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Fossil eggshell cuticle elucidates dinosaur nesting ecology

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The cuticle layer consisting mainly of lipids and hydroxyapatite (HAp) atop the mineralized avian eggshells, in contrast to the porosity, is a protective structure that prevents the egg from dehydration or microbial invasions. Previous ornithological studies have revealed that the cuticle layer also involves in modulating the reflectance of eggshells in addition to pigments (protoporphyrin and biliverdin) and thermoregulation. Thus, the cuticle layer represents a crucial trait that delivers ecological signals. While present in most modern birds, direct evidence for cuticle preservation in stem birds and non-avian dinosaurs, is yet missing. Here we present the first direct and chemical evidence for the preservation of cuticle on dinosaur eggshells. We analyze several theropod eggshells from various localities, including oviraptorid *Macroolithus yaotunensis* eggshells from the Late Cretaceous deposits of Henan, Jiangxi and Guangdong in China and alvarezsaurid *Triprismatoolithus* eggshell from the Two Medicine Formation of Montana, United States, with the scanning electron microscope (SEM), electron probe micro-analysis (EPMA), and Raman spectroscopy. The elemental analysis with EPMA shows high concentration of phosphorus on the boundary between the eggshell and sediment, representing the hydroxyapatitic cuticle layer (HAp). Depletion of phosphorus in sediment excludes the allochthonous origin of the phosphorus in eggshells. The chemometrical analysis on Raman spectra deduced from fossil and extant eggs provides further supportive evidence for the cuticle preservation on oviraptorid and alvarezsaurid eggshells. In accordance with our previous discovery of pigments preserved in Cretaceous oviraptorid dinosaur eggshells, we validate the cuticle preservation on dinosaur eggshells through deep time and offer a yet unexplored resource for chemical studies targeting the evolution of dinosaur nesting ecology. Our study also suggests that the cuticle structure can be traced far back to maniraptoran dinosaurs and enhance their reproductive success in a warm and mesic habitat such as Montana and southern China during the Late Cretaceous.

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Abstract

The cuticle layer consisting mainly of lipids and hydroxyapatite (HAp) atop the mineralized avian eggshells~~, in contrast to the porosity,~~ is a protective structure that prevents the egg from dehydration or microbial invasions. Previous ornithological studies have revealed that the cuticle layer also involves in modulating the reflectance of eggshells in addition to pigments (protoporphyrin and biliverdin) and thermoregulation. Thus, the cuticle layer represents a crucial trait that delivers ecological signals. While present in most modern birds, direct evidence for cuticle preservation in stem birds and non-avian dinosaurs, is yet missing. Here we present the first direct and chemical evidence for the preservation of cuticle on dinosaur eggshells. We analyze several theropod eggshells from various localities, including oviraptorid *Macroolithus yaotunensis* eggshells from the Late Cretaceous deposits of Henan, Jiangxi and Guangdong in China and alvarezsaurid *Triprismatoolithus* eggshell from the Two Medicine Formation of Montana, United States, with the scanning electron microscope (SEM), electron probe micro-analysis (EPMA), and Raman spectroscopy. The elemental analysis with EPMA shows high concentration of phosphorus on the boundary between the eggshell and sediment, representing the hydroxyapatitic cuticle layer (HAp). Depletion of phosphorus in sediment excludes the allochthonous origin of the phosphorus in eggshells. The chemometrical analysis on Raman spectra deduced from fossil and extant eggs provides further supportive evidence for the cuticle preservation on oviraptorid and alvarezsaurid eggshells. In accordance with our previous discovery of pigments preserved in Cretaceous oviraptorid dinosaur eggshells, we validate the cuticle preservation on dinosaur eggshells through deep time and offer a yet unexplored resource for chemical studies targeting the evolution of dinosaur nesting ecology. Our study also suggests that the cuticle structure can be traced far back to maniraptoran dinosaurs and enhance their reproductive success in a warm and mesic habitat such as Montana and southern China during the Late Cretaceous.

Key words

Chemometrics; Dinosaur reproduction; Soft tissue preservation; Elemental analysis; Raman spectroscopy

Introduction

Cuticle structures and functions

An avian egg is an evolutionary invention that protects the developing embryo against mechanical damage, dehydration and microbial invasion (Romanoff & Romanoff, 1949; Board & Fuller, 1974). This multi-functionality is contributed by both mineralized and non-mineralized, organic layers, which exhibit unique biomaterial properties. The avian eggshell forms inside the uterus. The layer deposited at last, the eggshell cuticle, represents the interface between the embryo inside and its outside environment. The eggshell cuticle is often described as "waxy" due to its high amount of lipids, which keep the internal fluids from evaporating and therefore protect the encased embryo from desiccation. In addition to lipids, the avian cuticle contains proteins, polysaccharides, calcium carbonate (vaterite), calcium phosphates (hydroxyapatite, HAp), and pigments (Wedral et al., 1974; Nys et al., 1991; Packard & DeMarco, 1991; Dennis et al., 1996; Mikhailov & Ornithologists' Club, 1997; Fraser, Bain & Solomon, 1999; Cusack, Fraser & Stachel, 2003). Among these components, the vaterite and HAp in avian cuticle are stored in the form of nanospheres (Dennis et al., 1996; D'Alba et al., 2014).

The cuticle layer varies in thickness (up to 12μm) and has a patchy distribution on the eggshell (Board & Halls, 1973). Basically, the cuticle is divided into two distinct layers, including an amorphous HAp inner layer and a proteinaceous outer layer (Simons, 1971; Dennis et al., 1996; Fraser, Bain & Solomon, 1999).

A recent study suggested that the circular mineralic nanospheres on eggshells serve an antimicrobial function for species nesting in humid environment (D'Alba et al., 2016). D'Alba et al. (2016) also proposed that eggs in humid environments tend to have more cuticular nanospheres to prevent flooding and bacterial invasion. Conversely, in arid environments, lacking a cuticle layer would be benign for embryos in eggs with low conductance, as Deeming (1987) suggested. Besides, the intactness of the cuticle is an indicator for the freshness of an egg (Rodríguez-Navarro et al., 2013). Several studies suggested that the cuticle functions as a lubricant that facilitates egg rotation in the uterus (Rahman et al., 2009). Besides, a recent study shows that the cuticle modulates ultraviolet reflectance of avian eggshells (Fecheyr-Lippens et al., 2015). For instance, an extremely smooth cuticle produces glossiness and iridescence in tinamous eggs (Igic et al., 2015).

