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RELATIONSHIPS BETWEEN EGGSHELL PIGMENTATION, ULTRASTRUCTURE AND WATER VAPOUR CONDUCTANCE IN THE HOUBARA BUSTARD (*CHLAMYDOTIS UNDULATA MACQUEENII*)

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Introduction

Typically, aviculture of the Houbara bustard (*Chlamydotis undulata*) is based around artificial insemination of females and artificial incubation of eggs. Success rates under artificial incubation are not always as good as is expected (Hémon *et al*., 2000). The reasons why hatchability is poor are not clear although eggshell quality could be important.

Houbara eggshells exhibit variability in the degree of pigmentation from almost white to dark brown, heavily spotted but how this affects ultrastructure is not known. In pheasants (*Phasianus colchinus*) colour of the eggshells is correlated with their ultrastructure (Richards and Deeming, 2001). Blue eggshells are significantly thinner and have ultrastructural defects compared with more typical olive green–brown shells. Blue eggs lose more water vapour under standard incubation conditions suitable for olive-brown eggs and hence have a significantly lower hatchability (Richards and Deeming, 2000). However, surface pigmentation of eggshells of the red-legged partridge (*Alectoris rufa*) do not correlate with the presence of shell accessory material (Fraser *et al*., 1999).

Weight loss of Houbara (*C. u. macqueenii*) eggs incubated by females is higher than previously recorded in an incubator (Deeming and NWRC Unpublished data). This suggests that there is a change in the functional porosity of the eggshell during natural incubation. Natural increases in the porosity are known in several species (Deeming, 2002). Removal of shell accessory material (SAM) significantly increases water vapour conductance of eggshells of penguins (Handrich, 1989; Thompson and Goldie, 1990), the Mandarin duck (Baggott and Graeme-Cook, 2001), and some domestic poultry (Deeming, 1987). Other changes in water vapour conductance are associated with thinning of the eggshell by removal of calcium carbonate by the embryo (Booth and Seymour, 1987; Booth, 1989). It is unclear whether changes in weight loss in Houbara eggs are related to abrasion of SAM during incubation in a sandy nest, or due to thinning of the eggshell.

In this study, ultrastructure of Houbara eggshells was investigated in relation to 1) degree of pigmentation and water vapour conductance, and 2) effects of natural incubation on external structure.

Methods

Eggshells were graded on the basis of the degree of their pigmentation: ''Very light'' (almost white), ''Light'' (pale buff, few spots), ''Mid'' (buff with some dark spots) and ''Dark'' (dark buff with widespread dark spots). Pieces of shell were taken from the equatorial region of each egg and set in *Leit-C* carbon-based cement on aluminium microscope stubs. They were set either flat on the stub surface with the external surface uppermost, or set vertically into the cement so presenting the fractured surface uppermost. Specimens were gold-coated using a sputter-coater (*Emitech*) for 3- minutes. Microscopy was carried out using a *Hitachi S-570* scanning electron microscope operating at 20 kV.

For each of the eggshells a photographic image captured on celluloid was taken of both the external surface and the radial fracture surface, with the microscope scale bar calibrated at 100-µm. Micrograph images were scanned, digitised and analysed using *ImageJ* image analysis software.

Prior to determination of shell thickness of other shell fragments, organic shell membranes and SAM were removed by treatment with sodium hypochlorite (Kern *et al*., 1992). Shell thickness was measured using a *Mitutoyo* ball-head micrometer measuring to 1 µm. Pore counts were taken on shell pieces which were painted on the inside surface with Evans blue solution. Penetration of the dye through the pores allowed them to be counted on the outer surface using a stereomicroscope at a magnification of x32 (an area of 21.65 mm^2). Two replicates of between 4–10 samples of shell from each egg were counted for pores and the average pore per cm^2 calculated from the total area examined. Pore diameters were measured at the narrow point of the canal from micrograph images.

Values for pore diameter and pores per cm^2, in conjunction with the surface area (calculated from initial egg mass [IEM]) allowed for functional pore area and water vapour conductance (GH_2O) to be calculated for each eggshell.

The total area of shell deemed to be free from SAM was expressed as a proportion of the total area of the image. Coverage of the shell by SAM was determined by subtraction from one. At least two images of each eggshell were analysed and values for SAM coverage were averaged.

One-way analysis of variance (*Minitab* version 12.2) was carried out on square root transformed and compared values based on 1) shell colour and 2) time under the female. Tukey's pairwise comparisons had an individual error rate of 0.05.

Results

Ultrastructural characteristics of the Houbara eggshells were comparable to many other bird eggshells (Mikhailov, 1997). Pore morphology was variable with some pores having narrow canals and narrow, shallow orifices at the outer surface whereas others had a broad canal with a wide orifice extending deep into the palisade layer of the shell (Figure 1A). SAM was an amorphous mass of an organic (probably protein-based) material (Figure 1B).

