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The blue-green eggs of dinosaurs: How fossil metabolites provide insights into the evolution of bird reproduction

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J.W., T.-R.Y., P.N.S. and P.M.S. wrote the article; J.W., P.N.S. and C.E.M. designed and optimized the extraction, J.W. carried out the extraction. J.W. prepared the extracts for mass spectroscopy; M.E. and M.S. designed the ESI MS method and carried out the measurements. J.W., M.E., M.S. and C. E. M. analysed and interpreted the ESI spectra. T.-R.Y. worked out and provided the porosity measurements. J.W., T.-R.Y. and P.M.S. designed the idea, P.N.S. and S.K.-S. contributed in planning of the experiments.

Conflicts of interest

The authors declare no competing financial interests.

Supplementary information line

Supplementary Information will be available in the final version of this article.

Abstract

Open-nesting birds use biological pigments in eggshell to camouflage their unhatched offspring, varying the colour to account for the nesting environment and location. The tetrapyrrolic pigments protoporphyrin (PP) and biliverdin (BV), which both participate in the haem metabolism, are responsible for the reddish brown of chicken eggs and the brilliant blue of robin and emu eggs. However, eggshell pigmentation correlates with the nest type in a wide range of avian species and suggests that coloured eggs are basal to the avian lineage, extending back to their non-avian dinosaur origins. Detecting preserved eggshell pigments could thus shed light on dinosaur nesting behaviour. Using HPLC separation coupled to ESI-Q-TOF mass spectrometry, we here provide the first record of the eggshell pigments PP and BV preserved in fossils from three different localities, in 66 million year-old oviraptorid eggshell (*Macroolithus yaotunensis*). These eggs were presumably laid in at least partially open nests by the oviraptorid *Heyuannia huangi* and camouflaged by an originally blue-greenish egg colouration. Such a blue-greenish eggshell pigmentation hints at increased paternal care in *Heyuannia*. Shell porosity measurements, preserved clutches and parental animals support an open nesting behaviour for oviraptorid dinosaurs. Furthermore, the detection of PP, together with supporting microscopic observations, represents the first evidence for cuticle preservation in fossil eggshell. Our study demonstrates that molecular biomarkers, such as preserved metabolites, can be used to trace the evolution of modern avian traits, and to provide insights into dinosaur reproductive biology and the preservation of endogenous organic matter in fossil vertebrates.

Introduction

Birds offer some of the most diverse displays of colour and shape among modern vertebrates (Koschowitz, Fischer & Sander, 2014). Compared to the often flashy plumage, egg colouration has attracted little study. Avian eggs range widely in size and more importantly, in colour, from immaculate reddish brown and white in domestic chicken (*Gallus domesticus*), light beige with dark brown maculation (speckling) in the oystercatcher (*Haematopus ostralegus*), light blue in the American robin (*Turdus migratorius*) to the intensive bluish-green of emu eggs (*Dromaius novaehollandiae*).

While reptiles and monotreme mammals lack egg colouration because they bury their eggs, coloured eggs are present in most modern birds (Neornithes) and are considered synapomorphic for the group (Kilner, 2006), together with an open nest. Complete reduction of eggshell colouration is observed in many cave-breeding birds (Hewitson, 1864), hinting at the primary function of eggshell colouration: visual signalling. Intraspecific, positive signalling primarily concerns female quality (Cherry & Gosler, 2010), while negative signalling offers camouflage or crypsis (Wallace, 1890), and protection against brood parasitism (Newton, 1896). In addition to signalling, numerous other functions of egg colour pigments have been described, such as antimicrobial effects (Ishikawa, 2010), protection from solar radiation (Lahti, 2008), and eggshell reinforcement (Gosler, Higham & Reynolds, 2005).

Material & Methods

Institutional abbreviations

NMNS: National Museum of Natural Sciences, Taichung, Taiwan

PFMM: Paleowonders Fossils and Mineral Museum, Taipei, Taiwan

STIPB: Steinmann Institute of Geology, Mineralogy, and Palaeontology, Division of Palaeontology, University of Bonn, Bonn, Germany

ZFMK: Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, Germany

Extant eggshell material

The emu (*Dromaius novaehollandiae*) eggshells were from captive birds and stored in the ZFMK collections (ZFMK uncat.).