The cuticle is commonly observed on most calcified eggshells, but it shows different structures and characteristics between reptilian and avian eggshells (Ferguson, 1982). The cuticle on reptilian eggshells is no longer present after two weeks of laying (Ferguson, 1982); hence, ~~he~~ concluded that the reptilian cuticle is not equivalent of the cuticle present on avian eggshells. Ferguson (1982) hypothesized that a true cuticle layer is not present on alligator eggshell.

Conversely, avian eggshells have HAp granules along the inner periphery on calcareous eggshells, which are absent in reptilian eggshells. Several studies on extant eggshell have identified cuticle presence by means of SEM or TEM (Fink et al., 1993; Fraser, Bain & Solomon, 1999). Kusada et al. (2011) applied electron microscopy to map the P and calcium distribution in several extant avian eggshells and discovered that P is confined to the cuticle layer. Hence, P is a distinct marker for identifying the cuticle layer in case of P depletion in sediment. Although it is still not clear how P functions during the eggshell formation, a study suggested that P terminates eggshell growth since it competes with calcium during the eggshell crystallization (Cusack, Fraser & Stachel, 2003).

Discoveries of fossilized cuticle

Since the cuticle is composed of proteins, polysaccharides, lipids, calcium carbonate, calcium phosphates, and pigments, it is unlikely to preserve during the fossilization process. A previous report concerning spherical (*Oölithus spheroides*) and elongated (*Oölithus elongatus*) eggs from Laiyang, Shandong, China implied that the cuticle is preserved in elongated eggs, but not in spherical eggs (Chow, 1954). Another similar report on ornithoid eggshells from Naran-Bulak, Mongolia also described possible cuticle preservations (Sochava, 1971), but no diagnostic chemical data were obtained. Kohring & Hirsch (1996) studied the crocodilian and ornithoid eggshells from the middle Eocene of the Geiseltal, Germany, and proposed the possible fossilized cuticle and shell membrane preservation. Later, with the scanning electron microscopy (SEM), several studies on dinosaur eggshells, Cretaceous bird eggshells, and fossil moa eggshells showed the potential of the cuticle preservation (Mikhailov, 1991; Varricchio & Jackson, 2004; Jackson & Varricchio, 2010; Oskam et al., 2010). Although they provided a new avenue on the soft-part preservation, all previous studies described possible cuticle preservation only based on micro-structural comparisons with the cuticle of extant avian eggshells. Jackson and Varricchio (2010) described a new ootaxon – *Triprismatoolithus stephensi*, and reported a cracked and amorphous surface overlying the external layer, which might be a layer of cuticle. Nevertheless, they did not further investigate the possible cuticle layer by means of any other analyses. Therefore, all previous reports regarding the cuticle preservation were constrained to micro-morphological observations. The only chemical evidence supporting the fossilization potential of eggshell cuticle through deep time is offered by a study focusing on the two main eggshell pigments, biliverdin and protoporphyrin (Wiemann et al., 2017). The protoporphyrin is deposited predominantly in the cuticle layer (Schwartz et al., 1975; Nys et al., 1991; Mikšík et al., 2007; Nys & Guyot, 2011), suggesting indirect evidence of cuticle preservation based on the preservation of endogenous protoporphyrin discovered in the oviraptorid eggshells (Wiemann et al. 2017). We hence posited that the cuticle was completely, or partially, preserved on the oviraptorid eggshells.

Dinosaur nesting ecology

Dinosaur nesting ecology is a longstanding intriguing research topic. Among all dinosaurs, the nesting type of titanosaur, troodontid, and oviraptorid dinosaurs were comparatively well documented. These dinosaurs adopted different nesting strategies in the clutch arrangement, nest architecture, and nesting type. Deeming (2006) and Tanaka, Zelenitsky & Therrien (2015) roughly categorized two nesting modes in dinosaurs, including open and buried nesting modes. Most birds build open nests, while the buried nesting mode is observed in megapode birds. In dinosaurs, it was suggested that titanosaurs also built the buried nesting mode (Sander et al., 1998; Grellet-Tinner & Fiorelli, 2010; Sander et al., 2008; Vila et al., 2010; Hechenleitner, Grellet-Tinner & Fiorelli, 2015). The low porosity of titanosaur eggs from Auca Mahuevo, however, suggested that the titanosaurs from Auca Mahuevo did not bury their eggs (Jackson, 2007; Sander et al., 2008). The Auca Mahuevo exception demonstrates that the eggshell is a labile structure, which alters according to the environment. In comparison with the well-documented titanosaur nesting ecology, the nesting ecology of theropod has been remained uncertain until recent times. A nesting *Nemegtomaia* discovered in Mongolia indicated a semi-open nesting mode based on sedimentological evidence (Fanti, Currie & Demchig, 2012). The discovery of pigmentation in oviraptorid eggshells strongly suggested that the oviraptorid eggs were laid in an open nest (Wiemann et al., 2017). Yang et al. (2015) further supported the semi-open nesting mode according to the heterogeneous distribution of porosity in an oviraptorid egg and taphonomical evidence. While there is no evidence of pigmentation in troodontid eggshells because these have not been analyzed chemically so far, the heterogeneous distribution of porosity indicated that the troodontid dinosaurs might also have laid their eggs in a semi-open nest (Varricchio et al., 1997; Varricchio et al., 2013).

The goal of this study is to present further evidence of the cuticle preservation on dinosaur eggshells by means of the elemental analysis and Raman microscopy. In particular, we analyzed theropod eggshells from the Nanxiong Group in China and Two Medicine Formation in Montana, USA. Both Nanxiong Group and Two Medicine Formation present fluvial deposits, indicating a humid climate in both areas during the Late Cretaceous. Besides, nesting on the ground or in mounds increase the risk of microbial invasions, a cuticle layer should hence be prevalent on eggshells of semi-open nesting theropods.

Material and methods

Materials

In order to standardize our following analyses, domestic chicken eggshell (*Gallus gallus* *domesticus*) was obtained from a commercial source (supermarket). The fossil materials include oviraptorid and plausible alvarezsaurid dinosaur eggshells.