Initial egg mass (and hence calculated surface area) was not significantly different in the four different shell types. Other shell characteristics were different between the colour types. Compared with shell thickness predicted on the basis of egg mass (Ar and Rahn, 1985), ''Very light'' and ''Light'' shells were only 67–70% of the predicted thickness compared with 85–90% for the other two shells types (Figure 2A). ''Light'' shells had 101.6 (± 10.4) pores per cm^2 compared with values of 87.5 (± 4.8), 73.2 (± 4.6) and 82.6 (± 5.5) for ''Mid'', ''Dark'' and ''Very light'' shells respectively. This meant that ''Very light'' and ''Light'' shells had values for pores per shell comparable to that predicted values but the darker shells had much fewer pores per shell (Figure 2B). Pore radius averaged from all shell types was 11.9-µm giving an individual pore area of 109.3-μm^2. However, average pore radius was different between shell types: ''Very light'' – 10.9-µm, ''Light''– 13.5-µm, ''Mid'' – 11.8-µm, and ''Dark''– 11.3-µm. When the average pore radius was used to calculate total pore area (A_p) then values for A_p reflected the pore numbers but using shell-specific pore radii the A_p for ''Light'' increased considerably and approached that predicted on the basis of IEM (Figure 2C). These differences were reflected in the values for GH_2O calculated for the different shell types (Figure 2D). ''Mid'' and ''Dark'' shells had values for GH_2O only 40% of the predicted value compared with 60% for the ''Very light'' shells. When the average pore radius was used ''Light'' shells had a GH_2O only 75% of the predicted value but this increased to match the predicted value once shell-specific pore radius was used (Figure 2D).

Four of five ''Very light'' shells did not have SAM Hence the large SD in Table 1). The most external layer of the eggshell was assumed to be the vertical crystal layer composed of calcium

Figure 1. Scanning electron micrographs of (A) the radial face and (B) the external surface of a Houbara eggshell. In (A) note the pore with its broad, deep funnel opening on the outer surface and in (B) the plaques of shell accessory material that does not completely cover the crystalline shell (arrow). Scale bars = 100 µm

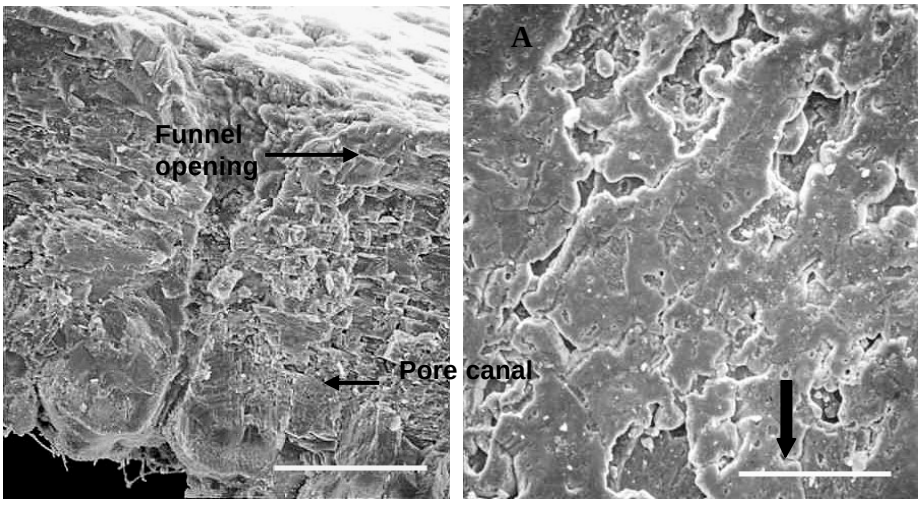

Figure 2. Comparison between measured means + SEM and predicted values (using equations from Ar and Rahn, 1985) for (A) Shell thickness (µm), (B) Total pores per shell, (C) total pore area (A_p, mm^2) and (D) water vapour conductance (GH_2O, $mgH_2O/D/Torr$). Values for A_p and GH_2O are shown for the average pore area and shell-specific pore area.

