA was obtained from individually caged pedigree Rhode Island Red sires (n=50), dams (n=421) and offspring (2066 hens) as described (Dunn *et al.*, 2004). Eggs were collected and transported in 8 batches for hens aged 38 to 45 weeks. Phenotypes of 2 (exceptionally 1, 3 or 4) intact eggs were measured per hen and the bird mean analysed. SNPs were detected by resequencing of a subset of sires or from *in silico* prediction. Assays were based on RFLP or the Amplifluor system (CHEMICON Europe Ltd. Hampshire, UK).

Phenotypic measurements: Eggs were weighed, measured and shape index (*SI*) defined as length/breadth. Dynamic stiffness ($K_{dyn,}$ N/m), damping ratio (*Damp*, %), Breaking strength (*Break*, N) , deformation at fracture (*Deform*, mm) and stiffness (*Stiff*, N/mm) defined as breaking strength/deformation at fracture, were measured as described (Dunn *et al.*, 2005).

Thicknesses of the mammillary layer (*Tmamm*, mm) and the combined palisade, vertical crystal layer and cuticle (*Teffective*, mm) and their sum, total thickness (*Thick*, mm) were measured by scanning electron microscopy (SEM) (Panheleux *et al.*, 1999) on 3 samples per egg.

Derived measurements: The elastic modulus (*Eshell*, N/mm^2) measures "material stiffness": E_{shell} = C ((SR)/T_e^2), where S=*Stiff*, R =the radius of curvature (breadth/2), mm, T_e=*Teffective*, and C is a dimensionless constant dependent on the shape index (*SI*), R, and T_e which is calculated as follows: C = A (0.408+(3.026 T_e)/R) where A = (-0.666 + (1.8666 × (SI)) - (0.907 × $(SI)^2$) + (0.153× $(SI)^3$))/0.444. The fracture toughness, (*KC*, N/mm$^{3/2}$) is defined by KC = K_{nd} (F/ $T_e^{3/2}$), where F =*Break* and K_{nd} = 0.777 $(2.388 + 29.934(6/R))^{1/2}$. A full explanation of the derivation of these formulae is given in Bain, (Bain, 1990).

For association analysis means were weighted inversely by the number of eggs measured. The effects of hatch/house (h), tier (t), row (r) and interaction and the marker genotypes (g) were fitted as fixed effects, together with sires (s) and dams within sires (d) and error (e) as random effects to the responses (y), as

$$y_{ijklmn} = s_i + d_j + h.t.r_{klm} + g_n + e_{ijklmn}$$

For haplotype models g is the haplotype effect. Birds with uncertain haplotypes were excluded. 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, and the dominance effect as the difference between the heterozygote mean and the average of the homozygote means. K_{dyn}, *Damp* and (200-*Stiff*) were log transformed (LK_{dyn}, *Ldamp* and *Lstiff*) and the reciprocal of deformation (*1/Deform*) was taken to give approximate normality and consistency of variances.

We examined possible effects of egg production on eggshell quality by fitting 26-50 week egg production as an additional covariate in the linear model for the significant traits, and similarly checked for effects of egg weight, with no change in conclusions.

Ten useable SNP markers were identified, two resulted in substitutions in the protein: Oc116_1336 (AF148716:p437.S>T); Ovocal32_1671 (CAC44378.2:p225L>M) (Table 1). Means ± se and heritabilities for several of the phenotypic traits are published (Dunn *et al.*, 2005). For additional measurements *Teffective* (0.28mm±0.02) , *Tmamm* (0.085mm±0.008), *Eshell* (19680±3089) and *KC* (667±91) are consistent with those given in (Bain, 1990) and the heritabilities are 0.29±0.10, 0.18±0.07, 0.06±0.03 and 0.02±0.03 respectively.

The most significant associations ($p<0.05$) are presented in Table 2. Analysis of haplotypes for *ovocleidin-116* and *RARRES1* suggested relationships with 2 traits per gene, but more specific comparisons between haplotypes were not significant.