We sampled three oviraptorid eggshells that were collected from three localities in China (Henan Province, Jiangxi Province, and Guangdong Province) and were housed at the NMNS and STIPB. Based on the eggshell microstructure, all eggshells were assignable to *Macroolithus yaotunensis*, which is laid by the oviraptorid *Heyuannia huangi* (Cheng et al., 2008). The *Macroolithus yaotunensis* is characterized by an undulating boundary between two crystalline layers, including outer prismatic layer and inner mammillary layer (Supplemental Information; Zhao, 1975). The first *Macroolithus yaotunensis* eggshell was sampled from specimen NMNS CYN-2004-DINO-05-I. The specimen NMNS CYN-2004-DINO-05-I was collected from the Late Cretaceous Tangbian Formation of the Hongcheng Basin in Jiangxi Province, China (Table 1). The Tangbian Formation is characterized by fine-grained brownish-reddish sediments with dispersed coarse clasts and conglomerates, representing a fluvial/alluvial environment with occasional stream flooding during the Campanian age (Chen et al., 2017). The second *Macroolithus yaotunensis* sample was collected from the Late Cretaceous Hugang Formation in the Liguanqiao Basin near Nanyang, southwestern Henan Province, China (Table 1). This sample has been housed in STIPB since 1985 and was described in Erben (1995). Hugang Formation is composed of dark grayish red breccia with interbeds of calcareous sandstone, indicating a fluvial environment. (Zhang, 2009). The third *Macroolithus yaotunensis* sample was obtained from the Late Cretaceous fluvial deposits of the Pingling Section of Shanghu Formation in the Nanxiong Basin, located in the northwestern part of Guangdong Province, China (Table 1). The NE-SW striking Nanxiong Basin, which is filled with dark purplish silty mudstone with interbeds of conglomerate, has produced numerous theropod egg clutches and eggshell fragments (Erben, 1995).

The *Triprismatoolithus stephensi* eggshells (MOR ES101), probably attributable to alvarezsaurids (Agnolin et al., 2012; Varricchio & Jackson, 2016), were collected in the lower portion of the Two Medicine Formation in the Sevenmile Hill outcrops, Teton County, Montana, USA (Table 1). The *Triprismatoolithus stephensi* eggshell has three crystalline layers, including external layer, prismatic layer, and mammillary later, from outermost to innermost (Varricchio & Jackson, 2016). The Two Medicine Formation is mostly composed of sandstone, deposited by rivers and deltas in the western shoreline of the Late Cretaceous Interior Seaway.

Chemical analyses

For the chemical analyses, first, we used a VEGA TS5130 LM (Tescan) SEM at the STIPB to locate and image plausible cuticle preservation on studied eggshells. The eggshells were naturally cut fragments and coated with gold for SEM observation. The operating conditions for the SEM were set as 20 kV accelerating voltage.

All samples were cleaned with ethanol before chemical analyses. We then performed elemental analyses using the EPMA equipped with a WDS at the Section of Mineralogy of the STIPB to elucidate the P distribution on the radial section of dinosaur eggshells. All samples were polished, fixed on a slide glass with araldite histological resin, and then coated with carbon for the elemental analysis with EPMA. The elemental analyses were performed with a JEOL SUPERPROBE 8900 EPMA at the same lab. The operating conditions for the EPMA were set as 1 µm beam diameter, 15 kV accelerating voltage, and 15 nA specimen current. The ZAF correction scheme was used. Natural and synthetic minerals were used as standards.

We performed two rounds of Raman spectroscopy using two Raman spectrometers at the Max-Planck Institute for Iron Research, Dusseldorf, Germany. For the first round, we analyzed the domestic chicken eggshell from a commercial source with a WITec Alpha300 system with 532 nm wavelength laser light and a confocal 50x objective at the MPIE, each for 20 seconds (hereinafter referred to as RI). Since the cuticle on the chicken eggshell was easily identified with the naked eyes, as well as under the SEM (Figs. 1C & 1D), the first round hence confirmed the detectability of Raman spectroscopy for identifying the HAp of the cuticle layer (Figs. 1E & 1F). Due to the incomplete preservation, we performed Raman surface mapping on the area where we found possible cuticle remains using the SEM and EPMA previously.

Raman analysis II (refereed to RII hereinafter) was performed using Raman spectrometer of Horiba Jobin Yvon GmbH, with a 632 nm laser. All results were obtained using the 10x objective of the integrated LabRam microscope. While the 10x objective is not strongly sensitive to rare compounds, it provided larger field of view for faster and broaden qualitative detection of HAp on eggshells than the RI. In order to distinguish HAp and calcite from sediments, we collected Raman spectra of the fresh chicken eggshell, the studied fossil eggshells and their associated sediments (Fig. 2A). All Raman spectra of eggshells and their associated sediments using the RII were imported into R and normalized with the R package “ChemoSpec” (Hanson, 2017). We performed principle component analysis (PCA) on the spectral area of 500 to 1500 cm^{-1} using the same package (Fig. 2B).

Results

Firstly, we analyzed fresh *Gallus gallus domesticus* eggshells for standardizing our following analyses on dinosaur eggshells. The cuticle layer on *Gallus* eggshell was easily observed with the naked eyes. The Raman spectrum (RI) from the *Gallus* eggshell shows a significant peak at 1087 cm^{-1} that arises from the calcite, which is the major component of eggshell (Fig. 1F). Another intense peak at 967 cm^{-1} was assigned to the $v_3PO_4^{3-}$ symmetric stretching vibrations (Walters et al., 1990; Gergely et al. 2010; Frost et al., 2014). Targeting the

967 cm^{-1} peak, the Raman surface scan shows the patchy distribution of the HAp layer overlying the calcitic eggshell (Fig. 1E). The result demonstrates detectability of the inner HAp layer of the cuticle by Raman spectroscopy.

For the outside of *Macroolithus yaotunensis* and *Triprismatoolithus stephensi* eggshell fragments, we also found peaks in these spectral regions (Fig. 2A). The analysis using RII showed two intense broad peaks that are located between 1063-1097 cm^{-1} and 972-986 cm^{-1} in the spectra. The spectral range of 1063-1097 cm^{-1} can be assigned to C-O bond of calcite. The 972-986 cm^{-1} spectral ranges arose possibly from the phosphate (PO_4^{3-}) or hydrogen phosphate (HPO_4^{2-}) (Crane et al., 2006; Sauer et al., 1994). Besides, three significant spectral ranges of 1326-1422 cm^{-1}, 1422-1476 cm^{-1} and 1535-1655 cm^{-1} were also seen, possibly corresponding to preserved pigments (Thomas et al., 2015; Wiemann et al., 2017). The chemometrical analysis using ChemoSpec suggests that all studied eggshells show similar spectral pattern between 800 cm^{-1} and 1200 cm^{-1} (Fig. 2B). In addition, the spectra from the surrounding sediments occupy different chemospaces from the ones of eggshells (Fig. 2B).

