2015

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The Auk
Ornithological Advances
www.aou.org

Volume 132, 2015, pp. 206–211
DOI: 10.1642/AUK-14-158.1

RESEARCH ARTICLE

Corrosion casts: A novel application of a polyurethane resin (PU4ii) for visualizing eggshell pore morphology

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Submitted July 4, 2014; Accepted September 25, 2014; Published December 10, 2014

ABSTRACT

Avian eggshells serve the dual purposes of protecting the developing embryo from the external environment while also facilitating the loss of water vapor and the required exchange of CO_2 and O_2 gases. Pores that span the eggshell enable the loss of water and trans-shell gas exchange. Although knowledge of the geometry of these spaces is necessary to generate accurate estimates of the rate of gas diffusion across the shell, few techniques exist to obtain these data. Estimates of gas conductance across eggshells are typically calculated from eggshell thickness and the size and number of the pores on the exterior eggshell surface; the trans-shell pore spaces are assumed to be cylindrical in shape. To enable the testing of this assumption, we devised a novel method to visualize the three-dimensional morphology of eggshell pores using PU4ii, a polyurethane-based resin. Casts of the pores of eggshells of the domestic chicken (*Gallus gallus*) and House Sparrow (*Passer domesticus*) were unbranched and varied in diameter throughout their length, while casts of the pores of eggshells of the Ostrich (*Struthio camelus*) revealed a complex network of interconnected spaces. The simplicity of this technique and the stability and resilience of the resulting casts provide opportunities to predict gas flux across the shell and to evaluate the morphology of eggshell pores among birds from different taxonomic groups.

Keywords: egg shells, *Gallus*, gas flux, *Passer*, porosity, *Struthio*

Moldes de corrosión: Una nueva aplicación de una resina de poliuretano (PU4ii) para visualizar la morfología de los poros de la cáscara del huevo

RESUMEN

La cascara del huevo de las aves tiene el doble propósito de proteger el embrión en desarrollo del ambiente externo, mientras que también debe facilitar la pérdida de vapor de agua y el intercambio requerido de gases de CO_2 y O_2. Los poros que cubren la cáscara del huevo permiten la pérdida de agua y el intercambio de gases a través de la cáscara. Aunque se necesita del conocimiento de la geometría de estos espacios para generar modelos precisos de la tasa de difusión de los gases a través de la cáscara, existen pocas técnicas para obtener estos datos. Las estimaciones de conducción de gases a través de la cáscara del huevo son típicamente calculadas a partir del espesor de la cáscara y del tamaño y el número de poros en la superficie externa de la cáscara; se asume que los poros que atraviesan la cáscara del huevo son de forma cilíndrica. Para evaluar este supuesto, ideamos un nuevo método para visualizar la morfología tridimensional de los poros de la cáscara usando PU4ii (vasQtec), una resina basada en poliuretano. Los moldes de los poros de la cáscara del huevo de *Gallus gallus* y *Passer domesticus* presentaron una forma no ramificada y variaron en diámetro a lo largo de su longitud, mientras que los moldes de los poros de la cascara del huevo de *Struthio camelus* mostró una compleja red de espacios interconectados. La simpleza de esta técnica y la estabilidad y resiliencia de los moldes resultantes brindan oportunidades para predecir el flujo de gases a través de la cáscara y evaluar la morfología de los poros de la cáscara del huevo entre aves de diferentes grupos taxonómicos.

Palabras clave: cáscara del huevo, flujo de gases, *Gallus*, *Passer*, porosidad, *Struthio*

INTRODUCTION

Avian eggshells serve the dual purposes of protecting the embryo from the external environment while also facilitating the loss of water vapor and the required exchange of CO_2 and O_2 gases (Wangansteen and Rahn 1970–1971). The rate at which gases diffuse across the eggshell is positively correlated with the embryo's rate of development for at least 161 avian species (Ar and Rahn 1985). Trans-shell gas diffusion is enabled by channels (i.e. pores) that span the mammillary and palisade portions of the eggshell (Board and Scott 1980, Mikhailov

1997). The morphology of these pores (e.g., size, distribution, presence of occluding materials, and degree of branching) varies greatly among avian taxa (Board et al. 1977).