The influences of effective (*Teffective*) and total thickness (*Thick*) were additive for Oc-116_310 (highly correlated traits; r = 0.96), and for Oc-116_1336 there was an association with elastic modulus (*Eshell*), which describes the contribution made by the eggshell material to the overall stiffness characteristics of the eggshell (Table 2). Ovocal32_626, had an additive association with mammillary thickness (*Tmamm*). The observed association of Oer_2571 with dynamic stiffness (LK_{dyn}) may be valuable, given the latter's proven benefits (Bain *et al.*, 2006) (Table 2). There are also associations of *SPP1* with fracture toughness (*KC*), a measure of resistance to fracture, and a large effect on shape index (*SI*) with Carb_1210.

The dominance associations, are principally over-dominance dominance and include large effects of *ovalbumin* with total and effective thickness (*Thick, Teffective*), deformation at fracture (*Deform*), and breaking strength (*Break*) (Table 2). This is logical as breaking

strength is positively correlated with thickness (*Thick, Teffect*: r = 0.45) and negatively with deformation (*1/Deform* :r = -0.54). It is, however, difficult to see how this result could be utilised, as the heterozygote represents the less desirable phenotype, which includes a thinner shell, lower breaking strength and greater deformation (Table 2). There are also over-dominance effects of Oc116_310 and 1336 on *SI* and 310 on *1/Deform*.

Because of the large number of statistical tests none of the significance probabilities exceed the Bonferroni correction for a 0.05 significance level (0.0002). However many of the traits are dependent and so this correction may be excessive(Allison and Beasley, 1998). Li and Ji (Li and Ji, 2005) uses the correlations to estimate an "effective" number of traits, six for our data, to reduce the Bonferroni multiple testing penalty, but still not sufficiently to allow any observed effects to be declared significant. For the list of associations in Table 2 the false discovery rate (Benjamini and Hochberg, 1995) is 0.71 at $p < 0.05$. Further experimental evidence is therefore needed.

Table 2 presents significant ($p<0.05$) REML estimates combining information from between and within full-sib families. Overall, within family information was the major contributor to the combined estimates and tests, as expected. Therefore it is surprising that the list of 7 significant additive trait-SNP pairs in Table 2, and a list of 9 that were significant for within family associations (not shown) had only 3 trait-SNP pairs in common. Dam variances were found to exceed sire variances for some traits, and they may have maternal or dominance effect contributions.

In conclusion we propose that some of the markers are 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. In particular there are possibilities for the different components of shell thickness, as well as dynamic stiffness and breaking strength.

Acknowledgement: Supported by EU project QLK5-CT-2001-01606 ‘EGGDEFENCE’.

Gene name	*SNP Location*	*SNP code*	*Location in gene*	*Primers name and sequence used in enzyme assay*		*Enzyme*	*Allele frequency %*	*Offspring in analysis*
Ovocleidin-116	AM076827:g310.C>T	Oc116_310	Upstream	5prime-OC116Ra	AAATGCCCCGATAAGGCC	MnlI	62	1895
				OC116-488r	CAGAGGCAGAGGCAGAAGAG			
Ovocleidin-116	AF148716:c1110.C>G	Oc116_1336	Exon	OC1180F	AGGGGAGAAGCGGACAGAG	PstI	66	1983
				OC1482R	CCACCTCTTGCTGGACTCTA			
Osteopontin (*SPP1*)	U01844:g668.A>T	Ospon	Exon	ospon570F	ACTGAGGGACATGGCTAGTG	BsmAI	64	561
				ospon849R	TCTGTAAGGGGTAGGGATGTG			
Ovotransferrin (*LTF*)	Y00407:g8685.C>T	Ovotrans	Exon	ovot8481F	AAACACGGTTGTGCCTGC	NlaIII	83	649
				ovot8777R	ACCTCTGGCCCCTTCTTTTT			
Ovalbumin	J00895:g8605.C>G	Oval	Exon	ovalb8505	GAGTCATCACACTGAAAAATGC	MnlI	63	788
				ovalb8703	CAGGAACACAGAGAACAAGCA			
Ovocalyxin-32 (*RARRES1*)	AM076826:g1671.C>A	Ovocal32_1671	Exon	Ovocal32_1614F	TCCAACACAGGTAAGAAAGCA	AlwnI	76	1425
				Ovocal 32_1821R	TCCCGATCCATCTTCGAG			
Ovocalyxin-32 (*RARRES1*)	AM076826:g626.C>T	Ovocal32_626	Intron	Ovocal32_586F	GTTCATTCTCCTCCCTGTTCA	AciI	75	1433
				Ovocal32_774R	ACAGTTGAAGATGGTCATGGG			
Ovocalyxin-36	AJ968387:c961.C>T	Ovocal36	Exon	Ovocal36_5F	ATGGGAGGCAGCATCATC	HaeIII	61	1931
				Ovocal36_227R	AATCAGGTGAGAGCCAGCAG			
Estrogen receptor (*ESR1*)	U60211:g2571.G>A	Oer_2571	Upstream	Oer1304f	TCTTTTCCTCGCTTTTTAATGT	HhaI	61	1819
				OerHhaI	ATCTCTCCCTGCTTGATTCA			
Carbonic anhydrase II (*CAII*)	NM_205317:1120.C>T	Carb_1210	Exon	Carb_17151F	TATGGTGATGTTTCTCTTGG	N/A	87	1910
				Carb_18270R	TGTCATTGAAACCTCACTCC			