Under the polarized microscope, the *Macroolithus* eggshell from the Hongcheng Basin in Jiangxi, China displayed distinct two crystalline layers covered by an enigmatic layer and surrounding sediments (Fig. 3A). These features are also present in the other two *Macroolithus* eggshells (see Supplemental Information). The uneven thickness of the prismatic layer in the Jiangxi *Macroolithus* eggshells was a result of unpolished natural cut section (Fig. 3B). Possible patchy and flake-like cuticle layer on the outer surface of the *Macroolithus* and *Triprismatoolithus* eggshells was observed with SEM (Figs. 3C & 4B). Furthermore, an enigmatic protruding tube-like structure was recognized in the *Triprismatoolithus* eggshell (Figs. 4C & 4D). The diameter of the tube-like structure is approximately 3 μm.

We performed ten elemental line-mappings on each eggshell with the EPMA and targeted phosphorus of the HAp. Of in total 30 line mappings on the three *Macroolithus yaotunensis* eggshell, only two of them, which were from the Jiangxi and Guangdong specimens, show a significant peak for rich phosphorus on the boundary between the eggshell and surrounding sediment (Figs. 3D & 3F). The elemental analysis also indicates the scarcity of the phosphorus in surrounding sediment. Besides, the P concentration increases from the mammillary layer to prismatic layer in all analyzed eggshells.

In the EPMA line-scan on the *Triprismatoolithus stephensi* eggshell, three significant peaks for P were observed. The peak on the boundary between the eggshell and surrounding sediment indicates possible cuticle preservation (Figs. 4E). However, another two peaks were seen in the sediment and on the boundary between the external layer and prismatic layer. The variation of P between different eggshell layers was also observed in *Triprismatoolithus stephensi* eggshell. Specifically, the P concentration increases from the mammillary layer to the prismatic layer but decreases from the prismatic layer to the external layer.

Discussion

Elemental analysis of theropod eggshells

While previous studies have reported possible preservation of cuticle and *membrane testacea* (Chow, 1954; Kohring & Hirsch, 1996; Grellet-Tinner, 2005; Jackson & Varricchio, 2010; Reisz et al., 2013; Yang et al., 2015), none of them performed chemical analyses to test this hypothesis. In this study, elemental analysis suggested the preservation of the inner hydroxyapatitic cuticle layer based on the high concentration of P at the boundary between eggshell and sediment. The elemental analysis also shows that P is rare in the surrounding sediment from both the Nanxiong Group of China and Two Medicine Formation of Montana, United States. The deficiency of P in the sediment thus argues against an allochthonous origin of the P in the outermost part of the eggshell (Figs. 3D-F & 4E). The chemometrical comparison of the spectra derived from sediments also suggests the depletion of phosphate minerals in the sediments. Therefore, the peak in the sediment surrounding the *Triprismatoolithus stephensi* eggshell might be derived from the eggshell specimen itself.

The elemental analyses cannot provide evidence for the preservation of the capillary vessel of the chorioallantoic membrane. The SEM images of the vessels in the chorioallantoic membrane on modern chicken eggshells sizes from 3 to 10 μm (Maiber et al., 2015). It is therefore possible that the enigmatic tube-like structure in the *Triprismatoolithus* eggshell represents a fossilized vessel in the chorioallantoic membrane. However, no features of chorioallantoic membrane was observed. The preservation of the vessel in the chorioallantoic membrane is hence pending for more supporting evidence.

Variation of P concentration in eggshells

The elemental analyses show that P concentration increases from the mammillary layer to the prismatic layer of both *Macroolithus yaotunensis* and *Triprismatoolithus stephensi* eggshells (Figs. 3D-F & 4E). However, in *Triprismatoolithus stephensi* eggshell, P concentration decreases from the prismatic layer to external layer (Fig. 4E). This enigmatic pattern of P distribution has not been reported previously, which could be due to paucity of chemical studies on dinosaur eggshells. The deficiency of P in the surrounding sediments demonstrates that the distribution pattern of P in the studied eggshells are autogenous. Cusack, Fraser & Stachel (2003) also observed similar pattern of phosphorus distribution in *Gallus gallus domesticus* eggshells. Moreover, their study also revealed that the P concentration increases more sharply in the eggshell of younger birds than in older birds.

Since ornithological research had suggested P is an inhibitor of calcite growth during eggshell formation (Fink et al., 1993; Dennis et al., 1996; Fraser, Bain & Solomon, 1999; Cusack, Fraser & Stachel, 2003), it appears likely the non-avian theropod dinosaurs studied by us already possessed a similar eggshell formation mechanism to that of extant birds. Furthermore, the difference in P distribution between young and older birds can probably help paleontologists decipher if an oviraptorid clutch was laid by a single female or several females. Varricchio et al. (2008) compared the ratio of clutch size to adult volume and proposed that an oviraptorid clutch was produced by several females. Yang et al. (2016) has provided preliminary supportive results based on statistical analyses of P distribution in, and external morphology of, oviraptorid eggshells from the same clutch.

Interpretation of Raman spectra

In the Raman study of the fossil eggshells, the peak at 967 cm^{-1} of *Gallus* eggshell was not seen in all spectra, but a broad peak ranging from 972-986 cm^{-1} was detected instead (Fig. 2A). Three hypotheses can be formulated to explain the different spectral patterns from 960 cm^{-1} to 980 cm^{-1} in extant cuticle vs. the fossil eggshells.

First, theropod dinosaurs might have used whitlockite, a phosphate mineral containing hydrogen phosphate anions (HPO_4^{2-}), to form the inner cuticle layer instead of HAp. Whitlockite is the second most abundant mineral in biological calcified tissues, accounting for around 20% of bone mineral (Driessens & Verbeeck, 1990; Jang et al., 2014; Jang et al., 2015). It is also possible that the HAp of the inner cuticle layer is in non-stochiometrical form, and hence part of the phosphate anions is occupied by hydrogen phosphate (HPO_4^{2-}) (Rey et al., 2011; De La Pierre et al., 2014).

Second, the original HAp of the inner cuticle layer could have been transformed to whitlockite. Jang et al. (2014) showed that HAp transforms into dicalcium phosphate dihydrate (DCPD, $CaHPO_4{\cdot}2H_2O$) at 70 ℃. The DCPD is then converted into whitlockite ($Ca_9(MgFe)(PO_4)_6PO_3OH$) by incorporating Mg^{2+} or Fe^{2+} when the pH decreases. However, in natural environments, such as the Nanxiong Formation and Two Medicine Formation, burial and diagenetic temperatures over 70 ℃ is unlikely except from a fluid intrusion of igneous origin. However, geological investigations did not provide any evidence for such fluid intrusion in the Nanxiong Basin in China (Erben, 1995). At the Sevenmile Hill outcrops in Montana, United States, Foreman et al. (2008) reported an altered volcanic ash layer (bentonite) that is depleted in P. However, the volcanic ash layer was deposited prior to the eggshells (Jackson & Varricchio, 2010).

