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SHELL QUALITY AND ULTRASTRUCTURAL CHARACTERISTICS OF EGGSHELL IN THE 15TH GENERATION OF CHICKENS SELECTED FOR EGG PRODUCTION TRAITS

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SUMMARY

Scanning electron microscopy was used to assess the effect of selection on eggshell ultra structure in Alexandria chicken. The observed changes were studied to understand the role of selection in eggshell strength. There was a significant difference for egg and eggshell weights between lines. Concerning shell breaking strength, selection improved the shell breaking strength (29.30 vs. 31.71 N). Indeed, toughness (380.9 vs. 411.9), stiffness (133.9 vs. 138.8) and elasticity (15592.3 vs. 15609.5) were improved in line selected compared control line.

Scanning electron microscopy (SEM) analysis indicated that there was a significant increase in total, palisade and caps thickness in eggshell from the selected line compared to the control line. Confluence was significantly increased in the selected line than the control references, with better attachment between cone and shell membranes in the selection line. It can be concluded that the ultra structure of eggshell measurements and/or the associated genetic markers may therefore prove to be useful in selection programs to improve eggshell quality.

***Keywords:** chicken, eggshell, ultra structure, selection*

INTRODUCTION

The most important goal in egg layer breeding is to produce high numbers of good quality eggs with low production and handling costs. Poor egg quality, i.e., poor eggshell strength and egg white thinning, are the major factors affecting egg stability; when common, poor egg quality causes economic losses at all production stages. Eggshell strength and egg white thinning are quantitative traits, i.e., the observed phenotypes are continuously distributed and reflect the interaction of QTL and environmental effects. Estimated heritability's for these traits vary between 0.0 and 0.6 (Washburn, 1990). The eggshell is identified most readily as the structure that defends the ovum from potential pathogens. This is certainly true if the eggshell remains undamaged and so its ability to withstand cracking is important. The eggshell is a complex structure composing organic and mineral components, which are normally laid down in a highly ordered manner (Nys *et al*., 1999). Our understanding of the process of shell formation has been improved by the identification of proteins and genes for the organic component of the eggshell, although it is not fully understood. The mechanism by which the matrix proteins achieve their effect is in part by influencing the rate of crystal formation and providing nucleation sites for crystal growth and attachment of the mineralized shell to the shell membranes at the mammillary bodies, and hence the mechanical strength of the eggshell (Nys *et al.*, 1999). The eggshell membranes delimit the region of mineralization. Ultra structural studies have demonstrated that the eggshell is comprised of three morphologically distinct calcified layers (Fraser *et al*., 1998). Bain (1991) suggested that the organization of the palisade columns is a major determinant of shell stiffness and therefore of shell strength. Shell strength is directly related to shell thickness (Khatkar *et al*., 1997) and the palisade layer comprises approximately two-thirds of the eggshell (Parsons, 1982). Therefore, it is likely that alterations in the thickness of the palisade layer, independent of structural reorganization of the palisade columns, could affect shell strength. Recent transmission electron microscopy (TEM) studies (Fraser *et al*., 1998) have revealed columnar calcite crystals in a vertically aligned matrix associated with the vertical crystal layer. This study examines the effect of selection on mechanical properties and eggshell ultra structural in Alexandria chickens.

MATERIALS AND METHODS

Two lines of Alexandria chicken (selected L1 and control L2) were used in this study. The individual selection program was applied for 15 consecutive generations; through 10 successive years from 2000 to 2010. Selection criteria for high egg production traits were adopted. The field work was done at the

Poultry Research Center, Faculty of Agriculture, Alexandria University, while the lab work including eggshell quality and scanning electron microscopy images were fulfilled at the Department of Poultry Production, Faculty of Agriculture, Ain Shams University. The data used to estimate these traits were collected from 500 females, during season 2009/2010. These females were weighed in grams at the first egg (BWM) and the age at sexual maturity (ASM) was recorded. Egg number and weight were recorded throughout the first 90 days after sexual maturity. The flock history and management in details were presented in Ghanem (1995) and El-Dlebshany (2004).

For selected egg line (L1): Selection for early age at sexual maturity and egg production traits during seasons from 1995 till 2008. However, selection for egg number was done from 2008 till 2010. Females had higher egg number through the first 90 days after sexual maturity than the mean of the line was taken as dams for the next generation.

For control line (L2): Random mating was applied for the same base population without selection for any trait.

