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ULTRASTRUCTURE OF THE EGGSHELL OF SELECTED PALAEOGNATHAE SPECIES – A COMPARATIVE ANALYSIS*

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Abstract

The study aimed at comparative analysis of the eggshell ultrastructure, indicating differences and similarities in its structure depending on the bird species. The study was carried out in ostrich, emu and rhea breeding flocks. The birds were kept under open system. The ostrich flock comprised 6 females and 3 males, the emu flock included 22 birds with equal sex ratio, while the rhea flock consisted of 16 females and 4 males. Emus and rheas were 5 years old and in their 3rd laying year, whereas ostriches were 4 years old and in their 2nd laying year. The analysis of eggshell ultrastructure and porosity was performed on the post-hatching eggshells being obtained after the incubation of eggs from around peak laying period of these birds. In total, 27 eggshells were evaluated, 9 of each species. Analysis of the ultrastructure of ostrich, emu and rhea eggshells showed their different architecture being dependent on the bird species. The cuticle in ostrich eggs adhered firmly to the vertical crystal layer, it mainly occurred around pore canal orifices in rhea eggs, while only its residual presence was observed in emu eggs. The percentage of the vertical crystal layer was similar in the ostrich and rhea eggshells (2–3%) but significantly higher in the emu eggshells (9%). The largest number of mammillae per unit of inner eggshell surface area was recorded in emus, while the broadest palisades in rheas. The ostrich eggshells were shown to be characterised by the least number of pores per unit of surface area, with significantly narrower pore canals.

Key words: ostrich, emu, rhea, eggshell ultrastructure, eggshell porosity

Numerous studies on egg quality including eggshell ultrastructure have been carried out in many poultry species (Burton and Tullett, 1983; Krystianiak et al., 2005; Michalak and Mróz, 2004; Soliman et al., 1994). However, such a range of knowledge has not been gained in the case of Palaeognathae which include, among others, ostriches, emus and rheas. These birds have been economically exploited recently for the purpose of using their meat, feathers and leathers as well as oil in the cosmetic and pharmaceutical industries (Horbańczuk, 2002; Minnaar and Min-

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naar, 1993). One of the reasons impeding the development of their farm breeding is a relatively low hatching rate. Limited number of scientific research causes that many questions referring to their reproduction remain unanswered. Among others, there is no information on eggshell porosity and its ultrastructure which affect incubation results. Eggshell structure in the birds belonging to the subclass Palaeognathae differs notably from that of the eggshells of other species being described in literature (Board and Tullett, 1975; Christensen et al., 1996; Tullett, 1984; Wiercińska and Szczerbińska, 2010), which is connected with a considerable weight of eggs in these birds. With a large egg weight, thick and appropriately structured eggshell, securing proper embryogenesis, is necessary. No detailed analysis of the ultrastructure of ostrich, emu and rhea eggshell, including successive layers that build it, has been carried out so far. These issues are very important not only due to cognitive reasons but also because of the interest in the breeding of productive ratites and a growing demand for their chicks. The technology of incubation of the eggs of these birds is being continuously improved. At present, thermal and humidity parameters for the ostrich, emu and rhea hatch are similar, which does not seem to be appropriate considering the differences in the egg size and the structure of eggshell surface.

It was therefore appropriate to characterise the eggshell ultrastructure, indicating differences and similarities in its structure depending on the bird species.

Material and methods

Comparative study of the eggshell structure was carried out in African ostrich (*Struthio camelus*), emu (*Dromaius novaehollandiae*) and rhea (*Rhea americana*) breeding flocks. The birds were kept under open system; ostriches and rheas at private farms, and emus at an experimental farm of the Department of Poultry and Ornamental Birds Breeding, West Pomeranian University of Technology in Szczecin. The ostrich flock comprised 6 females and 3 males, the emu flock included 22 birds: 11 females and 11 males, while the rhea flock consisted of 16 females and 4 males. Emus and rheas were 5 years old and in their 3rd laying year, whereas ostriches were 4 years old and in their 2nd laying year.