Fossil eggshell material

Elongatoolithidae Zhao, 1975

Macroolithus Zhao, 1975

Macroolithus yaotunensis Zhao, 1975

(Laid by the oviraptorid *Heyuannia huangi*) (Cheng *et al.*, 2008)

We retrieved several *Macroolithus yaotunensis* eggshell samples from the collections of the NMNS and the STIPB for chemical analysis and porosity measurement. The specimens were collected from three different localities in China, including the Liquangqiao Basin in Henan Province, the Hongcheng Basin in Jiangxi Province, and the Nanxiong Basin in Guangdong Province. The geological background is discussed in detail below (Fig. 1).

The eggshells collected from Upper Cretaceous Hugang Formation of the Liguangqiao Basin near Nanyang, southwestern Henan Province, China, have been housed in STIPB since 1983 and were described by Erben (1995). These eggshells are assigned to *Macroolithus yaotunensis* based on their linearituberculate ornamentation, angusticanalicate pores, and shell thickness.

The oviraptorid eggshells collected from the Nanxiong Basin, located in the northwestern part of Guangdong Province, China, can also be assigned to *Macroolithus yaotunensis* based on their macro- and microstructure. The NE-SW striking basin is located in northern Guangdong Province and has produced numerous theropod egg clutches and eggshell fragments. The Late Cretaceous strata of the basin are divided into the Yuanpu Formation and the overlying Pingling Formation. The Yuanpu Formation was dated at 67 Ma (Zhao *et al.*, 1991).

The *Macroolithus yaotunensis* eggshells from the Hongcheng Basin, Jiangxi Province, China, were used for porosity measurement in addition to the chemical analysis. The eggshell samples were taken from CYN-2004-DINO-05/I (Fig. 3A) housed in the NMNS and identified as *Macroolithus yaotunensis*. The egg was excavated from the Upper Cretaceous Nanxiong Formation of the Hongcheng area near Ganzhou City (southern Jiangxi Province, China). The sediments mainly consisted of tan-red terrestrial sandstone. Four pieces of eggshell were also taken from the blunt, middle, and acute parts of the egg for measuring the porosity and calculating the water vapour conductance. The eggs are ornamented with linearituberculate ridges along their long axis, which were considered as the possible pathway for gas exchange (Hirsch & Quinn, 1990; Sabath, 1991).

All aforementioned eggshells can confidently be referred to *Macroolithus yaotunensis* Zhao (1975) based on their identical micro- and macrostructure. In addition, they can confidently be assigned to the oviraptorid *Heyuannia huangi* because of the identity of their micro- and macrostructure with that of the eggshells of the paired eggs inside a female oviraptorid pelvis from the same locality (Sato, 2005) and with embryo-bearing eggs (Cheng *et al.*, 2008) of the same taxon.