Mechanical properties of eggshell:

Egg dimensions (length and width, mm), weight (g) and shell weight (g) were measured on all individual eggs (30 eggs for each line). Shell weight was measured after washing the shells and drying overnight and the percentage of shell was calculated as: Shell% = (shell weight/egg weight*100)

Shell index (g/100 cm$^{2\,(}$Sauveur, 1988) was calculated as: I =(C/S)*100 with C the shell weight (g) and S the shell surface (cm^2) where S = 4.68*P$^{2/3}$ when P = egg weight (g).

Shell mechanical stiffness (N/mm) and breaking strength (N) were measured by quasi static compression using an Instron (UK527, High Wycombe, UK) fitted with a 50N load capture at compression speed of 5 mm/min for breaking strength and 1 mm/min for stiffness measurements.

Stiffness (N/mm) was calculated as the mean value for three linear slopes of the forced deformation curves resulting from the applied load of 10 N on three points on the equator of each egg (about 120° from each other). Breaking strength was measured as the maximum force (N) required fracturing each egg. The shell elastic modulus or Young's modulus (Eshell) in N/mm2 and shell fracture toughness (Kc) in N/mm$^{3/2}$ were calculated for each egg using formulae developed by Bain (1990). The elastic modulus describes the contribution made by the shell material to the overall stiffness of the shell:

Eshell = C [(Sd*R)/T^2]

Where Sd = stiffness, R = radius of curvature (width/2) and C = A [0.408 + (3.026*2*T/width)]

Where A = [(0.153*L^3) - (0.907*L^2) + (1.866*L) - 0.666]/ 0.444 with L = length/width. In addition, as described by this author (Bain, 1990), toughness was calculated with the formula

Kc = Knd (F*T$^{3/2}$) where Knd = 0.777 * [2.388 + (29.934*12/width)]$^{1/2}$ and F is the breaking strength value (N).

Preparation of samples for ultra structural analysis using SEM:

At 30 weeks of age, twenty samples of eggshell were randomly taken from control and selected lines to investigate ultra structural variations. The specimens were prepared by cutting a piece (1 cm) of shell from the equatorial region. The shell membranes were carefully removed by first soaking in water. The loosely adhering membranes were then gently peeled from the edge of the sample inwards. To remove the remaining tightly bound membrane fibers, each sample was then immersed overnight in 6% sodium hypochlorite, 4.12% sodium chloride and 0.15% sodium hydroxide. Thereafter, the specimen was rinsed with water and left to dry at room temperature. Following these preparative treatments, two samples from each egg were mounted in inner side uppermost and in vertically manner on aluminum stubs, coated with gold for three min in an Emscope Sputter Coater. These samples were examined using JEOL JSM-T330A scanning electron microscopy at 15 Kv. The incidence of ultra structural variants at the level of the mammillary layer was assessed according to the methodology and terminology developed by the Poultry Research Unit, University of Glasgow (Bain, 1990, Solomon, 1991 and Bain, 1992). The cross-sectional lengths of palisade and mammillary layers were directly measured in µm using scaling software provided with the SEM at a magnification of x200. The total thickness of each specimen was measured as the distance from its' outermost surface to the point where the basal caps inserted into the shell membranes. The thickness of the mammillary layer was also assessed, this being the distance from the basal caps to the point at which the palisade columns first fused. Subtraction of these two measures provided a length of the palisade thickness or effective thickness (Bain, 1990 and Solomon, 1991). Triplicate measures were performed in each case and the mean values were used in the statistical analysis.

Statistical analysis:

Data were subjected to a one-way analysis of variance with line effect using the General

Linear Models (GLM) procedure of SAS User's Guide (2001).

RESULTS AND DISCISSION

The differences between lines were significant (P≤0.01) for each of age at sexual maturity, body weight at sexual maturity and egg number during the first 90 days after sexual maturity, in addition, difference between lines was significant (P≤ 0.05) for egg weight (Table 1).

Table 1. Means and standard errors for studied traits of egg lines and control

	Line			
Trait	**Control**	**Selected**	**Diff.**	**Prob.**
ASM	169.1± 18.5	154.3± 12.5	14.8	0.01
BWM	1521± 13.6	1448± 10.5	73	0.01
Egg number (90 days)	52.9± 1.8	63.7± 1.5	10.8	0.01
Egg weight	36.3± 0.12	39.4± 0.17	3.1	0.05

These results indicated that egg line hens (L1) were earlier for age at sexual maturity, lighter for body weight at sexual maturity, higher for egg number during the first 90 days after sexual maturity and bigger for average egg weight during the first 90 days after sexual maturity than the control line (L2). These results are in agreement with Ghanem (1995), El-Tahawy (2000) and El- Dlebshany (2004). It can be concluded that the differences between egg line and its control may be due to the genetic changes resulting from selection for 15 generations.