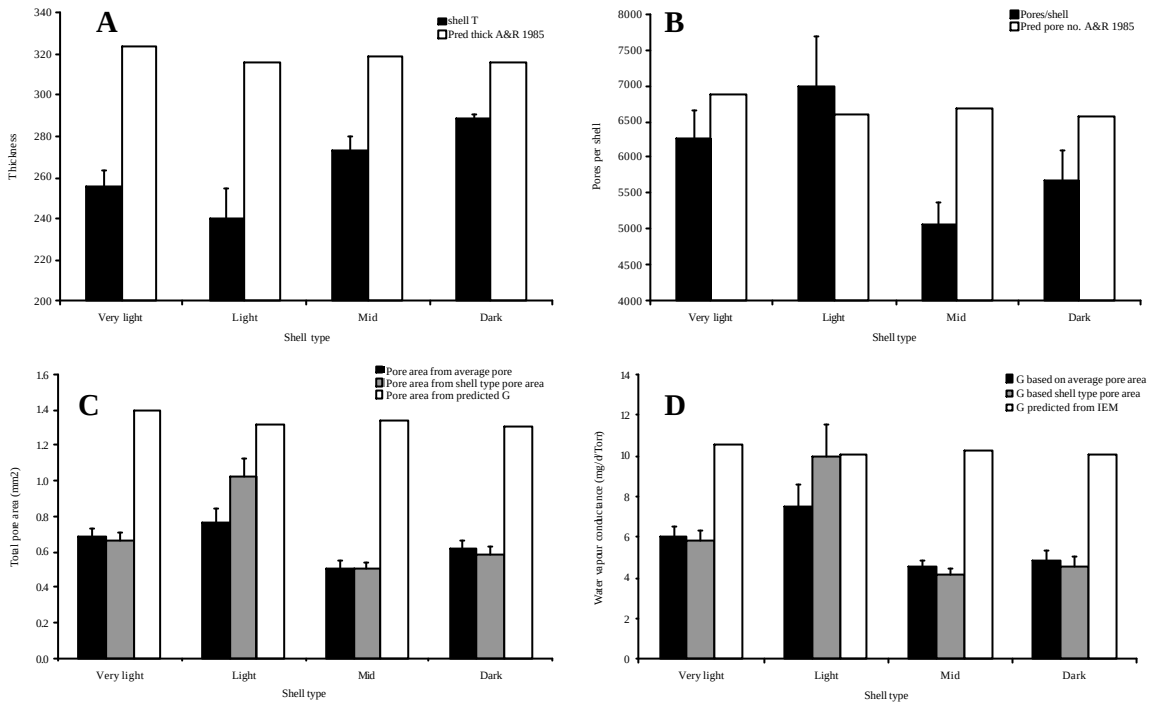

Table 1. Mean values for the % coverage by shell accessory material based on (A) shell pigmentation and (B) time spent under an incubating female.

Shell pigmentation	Number of shells	Mean % coverage	SD
''Very light''	5	17.98[a]	40.20
''Light''	6	83.47[a]	7.55
''Mid''	5	87.74[a]	7.68
''Dark''	5	87.28[b]	5.98
Days incubated under female			
O	5	87.74[a]	7.68
2	2	90.40[a]	2.69
7	5	80.72[a,b]	5.80
18	2	74.35[b]	1.77

carbonate and that was not continuous over the whole of the shell surface area. Other shell types exhibited a distinct layer of SAM that had a smooth texture. For large areas of ''Dark'' shells the external surface was a near continuous, smooth covering of areas of pigment crystals. There was a significant ($F_{3,20}$ = 15.88; p>0.001) effect of the shell pigmentation on the coverage by shell accessory material. This was entirely due to the very low average value for coverage in the ''Very light'' shells (Table 1A). For the other shells the coverage was not significantly affected by pigmentation.

Comparison of ''Mid'' eggshells that had been incubated under a female for 0 or 2-days showed little difference in the appearance of the SAM (Table 1B). By contrast, an incubation period of 7-days under a female caused the number and size of the holes in the SAM to increase considerably. After 18-days the number and size of holes in the SAM had not increased dramatically but the SAM appeared to be thinner. The number of days that an egg was incubated under a female did not significantly ($F_{3,13}$ = 3.35, p=0.064) affect the percentage coverage by the SAM (Table 1B). However, the decline in coverage was different for the shells incubated for 18-days compared with those incubated by the bird for 0 or 2-days (Table 2B).

Discussion

The ultrastructural characteristics of the Houbara eggshell are adapted to a produce low values for GH_2O that are lower than that predicted from egg mass but match the dry nesting environment normally encountered in the wild. Under artificial conditions, a low humidity will be required for ''Mid'' and ''Dark'' shelled eggs to achieve the correct weight loss during incubation. ''Light'' and ''Very light'' shells were

thinner, had a greater pore number and higher values for GH_2O. To prevent excessive weight loss these shells require a higher humidity to prevent dehydration. Therefore, eggshell colour will allow the appropriate starting humidity to be selected at setting.

Coverage by SAM was unaffected by shell pigmentation in most shell colour types although coverage in ''Light'' shells was a little lower that in the ''Mid'' and ''Dark'' shells. However, most of the ''Very light'' eggshells exhibited a complete absence of SAM. This may indicate that the egg was laid prematurely before all of the components of normal shell ultrastructure were deposited.

Although not statistically significant, incubation under a female did appear to cause a degradation of the SAM on the Houbara eggshells. Given the dry nesting environment in the wild nest it is likely that the SAM serves to reduce water loss during clutch formation and the early stages of incubation when there is a danger of loss of embryonic viability through dehydration. Turning of eggs in the sandy nest would appear to rub away the SAM, presumably exposing the pore orifices to the nest air and increasing gas conductance (to what extent is still under investigation). The loss of SAM will not happen in artificial incubators and this may increase mortality of late-term embryos.

In conclusion, colour of Houbara eggs does indicate ultrastructural differences in the shell and these are reflected in GH_2O values. ''Very light'' eggshells typically lack SAM. Incubation by the female in a sand nest erodes the SAM from the shell surface thereby probably increasing gas conductance. Whether this is of physiological importance to the embryo and should be replicated in artificial incubation requires further investigation.

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