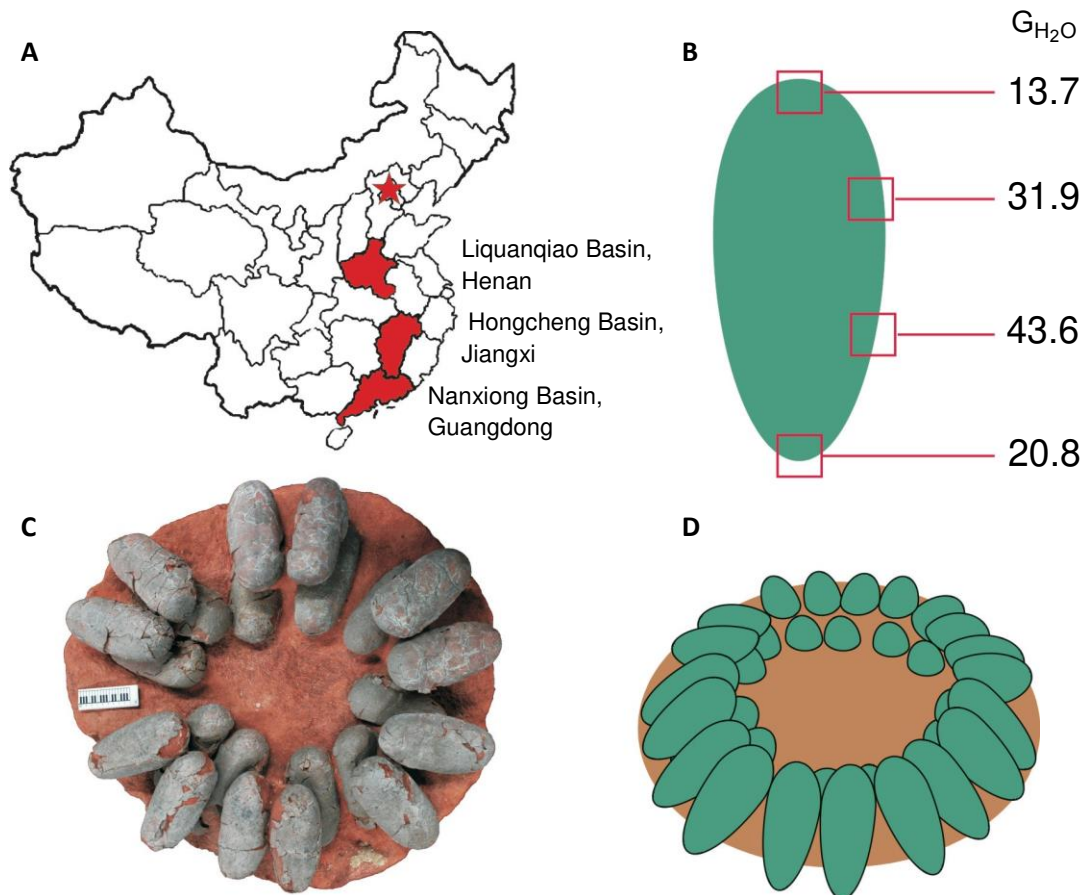


Figure 1 (A) Geographical map of China. The capital city, Beijing, is indicated by the red star. Red-filled provinces indicate the three different localities where the specimens were excavated, including the Liuanqiao Basin in Henan, Hongcheng Basin in Jiangxi, and Nanxiong Basin in Guangdong (see Supplementary Information). (B) The reconstructed colour and average zonal water vapour conductance of a *Heyuannia huangi* egg based on the biliverdin and protoporphyrin detection and porosity measurement. (C) Top view of an oviraptorosaurian clutch housed in the Paleowonders Fossil and Mineral Museum, Taiwan (Catalogue number: 0010403018). This clutch illustrates that the eggs are arranged in pairs with their blunt ends pointing to the clutch centre, as well as that the eggs are arranged in layers with sediment filled in between layers, indicating that the eggs were laid in an at least partially open nest. (D) An open-nesting model of the oviraptorosaurian nest. Note that the original dip of the eggs would have been much higher than what is preserved in (C) due to compaction during fossilisation.

Pigment Extraction and Detection

For the extraction of PP and BV, 500 μL of disodium EDTA solution (100 mg/mL), adjusted to a pH of 7.2, were added to aliquots of 180-562 mg eggshell fragments stored in Eppendorf tubes. The fragments were exposed for 5 min to this solution. During this time, the tubes were vortexed three times for 1 min each, interrupted by a 1 min pause with uncapped tubes. After removing the supernatants, this decalcification routine was repeated for BV, whereas one decalcification step sufficed for PP extraction. After each decalcification step, the eggshell-EDTA solution was centrifuged for 1 min at 15,000 g. Finally, 1 mL of acetonitrile – acetic acid (4:1, v/v) was added to the decalcified eggshell pellets for 10 min of exposure time, including 2 min vortex-mixing. After the end of exposure, the solution was centrifuged for 2 min, and the supernatants again were transferred to fresh tubes and stored in a dark environment at 4°C. The filtrated extracts were not stored longer than 24 h before they were injected into an HPLC Dionex Ultimate 3000 instrument (Thermo Scientific) onto a EC50/2 Nucleodur C18 Gravity 3 μm column (Macherey-Nagel), coupled to a micrOTOF-Q mass spectrometer (Bruker) with an electrospray ionisation (ESI) source for positive electrospray ionization of PP and BV.