Data presented in Table (2) show that the effect of selection on egg weight and biomechanical properties of Alexandria chicken. It could be observed that the selected line produced significantly heavier egg weight by about 2.6g, compared to the control line. In contrast, there was no significant difference either between lines for length or breadth of eggs, nor for shell index and eggshell percentage. Concerning shell breaking strength, selection improved the shell breaking strength (29.30 *vs.* 31.71 N). Indeed, toughness (380.9 *vs.* 411.9), stiffness (133.9 *vs.* 138.8) and elasticity (15592.3 *vs.* 15609.5) were improved in L1 compared L2. The data show that there is improvement in the mechanical properties of shell the Alexandria line selection. Both environmental and genetic factors affect the strength or quality of eggshell, and hence its likelihood of cracking during normal egg handling processes. To some extent the environmental factors could be controlled, for example, through improvement in bird management and nutrition. Nevertheless, genetics remain an important way to reduce eggshell breakage, as it is both permanent and cumulative. For decades, breeding companies have used laboratory-based measurements such as shell breaking strength, non destructive deformation (toughness, stiffness and elasticity) and ultra structure and organic matrix of eggshell in their selection programs. Coucke (1998) and Coucke *et al.* (1999) devised a simple acoustic resonance test, which can be used to calculate the mechanical or dynamic stiffness of intact eggs.

Table 2. Effect of selection (mean±SE) on egg weight and biomechanical properties of the Alexandria chicken

	Line			
Trait	**Control**	**Selected**	**Diff.**	**Prob.**
Egg weight (g)	51.13±4.04	53.76±3.20	+2.63	0.01
Egg length (mm)	54.24±1.65	54.71±1.47	+0.44	NS
Egg width (mm)	42.03±2.82	41.99±0.81	-0.04	NS
Shell index (g/100m^2)	7.61±0.55	7.73±0.28	+0.12	NS
Shell weight (g)	4.96±0.52	5.15±0.25	+0.19	0.02
Shell (%)	11.40±0.77	11.35±0.73	-0.05	NS
Breaking strength (N)	29.30±2.83	31.71±2.14	+2.41	0.01
Toughness (N/mm$^{3/2}$)	380.91±37.74	411.94±27.62	+31.03	0.001
Stiffness (N/mm)	133.85±6.16	138.82±3.84	+4.97	0.05
Elasticity (N/mm^2)	15592.30±359.21	15609.49±351.61	+7.19	0.03

This measurement has subsequently been found to correlate well with other measures including static stiffness (r=0.90) and eggshell thickness (r=0.78) (De Ketelaere *et al.*, 2002). Dunn *et al.* (2008) reported that the dynamic stiffness measurement also has both a high heritability (0.53) and a high genetic correlation with eggshell breaking strength (0.49). These results indicate that these measurements could be used successfully in a breeding program to improve eggshell quality. Genetic progress in eggshell quality, however, depends not only on having a measurement whose variance contains a substantial genetic component; it must also be shown to relate to the incidence of breakages in the field.

The cross-sectional lengths of palisade and mammillary layers were directly measured in µm using scaling software provided with the SEM at a magnification of X 200 and the results are presented in Table (3). It could be observed that there is significant increase in total, palisade and caps thickness in selected line compared to control line. These result same as trend with Carnarius *et al.* (1996) found a significant correlation between the effective thickness of the shell and puncture force. The palsied layer is an important component of shell thickness.

Table 3. Effect of selection (mean ± SE) on Absolute and relative thickness of individual egg layer

	Line			
Trait	**Control**	**Selected**	**Diff.**	**Prob.**
Total thickness (µm)	282.50±15.75	315.50±18.89	+23.45	0.001
Palisade thickness (µm)	205.50±15.43	235.25±8.78	+24.01	0.01
Cap thickness (µm)	77.00±12.73	80.25±10.51	+2.28	0.001
Palisade (%)	72.77±3.90	74.66±1.81	+2.46	0.04
Cap (%)	27.23±3.90	25.34±1.81	-3.05	0.01

