Egg pigmentation probably has an early Archosaurian origin

Matthew D. Shawkey¹* & Liliana D'Alba¹

ARISING FROM J. Wiemann et al. Nature https://doi.org/10.1038/s41586-018-0646-5 (2019)

Colours have crucial roles in the lives of organisms, from camouflage to mate and pollinator attraction. Although the colours of animals known only from fossils were once thought to be unknowable, recent advances have enabled some to be reconstructed, with important implications for their (Palaeo) ecology and evolution¹. A recent paper² used Raman spectroscopy to show evidence of preservation of the two colour-producing pigments in avian eggs (biliverdin and protoporphyrin IX) in fossil dinosaur eggs. The authors used these data to infer the colours of these eggs and, based on their presence and absence in extinct and extant clades, to suggest that colourful eggs had a single evolutionary origin in the Eumaniraptora, or bird-like dinosaurs². This is clearly an exciting finding, and the potential detection of these pigments could indeed enable considerable advances in our understanding of the evolution of egg pigmentation. However, the presence of these two pigments in white avian eggs and crocodile (non-eumaniraptoran) eggs draws their conclusions into question.

The presence of biliverdin and/or protoporphyrin IX does not inevitably mean that an egg is coloured. Both pigments are widespread, and biliverdin can be found in (among many other places) the blood of fish³ and the yolk of frog eggs⁴. When found in eggshells, they can cause brown or blue-green coloration, but many white avian eggs (for example, of chickens (Gallus gallus)⁵, white storks (Ciconia ciconia), wood pigeons (Columba palumbus)⁶ and swifts (Apus apus)⁷) contain the pigments in low levels. Indeed, an extensive survey concluded that pigment-free white eggs are rare⁶. An egg containing these pigments could thus either be coloured or not, depending on concentration. Raman data on organic signals, as implemented², can only be interpreted in relative terms, and thus give no information on absolute concentration that in turn would inform on coloration. Furthermore, none of the fossil eggs (with the possible exception of the previously reported Heyuannia⁸) shows any evidence of preserved colour. A recent study⁹ suggested that coloured eggs become brown during diagenesis, but did not examine the fate of white eggs. Without such data, it is not possible to infer with certainty that a fossil egg was coloured. Nevertheless, all eggs with any potential signal of preserved pigment were classified as fully coloured and/or colour patterned².

Apparent patterning of these pigments into maculation and spotting, as well as distribution in the shell cross-section reminiscent of those in modern bird eggs could be argued to further support the hypothesis that fossil eggs were coloured. However, these analyses were only performed on fossil eggs already classified as pigmented, making this argument circular. Fossil eggs classified as unpigmented, or white eggs with small amounts of pigment, lacking maculation patterns under the Raman surface-mapping analyses would obviously support the hypothesis that these eggs were coloured. But these critical negative controls were not done. The authors' argument for this omission is that Raman imaging data would only show patterns of background noise. But the same may be true for eggs classified as pigmented. This possibility is particularly likely given that (1) the wavelengths chosen for Raman imaging analysis $(1,160 \text{ cm}^{-1} \text{ and } 1,350 \text{ cm}^{-1})$ are outside of the diagnostic Raman 'fingerprint' region, and (2) all 'unpigmented' fossil eggs show strong peaks at $1,160 \text{ cm}^{-1}$, whereas most 'pigmented' eggs have valleys, rather than peaks, at $1,350 \text{ cm}^{-1}$ (see Extended Data Fig. 1). Thus, these imaging data, although intriguing, provide no further support for colouring of fossil eggs. It could be argued that the low concentrations of pigments found in white eggs are unlikely to be preserved, but this needs to be tested directly and was not done in a related recent study⁹.

Even if we do not definitively know that dinosaur eggs were coloured, it is still interesting and relevant that they may have been physiologically capable of depositing pigments in their eggs. However, the authors' titular conclusion, that this capability had a single origin at the base of eumaniraptorans, is challenged both by the above and even more directly by the recent detection of protoporphyrin in white Siamese crocodile eggs¹⁰ (Fig. 1). Crocodiles are phylogenetically distant from eumaniraptorans, and a new maximum likelihood ancestral state reconstruction that includes them indicates a 67% probability of egg



Fig. 1 | **Maximum likelihood ancestral reconstruction tree showing probabilities of pigment deposition (green sections of nodes) in eggs of archosaurs.** Species and pigment presence data are the same as Wiemann et al.², with the exception of *Crocodylus siamenensis*, which is taken from Mikšík et al.¹⁰.

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pigmentation at the base of archosaurs (Fig. 1). Whether extinct crocodilian eggs were coloured or not awaits more quantitative and thorough sampling, but this finding has important implications for the evolution of egg pigmentation. Perhaps deposition of protoporphyrin arose concurrently with hard-shelled eggs, to strengthen shells or serve some other crucial function. Colour (and perhaps biliverdin deposition) may have then arisen later.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

All data were taken from Fig. 1 of Wiemann et al.² with the exception of the data point on Siamese crocodiles, which was based on data from Mikšík et al.¹⁰.

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Author contributions M.D.S. and L.D. conceived the study, L.D. analysed the data, M.D.S. wrote the manuscript, and both authors edited the manuscript.

Competing interests The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41586-019-1282-4.