Differences in the number and size of eggshell pores are thought to contribute to variation in gas conductance across eggshells both within and among species (Massaro and Davis 2005, Zimmermann and Hipfner 2007, Clark et al. 2010). For example, eggs of Snares Penguins (*Eudyptes robustus*) showed intraclutch differences in eggshell porosity (total pore area ÷ eggshell thickness), and embryos in eggs with greater eggshell porosity developed faster than those in eggs with lower eggshell porosity (Massaro and Davis 2005). The shorter incubation period was linked to a hypothesized increase in oxygen consumption that presumably was facilitated by greater eggshell porosity. Variation in pore dimensions may also serve to protect against microbial ingress (e.g., Gantois et al. 2009, Jonchère et al. 2010).

Earlier workers have estimated gas flux across the eggshell from counts and size measurements of pores visible on the convex side of the shell, eggshell thickness, and environmental conditions (e.g., Ar and Rahn 1985, Clark et al. 2010, Jaeckle et al. 2012). Estimates of gas conductance calculated from physical characteristics of the eggshell are usually based on the assumption that pores are cylindrical in shape (Ar and Rahn 1985). However, plastic casts of eggshell pores of eggs of the domestic chicken (*Gallus gallus*) revealed unbranched pores shaped as elongated cones that varied in diameter throughout their length, with the widest portion typically located at the convex surface of the eggshell (Tyler 1956, Board and Scott 1980). Consequently, gas conductance predictions based on the diameter of pores at the eggshell surface are likely to overestimate the rate of trans-shell gas flux. To accurately estimate rates of gas flux through eggshells, an understanding of the morphology of the entire pore is necessary.

Corrosion casting is a technique used to visualize the morphology of hollow spaces by infiltration and subsequent polymerization of liquid resins in situ. The material surrounding the resin is removed and the resulting three-dimensional structure can be viewed using techniques such as scanning electron microscopy (Schneider et al. 2009). Earlier studies (e.g., Tyler 1956, Board and Tullett 1975) used methacrylate resins to create corrosion casts of pores within eggshells. Apparent inconsistencies in resin polymerization made replication of these earlier results difficult (Board and Tullet 1975, Bunk and Combs 1979). While other studies have used low-viscosity, epoxy-based resins (e.g., Spurr's resin; Tompa 1980), few reliable protocols and three-dimensional depictions of avian eggshell pores exist within the literature (but see Riley et al. 2014).

PU4ii (vasQtec, Zürich, Switzerland) is a polyurethane-based resin developed for corrosion casting of vascular networks. While polymerized methacrylate resins may shrink or sublime at the low pressure required for scanning electron microscopy (Bunk and Combs 1979), PU4ii resin is robust, possesses a sufficiently low viscosity to allow infiltration into small spaces, polymerizes quickly, and exhibits minimal shrinking (Krucker et al. 2006, Meyer et al. 2007, Schneider et al. 2009). We describe here a method using PU4ii resin that produced consistent, resilient corrosion casts of the pore spaces within bird eggshells. We developed, optimized, and tested this method by producing casts of simple (domestic chicken and House Sparrow [*Passer domesticus*]) and branched (Ostrich [*Struthio camelus*]) eggshell pores.

METHODS

Domestic chicken eggs ($n = 4$) and Ostrich eggshell pieces ($n = 6$) were purchased commercially, while eggs of the House Sparrow ($n = 3$) were collected in Cook County, Illinois, in May, 2013. Prior to use, eggs of the domestic chicken were stored at 5°C, while eggs of House Sparrows were stored at −20°C. All eggshells were broken into fragments with a surface area of $\sim$30 mm^2. Inner and outer shell membranes on the inside surface of each fragment were removed with 10% NaOH (80°C, 20 min) and fragments were then washed in distilled water. To displace any material occluding the pores, the fragments were placed in distilled water and exposed to ultrasonic waves (40 KHz; Model FS9, Fisher Scientific, Pittsburgh, Pennsylvania, USA) for 300 s. The treated eggshell fragments were then air-dried for $\geq$24 hr.