The analysis of eggshell ultrastructure and porosity was performed on the post-hatching remnants being obtained after the incubation of eggs from around peak laying period of these birds. In total, 27 post-hatching eggshells were evaluated, 9 of each species. Observations of the eggshell ultrastructure were conducted according to the methods given by Christensen et al. (1996). Eggshell fragments from the blunt, central and pointed parts of egg, about 2 cm^2 in size, were prepared in 3% glutaraldehyde, dried and then covered with a gold and palladium alloy (AuPd40) by vacuum deposition. The samples (eggshell fragments) prepared this way were observed under a scanning electron microscope (Jeol JSM 6100, 20 KV). Measurements were made on 1120 microscopic images, using Jeol Semafor 4.0 software. The method of pore identification and counting was adopted after Tyler (1953) with necessary modifications. The eggshell fragments from the blunt and pointed egg ends and its central part, about 2 cm^2 in size, were boiled in 5% sodium hydroxide for 40 minutes (ostriches), 30 minutes (emus) and 25 minutes (rheas). Afterwards, the eggshells were rinsed with distilled water, dried at room temperature and stained with methylene blue. The eggshell porosity was determined using a stereoscopic microscope, at a fourfold magnification, on a surface area of 0.25 cm^2. After the data were completed and entered into a Statistica 7.1 database, mean values and standard deviations for all eggshell parameters being studied were calculated. The results were analysed with one-way analysis of variance (ANOVA). Significance of differences was verified by Duncan's test.

Results

In the eggshells under analysis, the outer shell membrane was formed by a layer of intersecting keratin-collagen fibres being covered by numerous small protein deposits (Figure 1). The thickness of these fibres was similar and ranged from 1.2 µm in ostriches to 1.8 µm in rheas, being not significantly different (Table 1). The outer shell membrane fibres were rooted in the tips of mammillae, forming "anchor points" (Figure 2).