According to Ruiz and Lunam (2000) the palisade layers provides the stiffness characteristics of the shell and thereby shell strength. Thus, a reduction in its relative thickness could compromise shell strength leading to a higher incidence of breakage. In addition, Bain *et al*. (2006) reported that the eggshell consists of several different layers and proposed that each of these different layers must variously contribute to the eggs performance under load. Solomon (1991) and Bain (2005) described 12 structural variations in the mammillary layer of weak and poor quality eggshells. Watt and Solomon (1985) found that there were a high proportion of structural abnormalities in the cone layer of those eggs which were cracked or broken. Information from Table (4) clarified the various features present in the interior surface of eggshell after removing shell membranes the mammillary layer, which can affect eggshell stiffness. Data revealed that there were no significant differences between lines regarding depression, erosion, cubics, aragonite, type A's and changed membranes. However, the present result, showed that eggshell of each line were significantly deferent in confluence, cuffing and type B. Figure (1) presented an example of confluence appearance for the selected line, showing good mammillary cap confluence and extensive confluent caps, which provided good attachment to the shell membranes and consequently increases strength eggshell, compared to the control line. Solomon (1999) found that good shell ultra structure benefited high confluence reflecting good attachment with membranes. Table (4) displayed a cuffing form noticed in selected line eggshells. This material had a useful function with increasing cohesion and merging of the calcified columns, thus increasing eggshell strength. Cuffing appears as secondary crystallization between the cones and is believed to be formed at some point after the mammillary knobs have begun to fuse (Bain, 1990). A significant difference between lines eggshell was observed for type B's structures. Figure (2) showed type B's in control eggshell, which reduced the eggshell quality as a harmful affecting adjacent columns adhesion.

Table 4. Effect of selection (mean ± SE) on ultra structural variants of eggshell mammillae

	Line			
Trait	**Control**	**Selected**	**Diff.**	**Prob.**
Confluence	1.80±0.33	4.60±0.47	+2.80	0.01
Fusion	2.20±0.68	1.40±0.14	-0.80	NS
Cuffing	4.20±1.53	3.00±0.21	-1.20	0.02
Alignment	1.80±0.30	1.00±0.00	-0.30	NS
Type B	6.20±1.60	3.80±0.79	-2.40	0.01
Depression	1.60±0.12	1.00±0.00	-0.60	NS
Erosion	1.00±0.00	1.00±0.00	0.00	NS
Cubic	1.80±0.30	1.00±0.00	-0.80	NS
Aragonite	1.80±0.52	1.00±0.00	-0.80	NS
Caps	2.20±0.71	1.00±0.00	-1.20	NS
Type A	2.20±0.28	1.40±0.14	-0.80	NS
Total score	26.80±2.51	20.20±2.16	-6.60	0.01