To test for any effect of ultrasonic treatment on pore number and size, we exposed eggshell fragments of the domestic chicken ($n = 18$) to ultrasonic waves for a total of 480 s. After ultrasonic wave exposures of 0, 30, 60, 120, 240, and 480 s each eggshell fragment was examined using light microscopy (400×) and pore number and pore size were determined.

PU4ii resin with a proprietary blue dye was prepared following the manufacturer's instructions. The cleaned eggshell fragments were then placed on top of plastic drinking straw slices (0.5 cm height × 0.5 cm diameter), which served to elevate the fragments and prevent adhesion to a surface during polymerization. Liquid resin was applied to the concave surface of each treated eggshell fragment using a glass pipette. Complete infiltration of the pores was indicated by the appearance of blue spots on the convex portion of the eggshell.

After full resin polymerization (5 days, 22°C), the $CaCO_3$ portion of the eggshell fragment was dissolved in 5% HCl (22°C) for 1–2 days, depending on eggshell thickness (the acid was replenished for fragments of

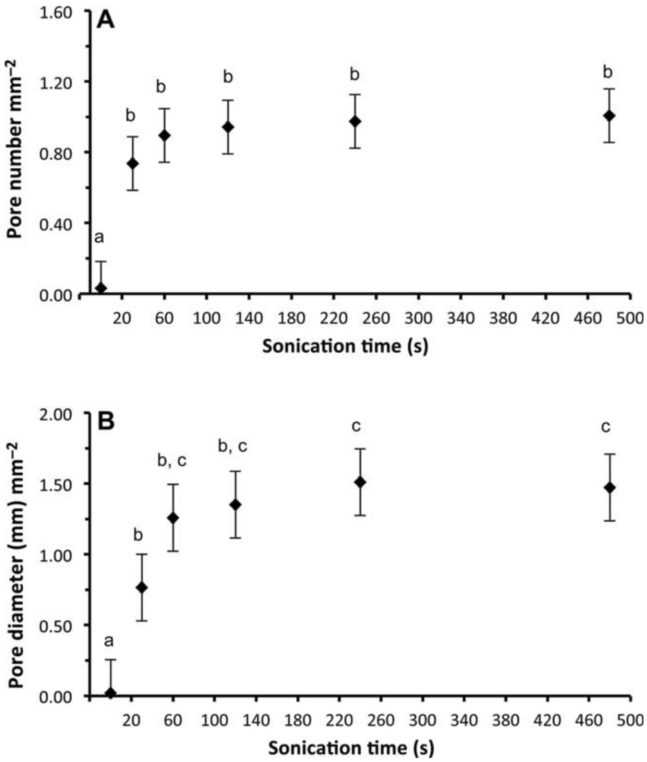

FIGURE 1. (**A**) Pore number per mm^2 (mean ± SE) and (**B**) Pore diameter (mm) per mm^2 (mean ± SE) of eggshell fragments ($n = 18$) of the domestic chicken exposed to ultrasonic waves for varying periods of time. Means identified by the same letter are not significantly different ($P > 0.05$).

thicker eggshells). The PU4ii casts were rinsed with distilled water and then placed in 10% NaOH (22°C) for 6 hr to remove any persisting organic material and to clean the polyurethane casts. The casts were then rinsed in distilled water. Rinsed casts were transferred to wells of a multiwell plate, covered by 500 μL of distilled water, and held at –18°C. The ice surrounding each cast was removed via lyophilization at ~500 mTorr for the minimum time necessary for complete sublimation (~5 hr). Dried resin casts were coated with a 20:80 mixture of gold:palladium using a PELCO SC-7 sputter coater (Pelco, Clovis, California, USA). Casts were examined and images were collected using a JEOL-5800 scanning electron microscope (JEOL, Tokyo, Japan; accelerating voltage = 5 kV).

Statistical Analyses

All statistical tests were performed using SPSS (SPSS 2013). We compared pore number and pore size among eggshell fragments exposed to ultrasonic waves for different periods of time using a repeated measures ANOVA following Field (2009). Differences among exposure times were detected and evaluated using the Bonferroni correction. For each statistically significant difference in pore number and pore diameter among exposure times to ultrasonic waves ($P < 0.05$) an effect size (η^2) was determined.