Third, the P we detected might be derived from the surrounding sediments during diagenesis. However, the detection of preserved pigments from the Nanxiong Basin and absence of fluid intrusion in both localities suggests that the eggshells we studied experienced very little diagenesis (Erben, 1995; Foreman et al., 2008; Wiemann et al., 2017).

Pigmentation on theropod eggshells

Two pigments, the tetrapyrroles protoporphyrin and biliverdin, which are red-brown and blue-green in color (Kennedy & Vevers, 1976), contribute to the various coloration of the avian egg. Previous studies suggested that the cuticle layer is the main repository of protoporphyrin (Schwartz et al., 1975; Nys et al., 1991; Mikšík et al., 2007; Nys & Guyot, 2011). Recently, these two pigments were discovered in oviraptorid dinosaur eggshells from all the three localities where the eggshells for this study were collected (Table 1; Wiemann et al., 2017). Thus, discovery of protoporphyrin in oviraptorid eggshells also supports the preservation of cuticle and excludes a diagenetic origin of the P. Thomas et al. (2016) performed Raman spectroscopy and mass spectrometry to show the detectability of pigments in extant avian eggshells. Our current Raman analysis also demonstrate detectability of pigments preserved in fossil eggshells, while the peaks in the spectra are not completely correspondent with the ones shown in Thomas et al. (2016). Extensive studies using mass spectrometry on dinosaur eggshells are needed.

Nesting ecology of oviraptorid dinosaurs

As noted, two nesting mode are recognized in birds, open and buried nesting (Varricchio & Jackson, 2016). Most birds build open nests either on the tree or on the ground (ostriches or emus), while the megapode birds bury their eggs in a mound. Therefore, all open nesting birds exploit eggshells with low porosity (Tanaka, Zelenitsky & Therrien, 2015). However, an intermediate mode — semi-open nests in oviraptorid dinosaurs was proposed based on detection of pigments and porosity evaluation (Yang et al., 2015; Wiemann et al., 2017). This intermediate mode is consistent with the fluvial environment inferred from sedimentological investigations in the Nanxiong Basin (Erben, 1995). In such a humid fluvial environment, the risk of microbial invasions was much higher. Our detection of a cuticle in these eggs also supports this nesting ecology since the cuticle layer hinders microbial invasions (D'Alba et al., 2016).

However, it was suggested that Mongolian oviraptorid clutches were discovered in the sand dune deposits (eolian) such as Baruungoyot Formation or Djadokhta Formation (Norell et al., 1995). Given the arid sand deposits, the cuticle layer was hence unlikely present on Mongolia oviraptorid eggshells, superficially contradicting to the inferences of this study. Fanti, Currie & Demchig (2012) documented that *Nemegtomaia* were discovered from both Nemegt Formation and Baruungoyot Formation that represent mesic and xeric habitats, respectively. Moreover, they also provided the evidence for the lateral coexistence of mesic and xeric habitats in the Baruungoyot Formation and posited that *Nemegtomaia*, or Mongolian oviraptorid dinosaurs, nested near permanent or seasonal streams that favor cuticle-coated eggs.

While it is uncertain that the alvarezsaurid eggshells were pigmented prior to further chemical studies, it is possible that the alvarezsaurid dinosaur exploited similar nesting strategy as oviraptorid dinosaurs did.

Conclusions

Our study suggests preservation of the inner cuticle layer in eggs of the oviraptorid *Macroolithus yaotunensis* and the alvarezsaurid *Triprismatoolithus stephensi* based on elemental analysis and Raman spectroscopy. The potential co-evolution of a cuticle layer and semi-open nesting in humid environments may have allowed theropod dinosaurs to nest in fluvial environments, as does the cuticle layer of extant birds nesting in humid environment. In combination with the previous discovery of pigments in oviraptorid dinosaur eggshells, this study provides further evidence for preservation of fossilized tissues in the fluvial deposits, especially in the Cretaceous red bed basins of China. Further chemical studies on dinosaur eggshells will shed additional light on the reproductive biology of dinosaurs, as well as on taphonomical questions.

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Figure 1

Cross-sectional view, SEM images, and Raman imaging and spectrum of a *Gallus gallus domesticus* egg and the eggshell.

The Raman image and spectrum were collected using 532 nm excitation and a 50x objective with RI. (A) The anatomy of an egg. (B) The oviraptorid eggshell comprises two crystalline layers, including the mammillary layer and palisade layer. The cuticle layer overlying the calcareous eggshell is further divided to two layers, including a HAp inner layer and a proteinaceous outer layer. The shell membrane, namely *membrane testacea*, is also characterized by two layers. (C) SEM image of the cuticle on the surface of the *Gallus* eggshell, showing a patchy and cracking pattern. (D) SEM image of the radial section of the *Gallus* eggshell. The white arrow indicates the cuticle layer that lies on the calcitic eggshell. (E) Raman chemical image with peak targeting at 967 cm^{-1} which is attributed to HAp. The yellow area represents the patchy distribution of the inner HAp cuticle layer on the *Gallus* eggshell. The calcitic eggshell that is not covered by the inner HAp cuticle layer is shown in the brown area. The dotted circle corresponds to the spectrum shown in (F). (F) Spectrum collected in the dotted-circle area of the Raman chemical image shown in (E). Two significant peaks at 967 cm^{-1} and 1087 cm^{-1} are indicated by yellow bars, representing HAp and calcite, respectively.