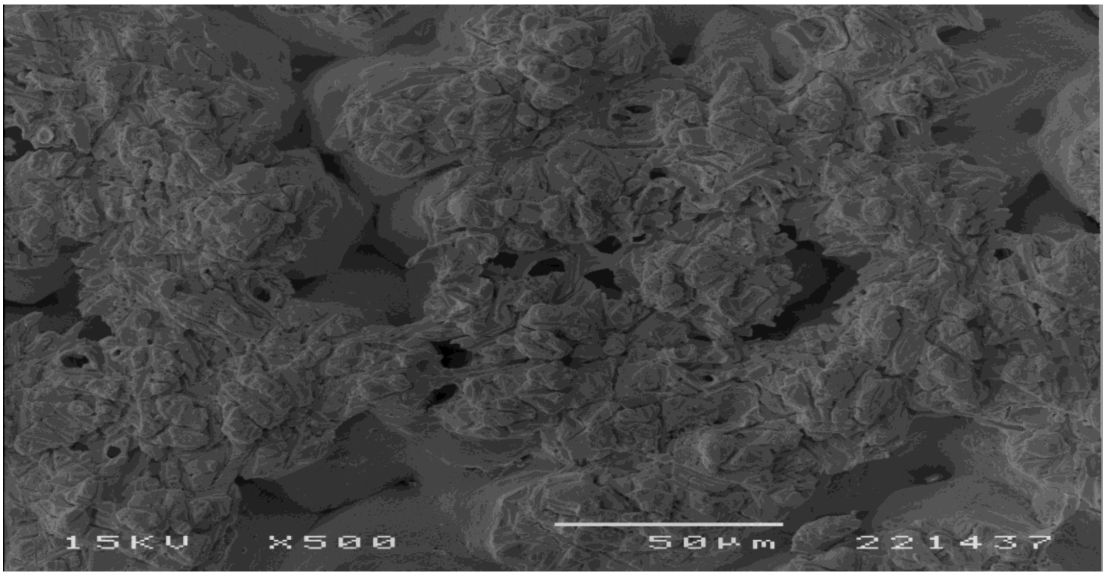

Figure 1. Good confluence and rounded caps in eggshell of selected egg line

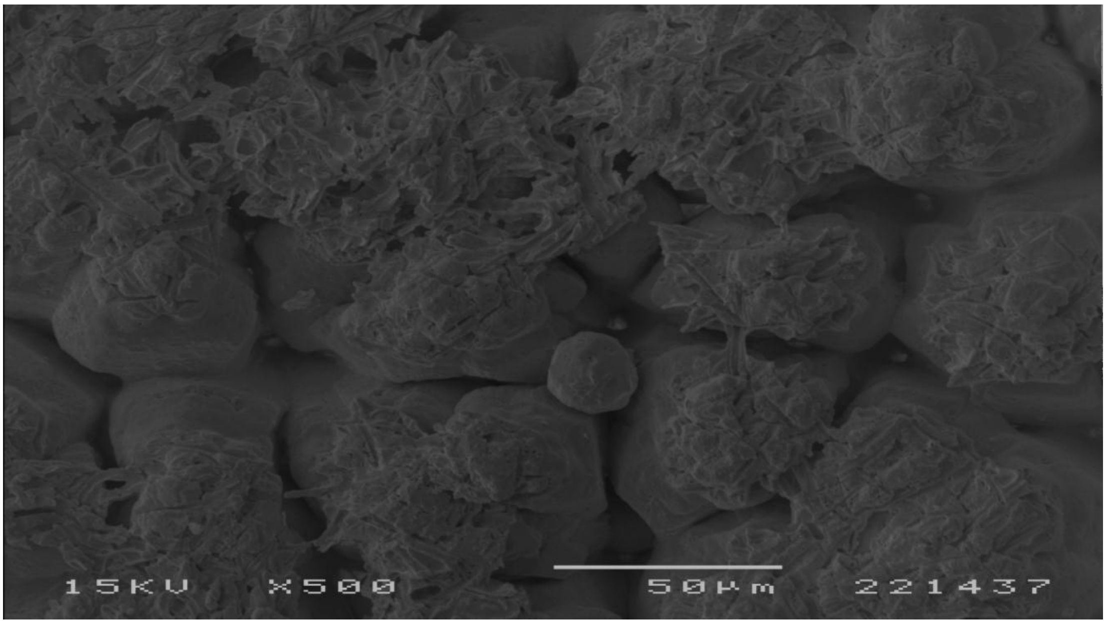

Figure 2: Type B's (poorly constructed mammillary layer) in eggshell of control line.

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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تنوع التركيب البنائى لقشرة البيض فى الدجاجات المنتخبة لزيادة انتاج البيض لمدة 15 جيل

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صممت هذة التجربة لتقييم الخصائص الميكانيكية والتركيب البنائى لقشرة البيض باستخدام المجهر الإلكتروني لتقييم تأثير الانتخاب على التركيب البنائى الدقيق لقشر البيض التركيبية في دجاج الإسكندرية. تم دراسة التغيرات الملحوظة لفهم دور الانتخاب على قوة قشرة البيضة للدجاج المنتخب لزيادة انتاج البيض. لوحظ وجود اختلافات معنوية عالية بالنسبة لوزن البيض ووزن قشرة البيض بين الخطوط المنتخبة والكنترول.اما بالنسبة لدراسة قوة تحمل الكسر لقشرة البيضة، اظهرت النتائج الى ان تاثير الانتخاب عمل على تحسين قوة تحمل قشرة البيضة بمقدار (31.71 N) مقابل (29.30.) بالتأكيد، تم تحسين لمتانة القشرة بمقدار(411.9&380.9)، وتصلب (138.8&133.9) ومرونة (15592.3&15609.5) بالنسبة للخط المنتخب مقارنة بخط الكنترول الغير منتخب.كما انة تم ملاحظة الزيادة المعنوية بالنسبة الى الطبقة الحسكية plisade layer والالتحام للاعمدة بالنسبة للخط المنتخب مقارنة بالخط الغير منتخب. وكذلك لوحظ زيادة معنوية للالتحام الجيد للاعمدة واغشية القشرة للخط المنتخب مقارنة بخط الكنترول. والخلاصة فقد اوضحت النتائج انة توجد اختلافات معنوية للدجاجات المنتخبة لمدة 15 جيل بتحسين التركيب البنائى لقشرة البيض بالمقارنة بالدجاج الغير منتخب.