RESULTS

Effects of Sonication

There were highly significant differences in the average pore number per mm^2 of eggshell with different exposures to ultrasonic waves ($F_{2.988,41.830} = 72.3$, $\eta^2 = 0.838$, $P < 0.001$; Figure 1A). Pairwise comparisons revealed that eggshell pore number per mm^2 increased after 30 s of exposure to ultrasonic waves, but with extended exposure there was no significant difference in the number of eggshell pores. There was also a highly significant increase in the pore diameter per mm^2 with ultrasonic wave treatment ($F_{2.729,46.393} = 24.0$, $\eta^2 = 0.586$, $P < 0.001$; Figure 1B). Pairwise comparisons of pore diameter per mm^2 showed significant differences among treatment groups (Figure 1B); however, there were no differences in pore diameter per mm^2 in ultrasonic wave treatments greater than 60 s.

Morphology of Pore Spaces

Domestic chicken. Casts of pores of the eggshells of domestic chickens had a minimum diameter toward the interior surface of the eggshell. These casts gradually widened toward the exterior surface of the eggshell and resembled the shape of an elongated cone (Figures 2A, 2B). The surfaces of the casts of eggshell pores were covered with bubble-shaped vesiculations ~0.24–0.43 μm in diameter.

House Sparrow. Casts of pores of the eggshells of House Sparrows varied in diameter throughout their length, with the widest diameter present on the exterior surface (Figures 3A, 3B). House Sparrow eggshell casts exhibited a biconical shape, as they widened again slightly on the interior surface. All casts of the pores of House Sparrow eggshells were covered with a large number of bubble-shaped vesiculations that ranged from ~1.4 to ~1.8 μm in diameter.

Ostrich. Casts of pores of Ostrich eggshells exhibited a complex, multibranched system, in which each branch was not consistently linear (Figures 4A, 4B). Some of these branches appeared to bifurcate multiple times in a seemingly stochastic fashion from the interior toward the exterior portion of the eggshell. Notably, some branches did not appear to extend to the exterior surface of the eggshell (Figures 4A, 4B). Branches that reached the exterior surface of the eggshell converged into a shallow depression. Pores of Ostrich eggshells did not have consistent diameters throughout their lengths, nor did pores exhibit a consistent change in diameter. Vesiculations that were ~0.75–1.50 μm in diameter were also present on the surfaces of these pore casts.

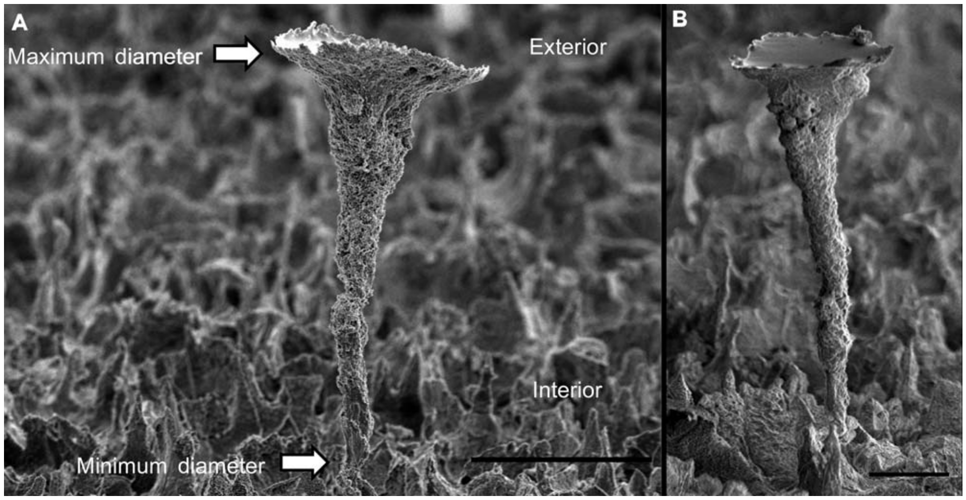

FIGURE 2. Images of PU4ii (a polyurethane-based resin) casts of pores of an eggshell of the domestic chicken. (**A**) Scale bar equals 100 μm. (**B**) Scale bar equals 50 μm. Exterior denotes the exterior eggshell surface, and Interior is the interior eggshell surface. Both images have the same exterior–interior orientation.