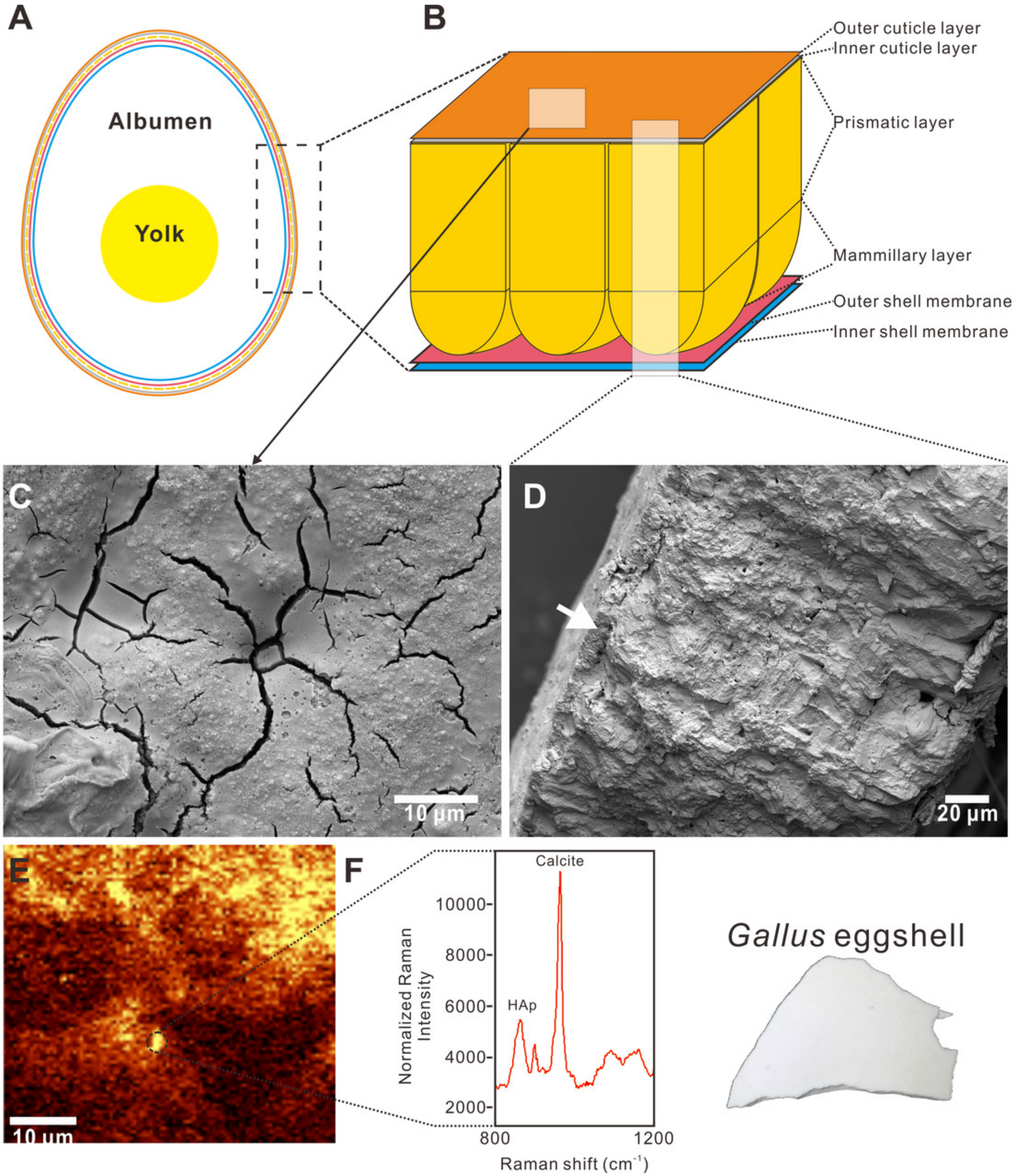
A
B
Albumen
Yolk
Outer cuticle layer
Inner cuticle layer
Prismatic layer
Mammillary layer
Outer shell membrane
Inner shell membrane
C
10 μm
D
20 μm
E
10 μm
F
Calcite
HAp
Normalized Raman Intensity
10000
8000
6000
4000
2000
800
1200
Raman shift (cm⁻¹)
Gallus eggshell

Figure 2

Raman spectra and chemometrical analysis.

(A) Raman spectra derived from chicken, fossil eggshells, and surrounding sediments. The peaks around 972-986 cm^{-1} and 1063-1097 cm^{-1} are marked by yellow bars, indicating the calcite and phosphate bonds. (B) The principle component analysis on the spectral area of 800-1200 cm^{-1} shown in (A) demonstrates a significant disparity between eggshells and sediments.