Results

Blue-green eggshell pigmentation

Here we present the phylogenetically most basal evidence for eggshell colouration, preserved in 66 million year-old (latest Cretaceous) oviraptorid dinosaur eggshell. We detected the endogenous, tetrapyrrolic pigments protoporphyrin (PP) and biliverdin (BV) for the first time in fossils, in three eggs from three different Chinese localities. These fossil eggs of the oospecies *Macroolithus yaotunensis* were produced by the oviraptorid *Heyuannia huangi* (Cheng *et al.*, 2008). The samples come from Upper Cretaceous localities in Henan, Jiangxi and Guangdong Provinces, China (Fig. 1). For comparison, we analysed recent emu (*Dromaius novahollandiae*) eggshell. Using reverse phase HPLC coupled to ESI-Q-TOF mass spectrometry (Gorchein, Lim & Cassey, 2009), we qualitatively detected PP, $[\text{M}+\text{H}]^+$ with 563.2623 m/z (calculated mass: 563.2653 g/mol), after 14 min retention time under the applied conditions. Additionally, we qualitatively detected BV which is the more polar oxidation product of PP. BV detection consisted of $[\text{M}+\text{H}]^+$ with 583.2520 m/z (calculated mass: 583.2551 g/mol), after 8 min retention time (Fig. 2). The retention times and spectra were confirmed by analysis of commercially available PP and BV standards and of emu egg

shell. Concurrent measurements of the sediment-free eggshell samples and of the sediment matrices confirmed the endogenous nature of the preserved PP and BV.

