ELECTRONIC APPENDIX

This is the Electronic Appendix to the article

Molecular preservation in Late Cretaceous
sauropod dinosaur eggshells

by

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Electronic appendices are refereed with the text; however, no attempt is made
to impose a uniform editorial style on the electronic appendices.
**APPENDIX 1. ANALYTICAL METHODS**

**Microscopy**

Sauropod eggshell and eggshell from extant taxa were embedded in resin (Silmar), and tangential sections were taken on a diamond blade. The wafers were mounted to microscope slides and then ground to transparency (~50-100 um) on decreasing aluminum grit papers, and polished with 0.5um aluminum grit powder, for use in transmitted light microscopy and in situ immunohistochemical analyses.

Fragments of sauropod eggshell were fresh-fractured, mounted to aluminum stubs with carbon tape, coated with approximately 20A of carbon film, and visualized at 15 KV with a JEOL scanning electron microscope. Elemental analyses were performed on the shell, infilling matrix, and small fragment of mineralized skin with a Voyager energy dispersive x-ray detector coupled to the electron microscope.

**Chemical Extraction Procedures**

Samples of dinosaur eggshell were first ground with a sterilized Dremel tool to remove all surface material that may have been exposed to contamination, either from sediment or potential human contact. Shell fragments were then ground to a fine powder and washed with either sterile distilled water or sterile 0.5M NaCl solution. Samples were centrifuged to pellet the bone powder, and wash water was removed. An extraction buffer consisting of 1.2g/ml guanidinium thiocyanate, 0.1M Tris/HCl, 2M EDTA (pH 8.0) and 26mg/ml Triton detergent was then added to the shell powder (Hoss and Paabo, 1993). Eggshell fragments were extracted in this buffer overnight at 60°C with gentle agitation; samples were then centrifuged to pellet the powder and supernatants collected and added to dialysis tubing (2000 MWCO). These solutions were dialyzed with several changes against distilled water at 4°C with constant stirring over a period of 3-5 days. The dialysates were collected and lyophilized under vacuum. The lyopholized material was waxy or oily in constitution, and impossible to remove from the lyophilization tubes, or to weigh out accurately. Therefore, for many experiments, extracts were first solubilized in 200ul of either sterile dH2O or sterile PBS, and then further diluted for assays. Corresponding fragments of chicken eggshell were treated in an identical manner, and sediments and buffer alone, incubated and dialyzed as described, provided negative controls. In some cases, it was impossible to remove all of the infilling sediment/calcite matrix from the eggshell proper as it was extremely adherent to the shell. Therefore, some of the calcite matrix deposits remained and were co-extracted with the dinosaur shell fragments.

**Antibody production**

To elicit polyclonal antibodies to chicken eggshell proteins, 100ug of chicken shell extract was solubilized in 500 µl of sterile dH2O and then emulsified with 500 µl of Freund’s Complete Adjuvant (FCA) for a total of 1 ml/injection for each rabbit. Extracted dinosaur shell was oily and difficult to weigh accurately, so the residue remaining on lyophilization tubes was suspended in 100 µl sterile dH20. 10 µl of this was added to 500 µl of sterile dH2O and emulsified with 500 µl of FCA. This was followed by two boosts of equal amounts of antigen at monthly intervals in Freund’s incomplete adjuvant. Blood collected from the rabbits prior to immunization (preimmune sera) provided negative controls.

**Immunoassays**

Tangential ground sections of eggshells (dinosaur, crocodile and chicken) and associated infilling matrix (described above) were subjected to *in situ* immunochemistry to localize antibody binding signal to tissues. Sections were etched three times for ten minutes in 0.5M EDTA (pH 7.5), washed 3 times for 15 minutes, then etched 3 times with sodium borohydride (NaBH₄) to expose any epitopes. Sections were blocked in 4% normal goat serum (NGS), then incubated with pre-immune or test sera overnight at 4oC. For pre-absorbance studies, anti-dinosaur serum was incubated overnight with dinosaur shell extract at a final concentration of 6.5mg/ml of antiserum to block antibodies in the serum specific for dinosaur “proteins”. This solution was added to the sections as described. Sections were washed 4 x 15’ in the above tween-20 buffer, then incubated with biotinylated goat anti-rabbit IgG for 4 hours to overnight. After washing as described, sections were incubated with fluorescent label (Avidin-FITC), washed, and visualized using a Leica TCS/SP laser scanning confocal microscopy system, interfaced to an SGI Octane work station. This instrument is equipped with mixed-gas argon/krypton and HeNe laser excitation sources, and incorporates a prism-based spectrophotometer head.
Lyophilized residue of extracts were solubilized in 200 µl of sterile PBS, then diluted further for ELISA assays. Assays were performed by incubating microtiter wells (Immuron 2, Titertek) 100 µl/ well with extracts of sauropod shell and calcite precipitate diluted to 1:50, and chicken shell extracts diluted 1:3000. Purified ovalbumin was added to wells at 10µg/ml. All antigens were allowed to incubate for four hours to overnight. Plates were blocked with PBS/1% bovine serum albumin, or with PBS/1% dried milk/ 0.1% Tween-20 (blotto), from 4 to 12 hours. Test antisera or preimmune sera were added to the wells at dilutions of 1:200, and incubated 4 hours to overnight. Sera were then removed and plates washed 6-12 times with PBS/0.1% tween-20. Alkaline phosphatase-conjugated anti-rabbit Ig (Zymed, San Fransisco CA) was diluted 1:1000 in blocking buffer, and plates were incubated with secondary antibody for 2 hours to overnight. Following washing as described above, the colorimetric substrate p-nitrophenyl phosphate (Sigma) was added at 0.5mg/ml, and the absorbance was monitored at 405nm at various timepoints.