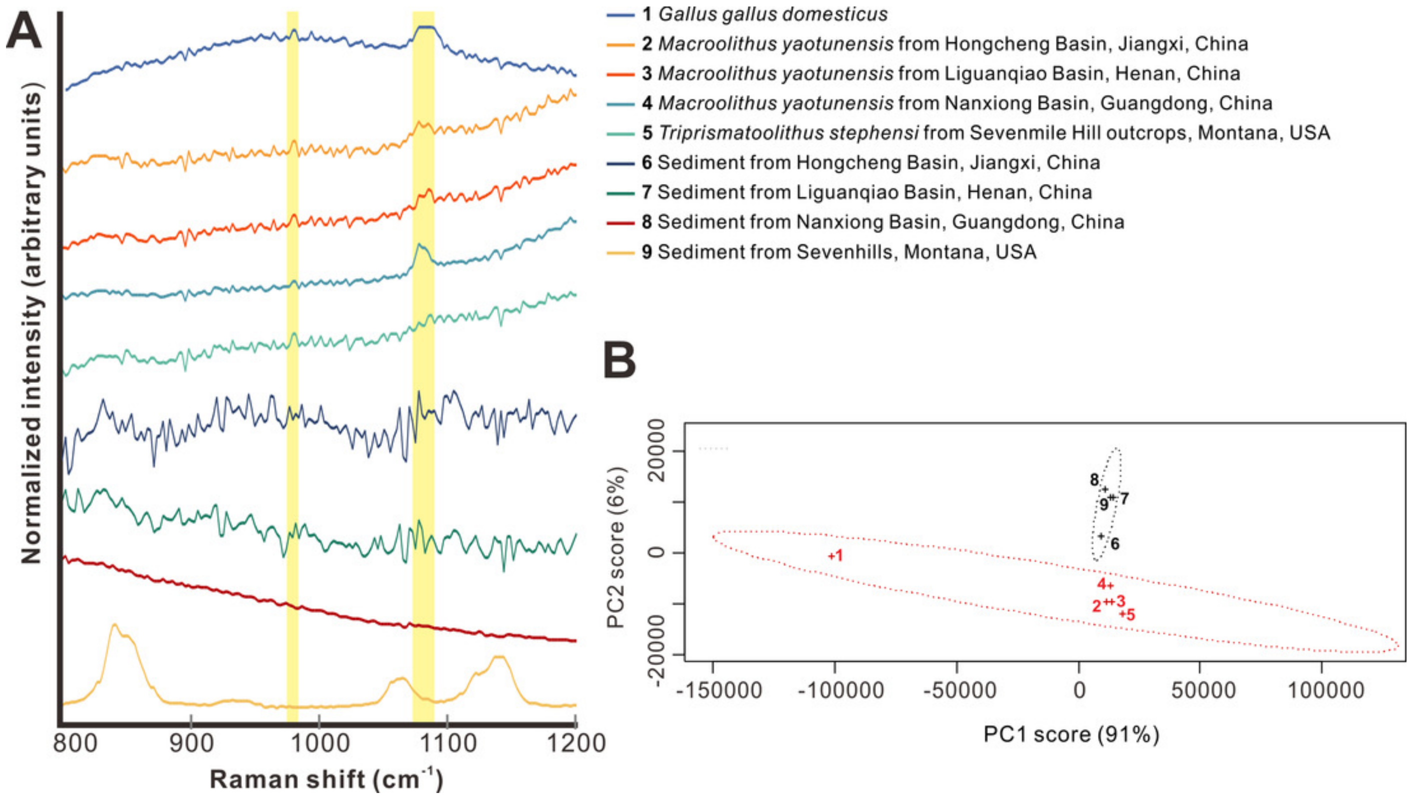

Figure 3

Microscopic images of *Heyuannia huangi* eggshells from Henan, Jiangxi, and Guangdong and their EPMA line-scan spectra.

(A) The *Heyuannia huangi* eggshell (NMNS CYN-2004-DINO-05-I) from Hongcheng Basin in Jiangxi Province of China under the polarized light microscope. The white dashed line indicates a possible boundary between the sediment and an enigmatic layer. The enigmatic layer was formed by the interaction between organics of the egg and surrounding sediment. The line-scan spectra with the EPMA were performed on the white solid line. In this eggshell image, a flat boundary between the prismatic and mammillary layers (PL and ML shown as dark and light zones) is clearly observed. (B) SEM image of radial untreated fracture of the *Macroolithus yaotunensis* eggshell. The boundary between the sediment and prismatic layer is marked by yellow dashed line based on their distinct structural features. The boundary between ML and PL is marked by the red dashed line from the overlying prismatic layer. The ML shows the distinct vertical mammillae structure. (C) Close-up image of the area in white box in (B). Possible preservation of cuticle is indicated by the white arrow, showing patchy and flaky structures similar to the cuticle on the modern chicken eggshell (Figs. 1C & 1D). (D) The spectra illustrate the distribution of the Ca and P across the eggshell from Hongcheng Basin in Jiangxi Province of China shown in (A), from innermost (left) to outermost as indicated by a black line in the microscopic image. The Ca spectrum clearly marks the extent of the calcitic eggshell. A significant peak that is marked by a yellow bar demonstrates a relatively high concentration of P, indicating the possible preservation of the cuticular HAp layer. (E) The spectra illustrate the distribution of the Ca and P across the eggshell from the Liguangqiao Basin in Henan Province of China, from innermost (left) to outermost. The Ca spectrum clearly marks the extent of the calcitic eggshell. A slightly richer amount of P near the interface between the eggshell and sediments is observed. (F) The spectra illustrate the distribution of the Ca and P across the eggshell from the Nanxiong Basin in Guangdong

Province of China, from innermost (left) to outermost. A significant peak that is marked by a yellow bar demonstrates a relatively high concentration of P, indicating the possible preservation of the cuticular HAp layer.

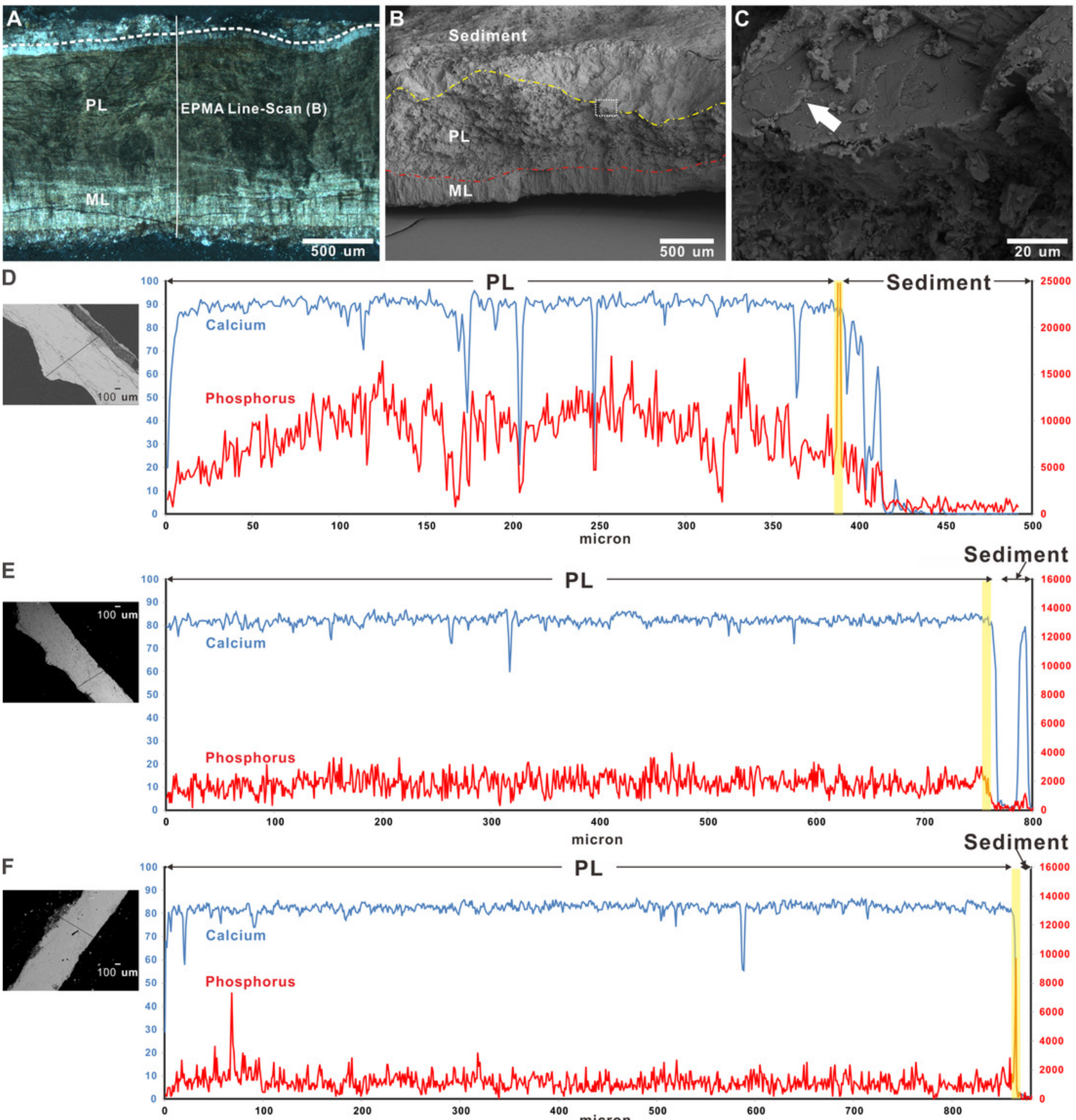

Figure 4

SEM images of the *Triprismatoolithus* eggshell and EPMA line-scan spectra.