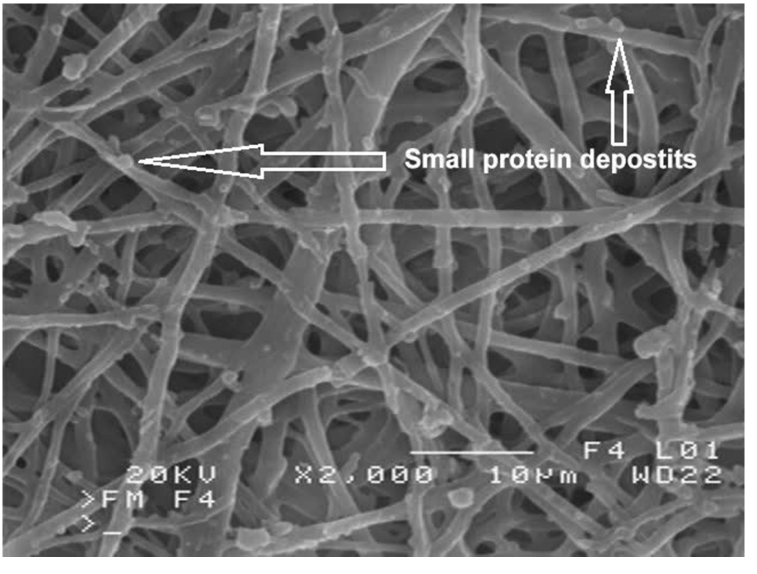

Figure 1. Outer shell membrane fibres in rhea eggs

The analysis of eggshell surface showed its large differentiation in respective ratite species. The most varied eggshell surface was observed in rhea eggs, being covered by oval and spherical structures or polygonal depressions. Considerable differences were also observed in the cuticle distribution on the eggshell surface. In ostriches, the cuticle adhered firmly to the vertical crystal layer, covering the whole surface of eggshell (Figure 3). Cracks in the mucin layer in the eggshells of these birds mostly formed quadrangular plates. The cuticle in the rhea eggshells mainly occurred around pore canal orifices (Figure 4). It was not observed either that the emu eggshells were entirely covered by that type of layer. Only in two microscopic images, small fragments of organic layer were present in depressions of the vertical crystal layer (Figure 5). Mammillae in the emu, ostrich and rhea eggshells were oval or took the shape of irregular polygons. Since the observations were made on post-hatching eggshells, clear changes were seen in the structure of mammillae due to demineralisation during embryogenesis (Figures 6 and 7). In rheas and ostriches, the mammillae were similar and larger in comparison to emu. These differences were confirmed statistically and amounted to 17.6 μm (Table 1). Interspecific differences were also observed in the number of mammillae. The largest number of mammillae per unit of inner eggshell surface area, being significantly different, was recorded in emus (Table 1).

Table 1. Characteristics of the eggshell ultrastructure ($\bar{x}$±SD)

Item	Species		
	ostrich	emu	rhea
Thickness of outer shell membrane fibres (μm)	1.2 a±0.2	1.7 a±0.3	1.8 a±0.3
Mammilla diameter* (μm)	104.5 a±27.2	88.9 b±17.3	108.5 a±28.8
Number of mammillae (mammillae/mm^2)	188.2 a±27.7	295.8 b±24.1	178.4 a±23.8
Palisade diameter (μm)	180.1 a±55.7	157.3 a±15.8	216.3 b±32.6
Thickness of the vertical crystal layer (μm)	36.9 a±11.0	79.0 b±18.9	22.9 a±4.8
(%)	1.99 a±1.1	8.66 b±2.0	2.83 a±0.9

*Mean value of two measurements – at the broadest and narrowest points.
a, b – mean values in rows marked with different letters differ significantly (P≤0.05).

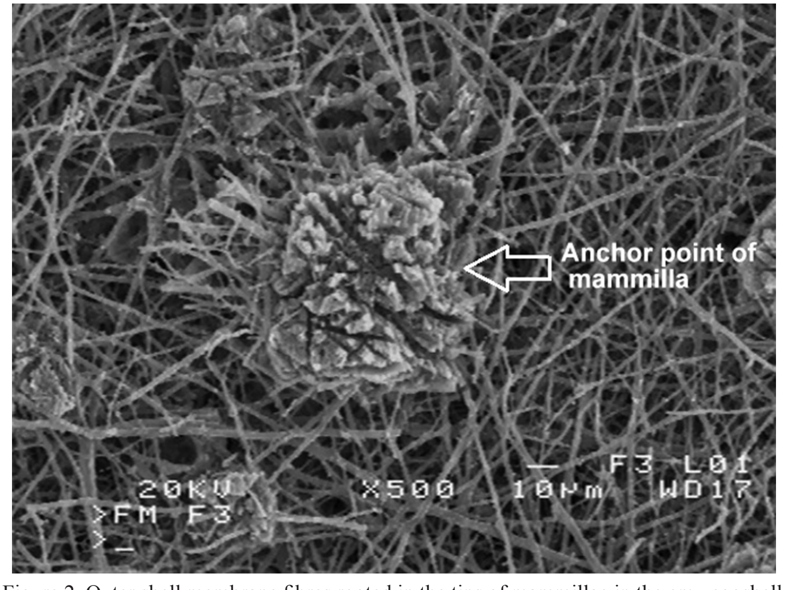

Figure 2. Outer shell membrane fibres rooted in the tips of mammillae in the emu eggshell