Table 1. Details of the SNPs used in this study including the position according to the human genome variation society nomenclature (SNP location), abbreviation used in the text (SNP code), primers used and the confirmatory enzymes used in diagnostic assays. The HGNC gene name is included where available however some of the genes are chicken specific and have no mammalian homolog. The frequency of the more abundant allele (Allele frequency) and the total number of offspring used in the analysis of each SNP are also presented.

Gene Marker	Trait	Geno[1] Type	Trait[2] mean	Effect[3] ±SE	Effect as % of the SD[4]	Probability[5]
Additive effects						
Oc116_1336	Eshell	GG	19583	366±132	12%	0.006
		CG	19525			
		CC	18851			
Oc116_310	Teffective	TT	0.2817	-0.0022±0.0009	8%	0.028
		CT	0.2842			
		CC	0.2860			
	Thick	TT	0.3673	-0.0023±0.001	8%	0.039
		CT	0.3700			
		CC	0.3718			
Ospon	KC	AA	627	-10.9±5.4	11%	0.042
		AT	632			
		TT	649			
Ovocal32_626	Tmamm	TT	0.0870	0.00065±0.0003	8%	0.040
		TC	0.0865			
		CC	0.0856			
Oer_2571	Lkdyn	AA	9.617	-0.012±0.004	9%	0.009
		GA	9.625			
		GG	9.641			
Carb_1210	SI	TT	1.282	0.011±0.004	18%	0.027
		CT	1.287			
		CC	1.302			
Dominance effects						
Oval	Thick	GG	0.370	-0.0044±0.002	15%	0.010
		CG	0.365			
		CC	0.370			
	Tmamm	GG	0.0841	-0.0012±0.0004	16%	0.010
		CG	0.0828			
		CC	0.0839			
	Teffective	GG	0.286	-0.0031±0.002	12%	0.044
		CG	0.282			
		CC	0.285			
	Break	GG	40.404	-1.096±0.44	15%	0.015
		CG	38.809			
		CC	39.406			
	1/Deform	GG	3.074	0.077±0.034	14%	0.023
		CG	3.189			
		CC	3.150			
Oc116_310	SI	TT	1.277	0.0061±0.002	11%	0.014
		CT	1.287			
		CC	1.285			
	1/Deform	TT	3.120	0.050±0.02	9%	0.044
		CT	3.163			
		CC	3.106			
Oc116_1336	SI	GG	1.281	0.0067±0.003	12%	0.021
		CG	1.286			
		CC	1.278			

Table 2. Egg shell quality trait means of marker genotypes with the estimated size of the effects±SE and their sizes relative to the trait standard deviation. Data presented have probabilities less than 0.05. [1]Genotypes represented by the SNP; [2]Trait means from the full-sib model given in the methods section. [3]Size of the effect: additive, (AA-aa)/2, dominance, aA-(aa+AA)/2. [4]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, side of battery and tier. [5]Probability from full-sib model.

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