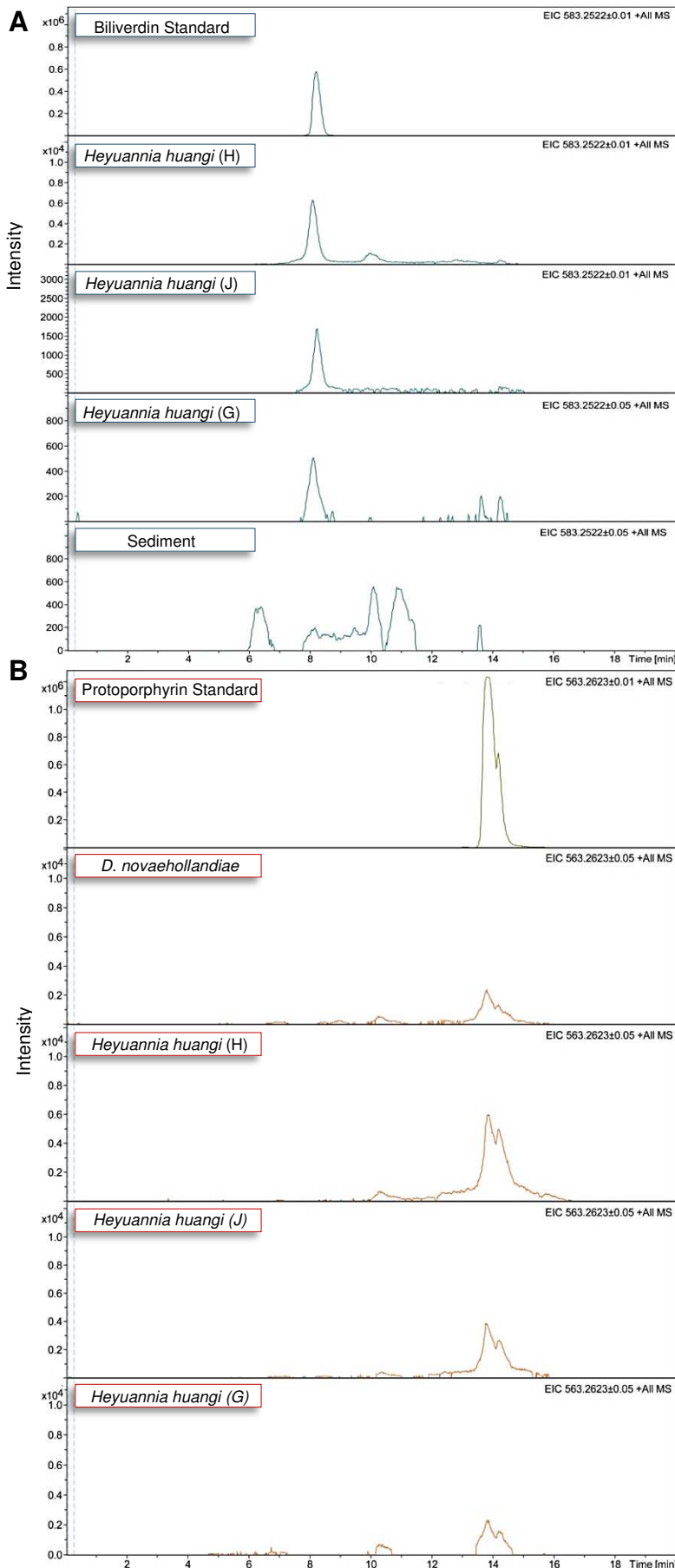


Figure 2 ESI (+) MS extracted ion chromatograms (EICs) for mass $583.2520 \pm 0.01/0.05$ m/z indicative of BV (A) and mass $563.2653 \pm 0.01/0.05$ m/z indicative of PP (B). We identified BV and PP by retention time, exact mass and isoform/tautomer separation. Assuming that peak height correlates with the pigment concentrations in the extracts, the best preservation of endogenous colour pigments (both BV and PP) occurred in the *Heyuannia* eggshell from the Liguangqiao Basin, Henan. A sediment sample was used as control for contamination, and its EIC shows no peak after 8 min retention time, thus we can assume, that it does not contain BV. **(A)** EICs for $583.2520 \pm 0.01/0.05$ m/z from top to bottom are for the commercial BV standard, extracts of *Heyuannia huangi* eggshell from Henan, Jiangxi and Guangdong Provinces, China, and Henan sediment. Unmodified BV elutes after 8 min retention time. **(B)** EICs for $563.2653 \pm 0.01/0.05$ m/z from top to bottom are for the commercial PP standard, extracts of fresh emu eggshell and *Heyuannia huangi* eggshell from Henan, Jiangxi and Guangdong Provinces, China. Unmodified PP elutes after 14 min. Note that in the dinosaur eggshell, PP is present in higher concentrations than in emu.

We found the highest amounts of PP in extracts from the initial surface decalcification of the eggshell fragments, suggesting that the PP resides in the outermost shell layer, the cuticle, as in modern eggs. On the other hand, the outermost layer of the fossils contained only minimal amounts of BV. Instead, BV was extracted successfully by decalcification of the deeper eggshell layers, suggesting a BV-based background colouration of the egg. Fossil PP and BV peaks seen in the extracted ion chromatograms (EICs) (Fig. 2) indicate the amounts of PP and BV in the final extract and were estimated to reach concentrations of > 1.0 nmol per gram eggshell.

These concentrations represent visible amounts of PP and BV. The estimate of preserved PP concentration in the specimen from Henan is 2 nmol per gram eggshell, while the estimate of preserved BV concentration in the same specimen is 6 nmol per gram eggshell. The visibility of these concentrations is indicated by comparison with modern eggshells, for example with *Sturnus vulgaris* eggs (BV amount 6.04 nmol/g, PP amount 0.24 nmol/g). While degradation behaviour and reactivity of PP and BV during fossilisation are unknown, we can safely assume much higher original pigment concentrations than what we were able to detect. The eggs of the dinosaur *Heyuannia* would thus have been perceived as strongly coloured.

The specific colour of an egg is determined by the ratio of PP to BV. PP is a reddish brown pigment, whereas a blue-greenish colour is caused by BV (Kennedy & Ververs, 1995). Due to the greater chemical stability of the macrocyclic tetrapyrrolic PP compared to the oxidised, linear tetrapyrrolic BV, the preserved pigment ratio is unlikely to reflect the original ratio.

An open nest: Porosity measurements

To further test the hypothesis of an open or semi-open nesting environment in the oviraptorid *Heyuannia*, we conducted zonal porosity measurements of a *Macroolithus yaotunensis* egg based on the protocol established for troodontid eggs (Varricchio *et al.* 2008; Fig. 1). The highest porosity in the middle part of the egg together with the nest structure (Fig. 1C) supports the hypothesis that oviraptorid dinosaurs built open nests with the blunt end exposed to the air.

We compared the result published by Mou (1992), which is $231 \text{ mg H}_2\text{O day}^{-1} \text{ Torr}^{-1}$ and was also cited by Deeming (2006), with our results. Mou (1992) assumed that the porosity is homogeneously distributed over the entire egg; however, this assumption proved incorrect for elongated eggs (Varricchio *et al.*, 2013). In the Mou's study, several pieces from the middle part of egg were taken for porosity estimation without considering the heterogeneous porosity

distribution. Our results show that the highest porosity is found in the middle zones, especially in zone 3, which is mainly covered by linearituberculate ornamentation (Fig. 3). The total volume of our egg model is 260.3 cm^3 . The egg's weight was estimated as 276 g by applying an average avian egg density of 1.06 g/cm^3 (Paganelli, Olszowka & Ar, 1974). The $G_{\text{H}_2\text{O}}$ of an avian egg of equivalent weight is $99.3 \text{ mg H}_2\text{O day}^{-1} \text{ Torr}^{-1}$ according to the regression equation proposed by Jackson *et al.* (2008). This value is slightly lower than our result ($108.66 \text{ mg H}_2\text{O day}^{-1} \text{ Torr}^{-1}$). Hence, the results of Mou (1992) overestimate water vapour conductance mainly because he applied the porosity of the middle part to the whole egg.