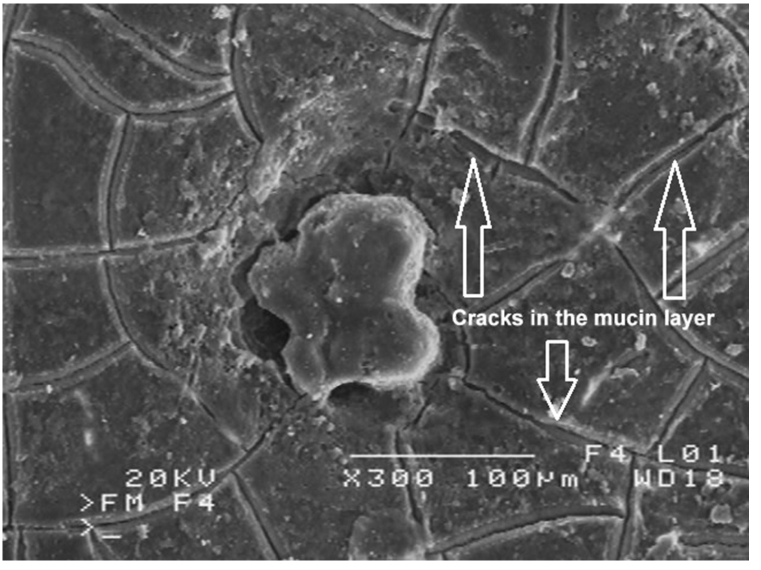

Figure 3. Cuticle on the eggshell surface in ostrich

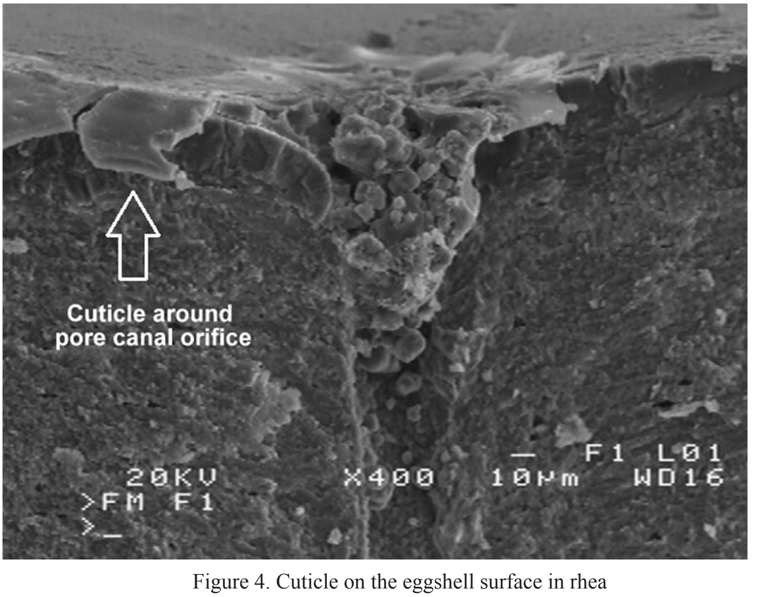

Figure 4. Cuticle on the eggshell surface in rhea

The palisade layer had very similar structure in all species under analysis. It was characterised by a compact structure, while calcium carbonate crystals in the form of plates – being arranged on top of each other – firmly adhered to each other, forming palisades of different size. Small air spaces were present between plates of irregular shape (Figure 8). Despite the similar structure, the breadth of palisades in respective species significantly differed, ranging from 157 µm in emus to 216 µm in rheas (Table 1).