DISCUSSION

Ultrasonic wave treatment of eggshell fragments increased the number and size of visible eggshell pores; however, there were no significant increases in either measure after 30 s and 60 s, respectively. It is likely that the 300 s of sonication time used to prepare our samples resulted in the clearing of any occluding material from all pores and allowed for accurate visualization of eggshell pore morphology. While it is possible that treatment with ultrasonic waves could have artificially increased the number or size of eggshell pores, the absence of a significant change in either eggshell character with treatments greater than 60 s suggests that this was not the case.

Creating corrosion casts of pores within eggshells using PU4ii resin, in conjunction with scanning electron microscopy, allowed for the visualization of their trans-shell shape, size, and interconnectedness. Although not addressed in this study, this method could also be used to document the shape, size, and frequency of other eggshell characteristics (e.g., incomplete pores and mammillary bodies). Examination of pore casts of eggshells of the domestic chicken and Ostrich revealed similarities with earlier descriptions (Tyler 1956, Board and Tullet 1975, Bunk and Combs 1979); we did not find any previous reports on the shapes of pores of House Sparrow eggshells. Distributions and sizes of the impressions of vesiculations in our casts were consistent with the descriptions of spherical air spaces within eggshells that were detected using scanning electron microscopy (Becking 1975, Pooley 1979). Shells of the domestic chicken had smaller and less numerous vesiculations than the shells of the House Sparrow and Ostrich. Becking (1975) suggested that these air spaces may contribute to gas flux across the shell or the structural integrity of the eggshell, but we did not examine these possibilities.

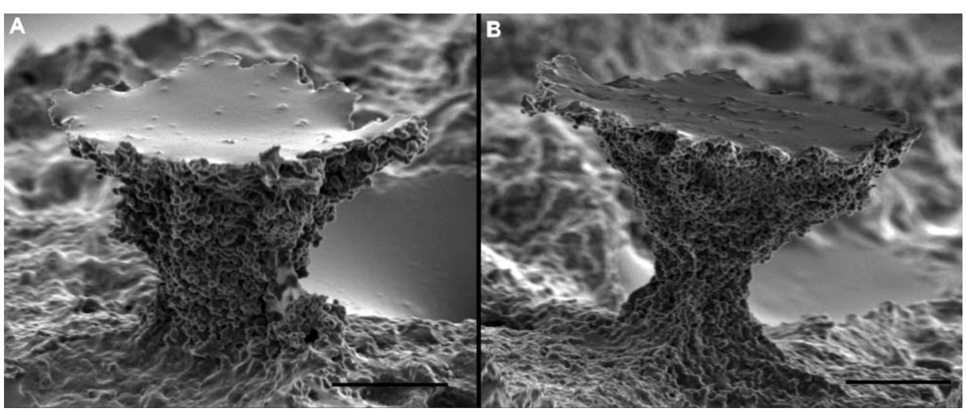

FIGURE 3. Images of PU4ii (a polyurethane-based resin) casts of pores of an eggshell of the House Sparrow. (**A**, **B**) Scale bars equal 25 μm. Images have the same exterior–interior orientation as in Figure 2.