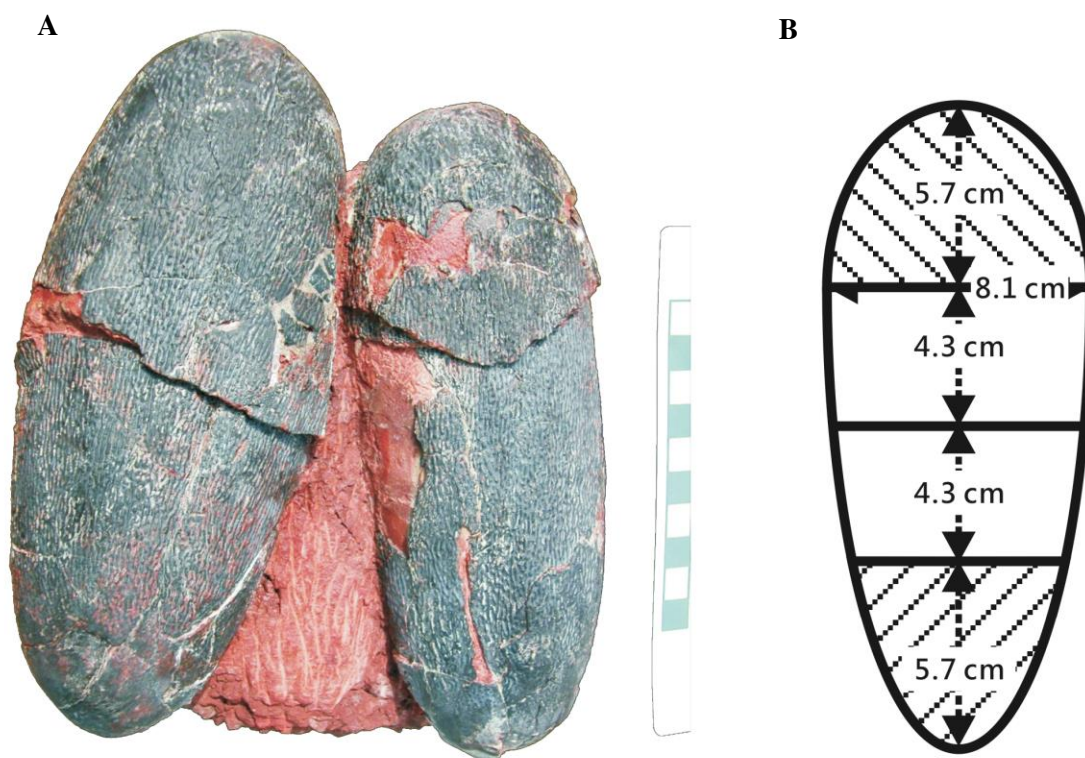


Figure 3 (A) Pair of *Heyuannia* eggs (NMNS CYN-2004-DINO-05), egg form genus *Macroolithus yaotunensis*, used for porosity measurements and calculations of water vapour conductance. One piece of eggshell from each of the four zones of the right egg (NMNS CYN-2004-DINO-05/I) was processed into a tangential thin section for porosity measurement under a polarised light microscope. Scale bar equals 10 cm. **(B)** Double half-prolate spheroids model applied to the egg to estimate the surface areas of the four zones for modelling of the egg.

Cuticle Preservation

Cuticle preservation in the *Heyuannia* eggshell is supported by microscopic observations of apatite crystals in the uppermost eggshell layer and a visual boundary between the putative cuticle and the lower crystalline zone (Yang *et al.*, 2015). Assuming a PP-storing cuticle on top of a BV-containing crystallite matrix, a two-tone eggshell colouration or even PP maculation may have been present in *Heyuannia*. Maculation is produced by the heterogeneous deposition of PP in the cuticle. Thus, while the precise colour of the dinosaur eggs cannot be reliably reconstructed, it likely would have been olive green in immaculate eggs or speckled brown on a bluish background if the eggs were maculated. However, we can assume that the overall colour impression of the *Heyuannia* eggshell ranged from blue to green.