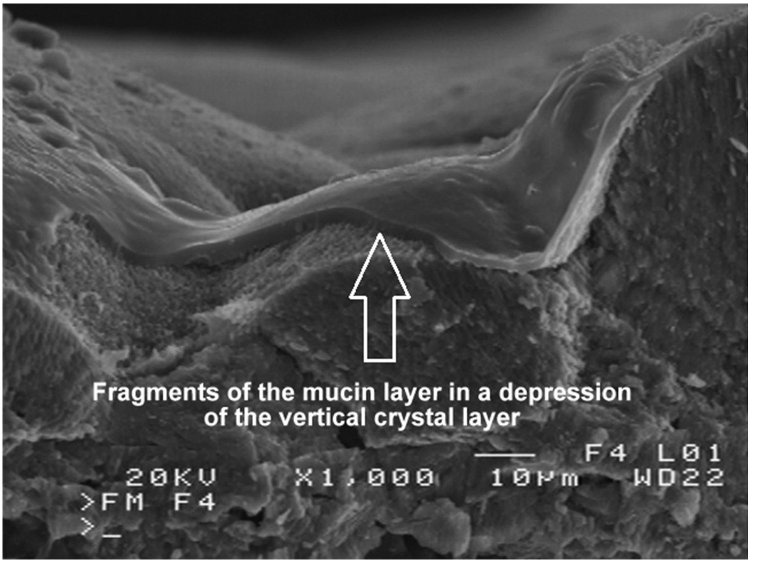

Figure 5. Cuticle on the eggshell surface in emu

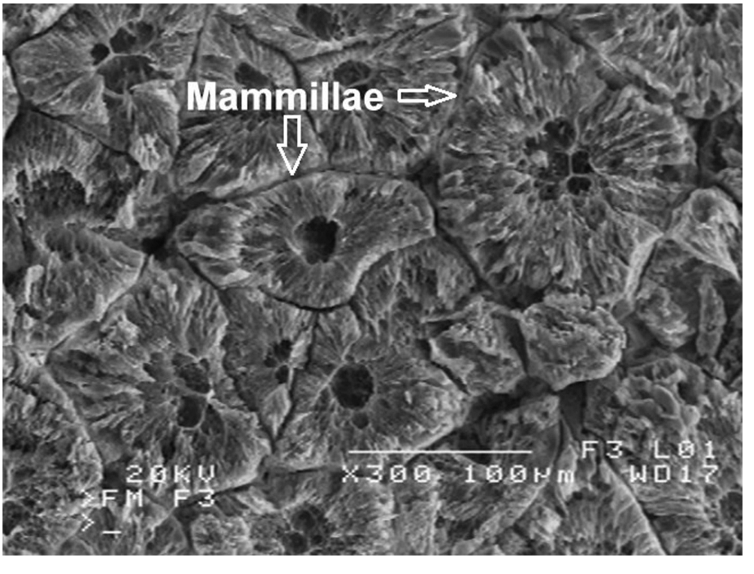

Figure 6. Mammillae in the emu eggshell

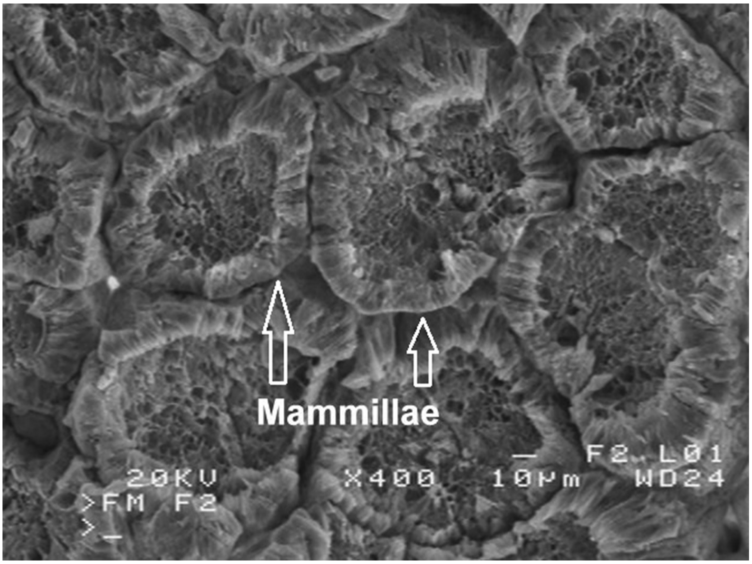

Figure 7. Mammillae in the rhea eggshell

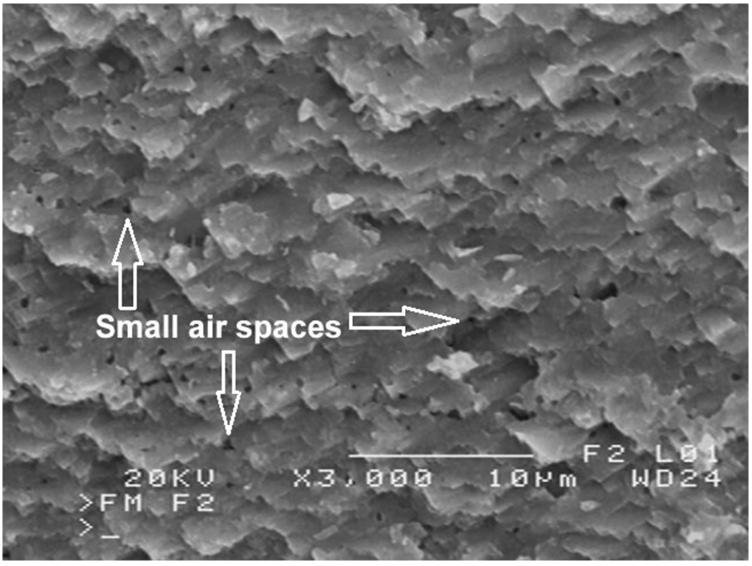

Figure 8. Air spaces in the palisade layer of the ostrich eggshell

The thinnest vertical crystal layer was observed in the ostrich and rhea eggshells. Significantly thicker one (by about 49 µm) was found in the emu eggshells. The percentage of the vertical crystal layer in the eggshell was the lowest in ostriches and amounted to nearly 2% whereas the highest in emus, more than 8% (Table 1). In the emu eggshells, it formed loaf-shaped prominences on the eggshell surface, contrary to other species (Figure 9).