(A) The radial cut section of studied *Triprismatoolithus* eggshell. (B) The plausible flake-like cuticle structure atop the eggshell, marked by white arrow. The red dotted line marks the outermost boundary of the prismatic layer. (C) An enigmatic structure (white arrow) protruding out of the mammillary layer, possibly a capillary vessel of the chorioallantoic membrane. (D) Enlargement of the possible capillary. (E) Line scans across eggshell in (A). The scan shows a high concentration of P on the boundary between the eggshell and surrounding sediment. Two other peaks of P were observed on the boundary between the external and prismatic zones, and in the sediment. The P concentration is homogeneous throughout the mammillary and prismatic layers, but decreases in the external layer. ML: mammillary layer. PL: prismatic layer. EL: external layer.

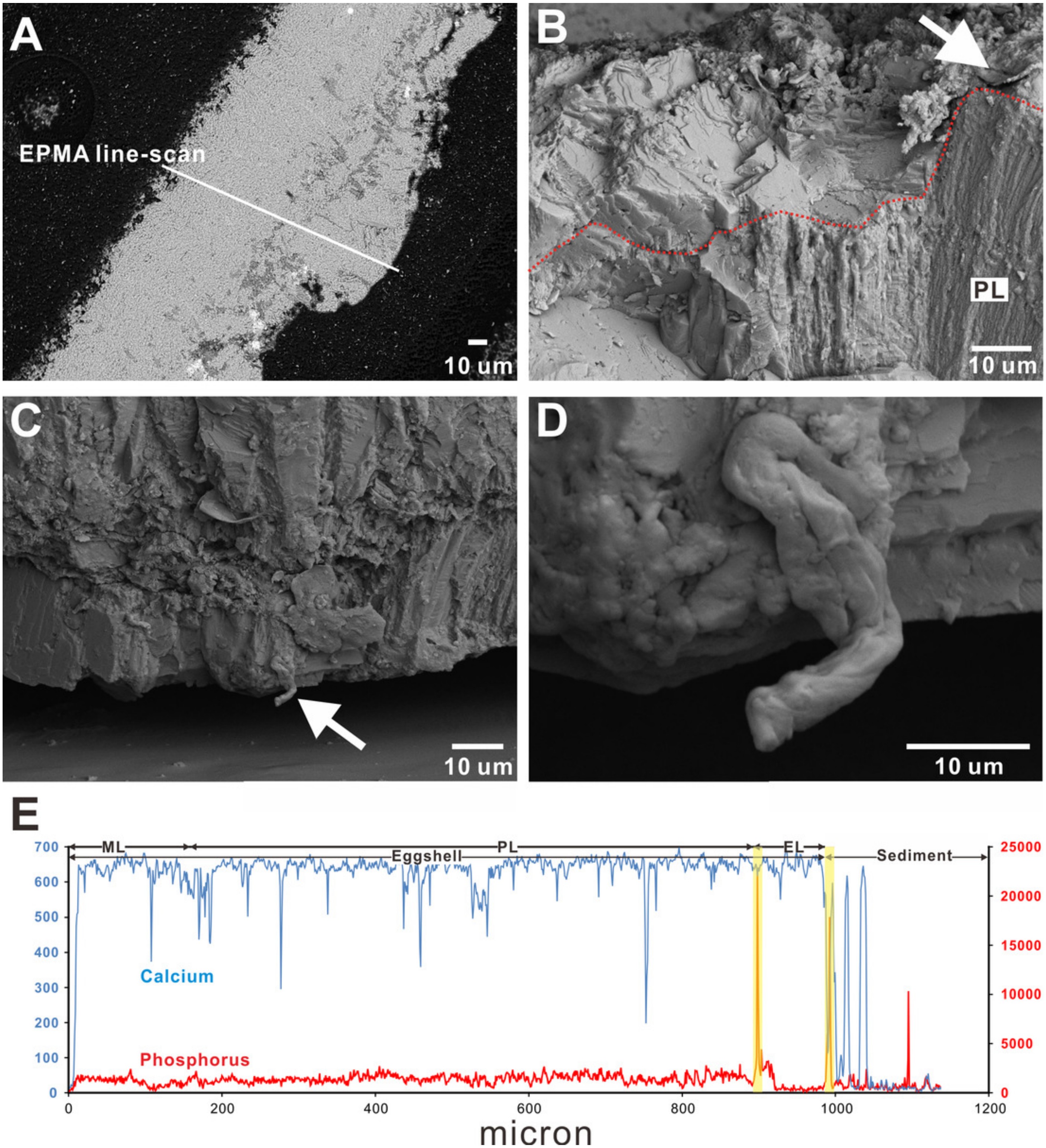
A
EPMA line-scan
10 um
B
PL
10 um
C
10 um
D
10 um
E
ML
Eggshell
PL
EL
Sediment
Calcium
Phosphorus
micron

Table 1 (on next page)

Eggshells analyzed in this study

The ootaxonomic/taxonomic assignments, stratigraphic and locality information, and catalog numbers of the eggshells analyzed in this study

Tables

Table 1 Eggshells analyzed in this study.

Oospeices or species	Stratigraphy	Locality	Catalog number	Reference
Macroolithus yaotunensis	Tangbian Formation	Hongcheng Basin, Jiangxi Province, China	NMNS CYN-2004-DINO-05-I	Wiemann et al. 2017
Macroolithus yaotunensis	Hugang Formation	Liquanqiao Basin, Henan Province, China	STIPB-E131	Wiemann et al. 2017
Macroolithus yaotunensis	Pingling Formation	Nanxiong Basin, Guangdong Province, China	STIPB-E66	Wiemann et al. 2017
Triprismatoolithus stephensi	Lower portion of the Upper Cretaceous (Campanian) Two Medicine Formation	Dave and Joel Site, Sevenmile Hill outcrops, Teton County, Montana	MOR ES101	Jackson and Varricchio 2010
Gallus gallus	-	Bonn, Germany	-	-