Discussion

Such a blue-greenish eggshell colouration confers selective advantages in an open nesting environment. The phylogenetically earliest evidence of open nesting is found in the most bird-like theropods, the maniraptoran dinosaurs (Fig. 4). All other dinosaurs had retained the primitive amniote pattern of complete burial of the clutch in the substrate. Among the most derived non-avian maniraptoran dinosaurs, at least partially open nesting has been documented in the troodontid *Troodon formosus* (Varricchio *et al.*, 1997; Varricchio *et al.*, 2013). The nesting mode of the less derived oviraptorids *Oviraptor* and *Citipati* remains controversial (Norell *et al.*, 1995; Dong & Currie, 1996). Furthermore, it has been documented that multiple ovipositions would have been required to lay an entire clutch of oviraptorid eggs such as *Macroolithus yaotunensis* (Sato *et al.*, 2005), implying an open nest. Mesozoic bird clutches and eggshell document open nesting as well (Mikhailov, Bray & Hirsch, 1996; Salvador & Fiorelli, 2011; Fernandez *et al.*, 2013; Kurochkin, Chatterjee & Mikhailov, 2013).

With the evolution of an open or partially open nest (Fig. 4), camouflaging egg colouration would be positively selected for because white, uncoloured eggs are easily spotted against a darker background by visually guided predators. We thus assume that eggshell colouration evolved in parallel with open or semi-open nesting behaviour. To provide effective egg crypsis in an open nesting environment, eggshell colour has to match the colour of the nesting background (Hewitson, 1864).

This camouflage would have afforded the parental animals more foraging opportunities away from the nest because there was no need to continuously brood or guard the eggs. Eggshell colouration may thus have increased reproductive success despite the need for *de novo* PP and BV synthesis in the shell gland wall (Wang *et al.*, 2009). Furthermore, blue-greenish eggshell colouration in recent birds often correlates with additional social adaptations (Morales, Torres & Velando, 2010), e.g., intensive parental investment like pair matching in nest care, or, in the most extreme case, paternal care like in emus (Coddington & Cockburn, 1995). Thus, the blue-greenish pigmentation reported here for oviraptorid eggs lends support to the hypothesis of paternal care in dinosaurs (Varricchio *et al.*, 2008). BV-coloured blue or green eggs can further be linked to the sexual signalling hypothesis (SSH), which posits that the state of health of a female bird is indicated by egg colouration intensity. Colouration intensity reflects the amount of BV in the eggshell, which thus is dispensable for the maternal metabolism (Moreno & Osorno, 2003). Intensively blue-greenish coloured eggs motivate the males to provide more intensive paternal investment, which leads to increased fitness of the offspring (Moreno & Osorno, 2003).

Both BV and PP take part in the haem metabolism. While PP is synthesized as a haem precursor, BV is formed as an oxidation product of haem degradation. The mechanisms responsible for pigment incorporation into eggshell and regulation of pigment secretion are not completely understood, but *de novo* synthesis based on the same precursor compound for both PP and BV in the shell gland seems most likely (Wang *et al.*, 2009). On a physiological level, such pigment incorporation implies that the reproductive biology of maniraptoran dinosaurs had more in common with modern birds than previously believed. Pigment incorporation, especially of BV, into the crystalline matrix implies a layered formation of the eggshell and a similar functional anatomy and physiology of the oviduct, or at least of the shell gland.