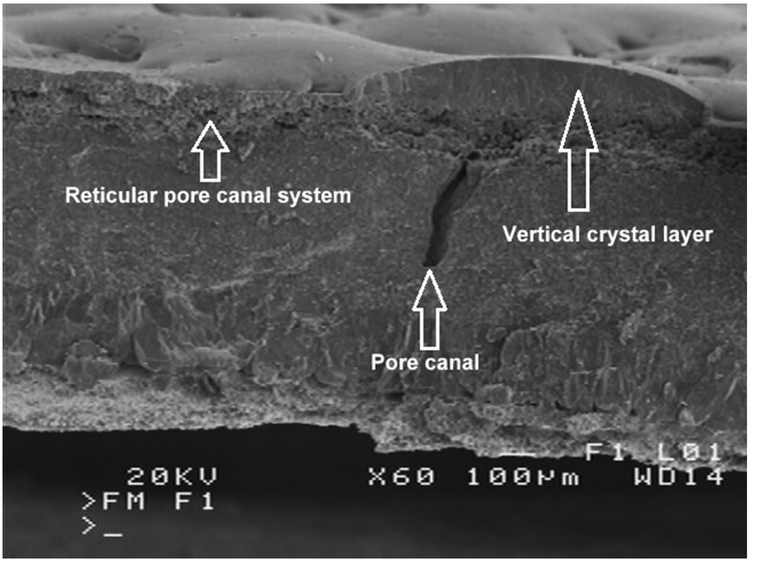

Figure 9. Vertical crystal layer in the emu eggshell

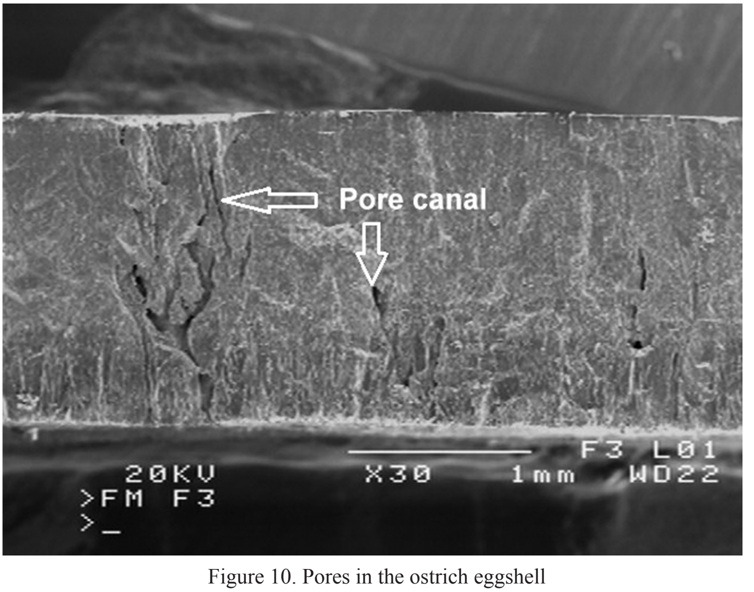

Figure 10. Pores in the ostrich eggshell

Major pore canals in the eggshells of all analysed birds started between mammillae but branched in successive layers, assuming different shapes depending on the bird species (Figures 10 and 11). The emu eggshells had reticular pore canal system, branching already in the palisade layer and then in the reticular one (Figure 9). Pores in the rhea eggshells were also branched, escaping to small depressions on the eggshell surface. In the rhea eggshells, organic deposits of different shape were observed at the orifice of pore canal which secured, together with the cuticle layer, pore canal inlet from outside (Figure 4).

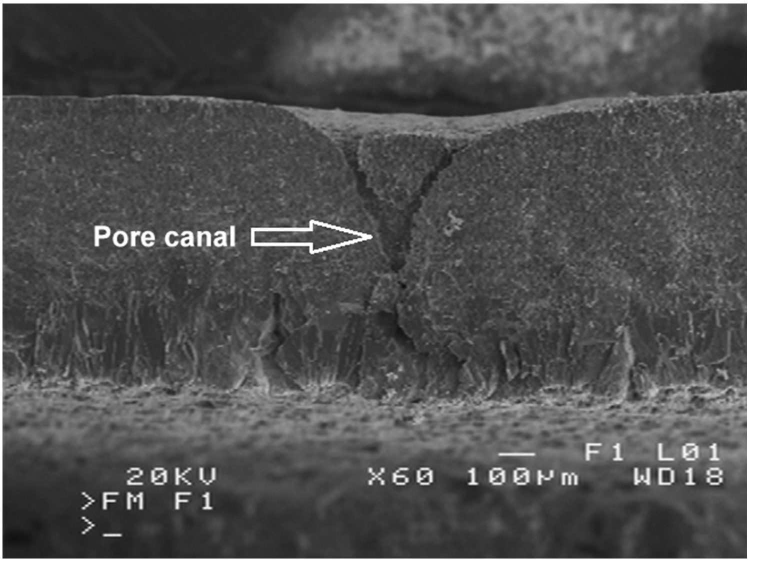

Figure 11. Pores in the rhea eggshell

The rhea eggshells proved to be the most porous (Table 2), whereas the ostrich eggshells were the least porous. The broadest pore canals were observed in emus and rheas, whereas the narrowest in ostriches.

Table 2. Number and breadth of the pore canals ($\overline{x}$±SD)

Item	Species		
	ostrich	emu	rhea
Number of pores (pores/cm^2)			
blunt end	22.6±7.4	43.7±12.6	49.2±13.6
pointed end	16.7±7.6	32.2±10.0	40.6±12.5
equator	21.4±6.6	33.1±13.7	44.2±14.5
mean value	20.2±7.2	36.3 b±10.1	44.6±13.5
Breadth of pore canals (µm)	35.3 a±10.1	53.3 b±11.8	48.4 b±4.8

a, b, c – mean values in rows marked with different letters differ significantly (P≤0.05).