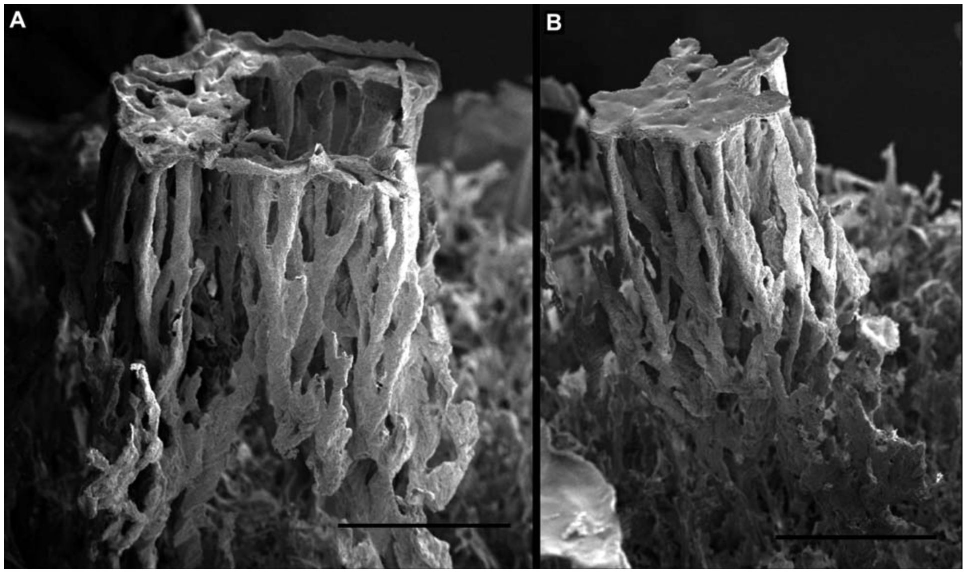

FIGURE 4. Images of PU4ii (a polyurethane-based resin) casts of pores of an eggshell of the Ostrich. (**A**, **B**) Scale bars equal 500 μm. Images have the same exterior–interior orientation as in Figure 2.

Using PU4ii we consistently produced structurally stable casts of pores within the eggshells of the domestic chicken, House Sparrow, and Ostrich. However, eggshell pore size, shape, and branching vary among avian taxa (Board and Scott 1980, Mikhailov 1997, present study), thus the general method described here represents a starting point for adjustments to optimize the production of accurate casts of eggshell pores.

Corrosion casting with PU4ii resin and subsequent imaging with scanning electron microscopy allows for measurement of the minimum diameter of each pore. In previous studies, predictions of gas flux through eggshell pores have been calculated using the surface area of each pore at the eggshell's exterior and assuming that all pores are cylindrical in shape (e.g., Ar and Rahn 1985, Clark et al. 2010, Jaeckle et al. 2012). However, Tøien et al. (1987) found that the minimum diameter of eggshell pores limits the rate of gas flux across the eggshell, and that the assumption of cylindrical pores in the eggshells of domestic chickens resulted in a predicted 59% overestimation of gas flux based on the dimensions of images of pore casts taken from Tyler (1956). We believe that any prediction of gas flux across the eggshell that is based on the outside pore diameter overestimates conductance. Further, the eggshell pores of some taxa are neither simple nor cylindrical, and any change in diameter or shape alters the resistance to gas flow (e.g., Board and Scott 1980). The assumption of a single opening on the convex eggshell surface per trans-shell pore for these groups would also lead to an overestimation of gas conductance (e.g., Portugal et al. 2014). Corrosion casting of eggshell pores provides a means to determine the minimum pore size and the topology of the pore channels that are necessary to make more accurate estimates of the rates of gas flux through pores.

Creating corrosion casts of eggshell pores with PU4ii resin allows for the assessment of eggshell pore characteristics as phylogenetically informative characters. For example, Board et al. (1977) found that the eggshell pore morphology of eggs produced by members of the Accipitridae and Falconidae are distinctly different. Shell ultrastructure (e.g., number and size of shell layers, size of squamatic units, and distribution of vesiculations) of members of Falconidae, Psittaciformes, and Passeriformes (Mikhailov 1997) are very similar. The distribution of such eggshell characters across these taxa is consistent with a recent molecular phylogeny of birds generated by Hackett et al. (2008), as well as with sequences of remigial molt within these taxa (Pyle 2013). Corrosion casting of representatives of different avian taxa would yield a dataset of character states that could be combined with the results of more recently published phylogenies of birds and other oviparous amniotes to explore the evolution of eggshell pore morphology within and among these groups.

ACKNOWLEDGMENTS

We thank vasQtec for providing us with PU4ii resin at a nominal cost and Eric P. Meyer for his help and advice. We also thank Nisa Blackmon for providing eggshells of the Ostrich and Joan Bruchman for providing eggshells of the House Sparrow that were used in this study. We are grateful to the reviewers who provided comments that improved the quality of this manuscript. We also thank J. Bates and C. Thompson.

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