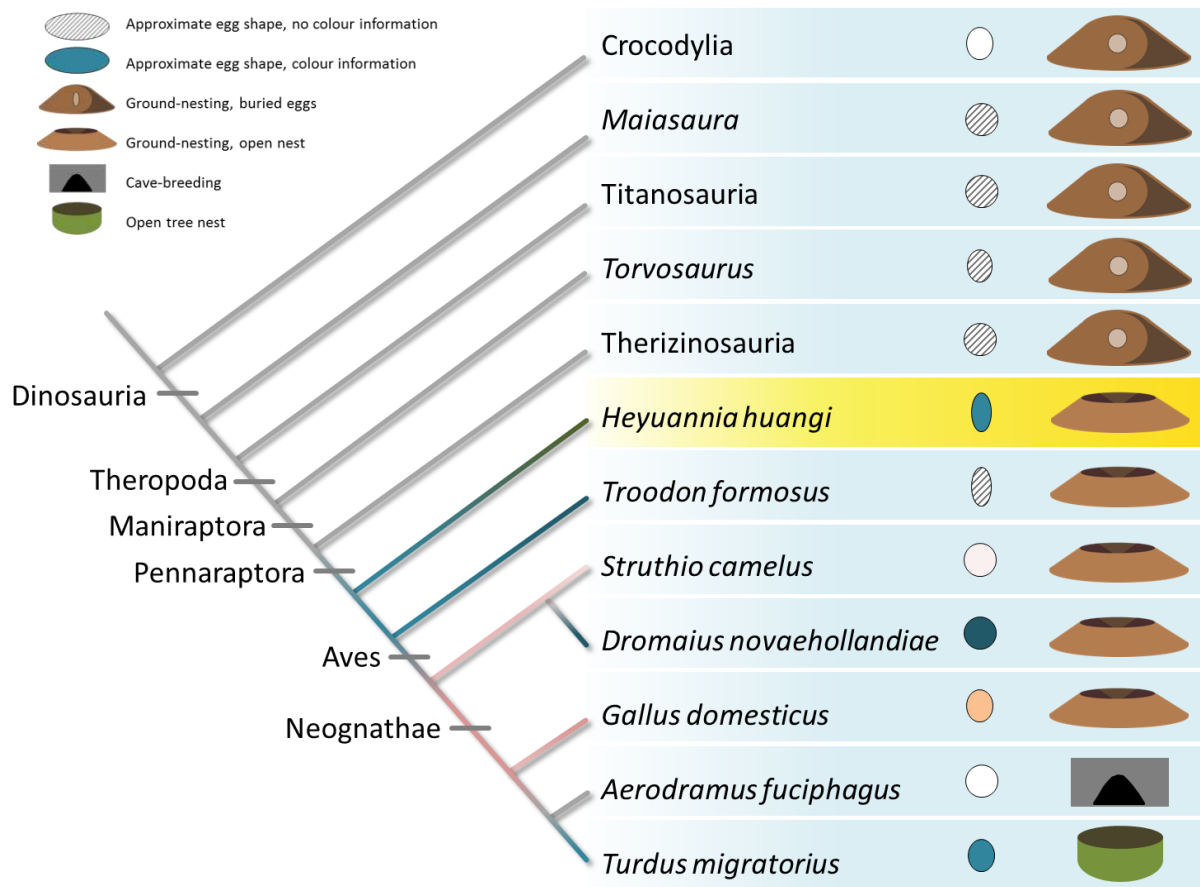


Figure 3 Evolution of egg colouration, egg shape, and nesting type in archosaurs, including birds. Fully calcified eggshell evolved in Dinosauria and is documented by successive outgroups to derived maniraptora, i.e., *Maiasaura*, Titanosauria, *Torvosaurus* and Therizinosauria. Porosity data and clutch structure indicate a fully buried nesting type for these dinosaur taxa. Oviraptorid dinosaurs, here represented by *Heyuannia huangi*, built at least partially open nests, concurrent with the phylogenetically most basal appearance of PP and BV in dinosaur eggshell. Phylogenetic inference, porosity data, and clutch structure suggest that nesting behaviour of *Troodon formosus* was similar to *Heyuannia*, and thus that *Troodon* also had coloured eggs. In modern birds, eggshell pigmentation depends on the nesting microenvironment. Presence and kind of eggshell pigment is represented by the colour of the branch.

Conclusions

The discovery of the tetrapyrrolic pigments PP and BV in fossil eggshell broadens the scope of molecular preservation in dinosaur fossils. Chemically stable, relatively small biological molecules such as PP and BV may be protected from degradation over millions of years in a carbonate biomineral matrix, such as eggshell, despite an oxidative chemical milieu in the embedding sediment. Similar biomolecule preservation should also be searched for in tooth enamel and bone mineral. Beyond ancient DNA and protein preservation in dinosaur fossils, we note that dinosaur metabolites invite further study because they can be easily detected by standard mass spectrometry and offer a new perspective on the biology of extinct taxa.

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