Discussion

The outer shell membrane in eggs of all bird species has a similar structure due to the functions it fulfils during embryogenesis (Solomon, 1997). A dense network of keratin-collagen fibres constitutes a mechanical protection which makes eggshell mineral structures more elastic. It also protects the egg contents and developing embryos against microorganism infections. Typically, fibres of the outer shell membrane in emus, ostriches and rheas root into mammillae, forming "anchor points", which have been previously described in other bird species (Solomon, 1997). Similar characteristics of the structure of egg membranes in the birds being phylogenetically distant from Palaeognathae were also reported by Puchajda et al. (2000) in geese and Solomon (1997) and Rosiński et al. (1993) in hens. However, in the studies cited above, protein fibres differed in their thickness and density.

Different structure of the eggshell surface may result from interspecific differences but also from adaptation of birds to specific living environment. The analysis of eggshells in this study showed essential differences in the cuticle distribution on their surface. Interestingly, the cuticle of ostrich eggshell had a similar structure to that of hen and turkey eggs (Michalak and Mróz, 2004; Solomon, 1997). Its cracks, forming polygonal plates, were already described in literature (Christensen et al., 1996; Heredia et al., 2005). Such a pattern of eggshell surface does not result from its primary structure but from the fact that the mucin layer dries out and cracks during storage. A paucity of information on the morphology of rhea eggs refers also to the structure of eggshell surface. No publications have been found which confirm the presence of cuticle in this species. It was not observed either that the emu eggshells were entirely covered by that type of layer. The results of this study confirm earlier suppositions (Szczerbińska, 2002) that it appears unlikely the emu eggs are being tightly covered with the cuticle due to very waved eggshell surface. It seems most probable that the eggshell cuticle in emus disappeared during the process of evolution. Due to specific eggshell structure, it stopped playing the protective role against microorganism penetration since its functions were taken over by a very developed vertical crystal layer as well as the reticular one, acting like a specific filter.

Mammillae in the Paleognathae eggshells did not differ from those in the eggshells of other bird species (Mikhailov, 1997; Panheleux et al., 1999). Similarly, the palisade and vertical crystal layer in the ostrich eggshells resembled in its structure those being described previously in literature (Christensen et al., 1996). In the earlier studies (Szczerbińska, 2002), the relative thickness of the vertical crystal layer of the emu eggshell was larger, ranging from 10.8 to 13.9%. In the emu eggshells, an additional layer was observed over the palisade layer which was not found in the eggshells of other bird species being analysed. It was characterised by a very loose structure, with numerous air spaces. The presence of this layer, being called the reticular layer, in emu and cassowary eggs was reported earlier by Board and Tullett (1975).

The exceptionally complex system of pores in the eggshells of all bird species under analysis is connected with a considerable egg weight which ranges from 600 g in emus (Minnaar and Minnaar, 1993) to 1500 g in ostriches (Horbańczuk, 2002). The developed pore canals, forming different types of branching, ensure proper gaseous exchange during embryogenesis. In our previous study (Wiercińska and Szczerbińska, 2010) referring to the hatchability of nandu eggs, the least number of pores was recorded in eggs with dead embryos. This confirms a significant role of eggshell porosity in embryogenesis. By contrast, a small number of pores with narrow canals being observed in the eggshell of ostriches is certainly connected with their life in the desert environment and egg protection against excessive water loss during embryogenesis. As shown by the analysis of Paleognathae eggshell structure, differences in porosity are most probably determined by birds' origin. This corresponds to the findings of Kożuszek et al. (2009) who demonstrated significant differences in the number of pores depending on the colour of pheasant eggshells.

In conclusion, it is possible to state that emu, ostrich and rhea eggshells have different structure. The most important interspecific differences being observed include:

– distribution of the cuticle, which covered the whole ostrich eggshell, while occurring mainly around pore canal orifices in rhea eggs and being rudimentary in emu eggs,

– differences in the breadth of palisades and vertical crystal layer and the density of mammillae. The emu eggshells were characterised by the highest percentage of vertical crystal layer and the highest density of mammillae, whereas the rhea eggshells by broadest palisades,

– the least number of pores per unit of surface area, with significantly narrower pore canals, was observed in the ostrich eggshells.

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