Supplementary information is available for this paper at https://doi.org/10.1038/s41586-019-1282-4.

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Extended Data Fig. 1 | Figure 1 from Wiemann et al.², with red lines indicating location of wavenumbers analysed using Raman imaging. Both white and coloured eggs have peaks at 1,166 cm⁻¹, and coloured eggs have valleys at 1,350 cm⁻¹.

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Software and code

olicy information about <u>availability of computer code</u>					
Data collection	Data were collected using RStudio Version 1.1.453				
Data analysis	All data were analyzed using the packages "ape" (Paradis et al. 2004) and "phytools" (Revell 2012) implemented in the software RStudio Version 1.1.453 (Team, R., 2015. RStudio: integrated development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com). - Paradis, E., Claude, J. and Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics, 20(2) pp.289-290. - Revell, L.J., 2012. phytools: an R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution, 3(2), pp.217-223.				

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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All data are taken from Fig. 1 of the criticized paper (Wiemann et al. (2018)) with the exception of one point (Crocodylus siamenensis), which was scored as positive based on data in Mikšík et al. (2018). References:

Mikšík, I., Paradis, S., Eckhardt, A. & Sedmera, D. Analysis of Siamese Crocodile (Crocodylus siamensis) Eggshell Proteome. Protein J. 37, 21-37 (2018).

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Study description	Our comment includes an ancestral reconstruction of egg pigmentation based on the data in Fig. 1 of Wiemann et al. (2018) and one additional data point taken from Mikšík et al. 2018.			
Research sample	All data are taken from Fig. 1 of the criticized paper (Wiemann et al. (2018)) with the exception of one point (Crocodylus siamenensis), which was scored as positive based on data in Mikšík et al. (2018).			
Sampling strategy	n/a			
Data collection	All data are taken from Fig. 1 of the criticized paper (Wiemann et al. (2018)) with the exception of one point (Crocodylus siamenensis), which was scored as positive based on data in Mikšík et al. (2018). All data scored by D'Alba.			
Timing and spatial scale	n/a			
Data exclusions	No data were excluded from analysis			
Reproducibility	n/a			
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Reply to: Egg pigmentation probably has an Archosaurian origin

Jasmina Wiemann¹*, Tzu-Ruei Yang^{2,3,5} & Mark A. Norell⁴

REPLYING TO M. D. Shawkey & L. D'Alba Nature https://doi.org/10.1038/s41586-019-1282-4 (2019)

In the accompanying Comment¹, Shawkey and D'Alba suggest that egg pigmentation probably has a single archosaur origin in response to our recent discovery of a single evolutionary origin of dinosaur egg colour². In our study², we analysed the preservation of blue (biliverdin) and red (protoporphyrin) colour pigment in fossilized eggshells of nonavian and avian dinosaurs based on Raman spectroscopy point analyses, protoporphyrin surface maps, and pigment depth profiles. Ornithischian, sauropod and North American troodontid eggs showed no detectable evidence for egg colour pigments, whereas almost all nonavian and avian eumaniraptoran eggs yielded pigment signals². Mapping these results onto an archosaur consensus phylogeny and performing a parsimony-based ancestral state reconstruction revealed a single evolutionary origin of egg colour in (the stem of) eumaniraptoran dinosaurs². Shawkey and D'Alba¹ dispute this conclusion based on several observations: that the presence of eggshell pigments does not necessarily indicate egg colour; that our Raman method cannot quantify pigments and therefore cannot distinguish between coloured and white eggs; that fossil eggs do not show visible evidence of colour; and that we excluded a potentially pigmented yet white Crocodylus siamensis eggshell in our ancestral state reconstruction of archosaur egg colour.

Certain eggs are white even if traces of biliverdin and/or protoporphyrin are present³—the intensity of colour reflects the concentration of eggshell pigment⁴. Therefore, as Shawkey and D'Alba¹ point out, dinosaur eggs that yield a pigment signal^{2,5} may have varied from white to intensely coloured (as we discussed and imaged in figure 2a of our Letter²). Raman spectroscopy cannot be used to quantify pigment concentrations^{2,6}, but a crucial amount is necessary to elicit a Raman signal, and the lower spectroscopic detection limit provides information on the concentration present⁶. Exploiting this requirement, we evaluated whether Raman spectroscopy can distinguish between traces of pigment in white eggs and increased amounts in coloured eggs. Additional white and lightly coloured extant eggs (Extended Data Table 1) were analysed under previously published conditions², and the resulting spectra (Fig. 1a) were subjected to two different types of cluster analysis (Fig. 1b). White eggs did not yield a pigment signal (Fig. 1a) because pigment traces did not exceed the detection limit for our Ramanbased approach⁶. All lightly coloured eggs, in contrast, produced a pigment signal (Fig. 1a). Both analyses clearly separated a cluster of white eggs from one of lightly coloured eggs (Fig. 1b). We infer that the fossilized dinosaur eggs that yielded a pigment signal² were coloured, as their pigment concentrations exceeded the spectroscopic detection limit⁶.

Shawkey and D'Alba¹ claim that our detection of eggshell spots and speckles using Raman-surface mapping, and our demonstration of pigment depth profiles through vertical sections of eggshell, provide no further support for colour in fossil dinosaur eggs¹. Protoporphyrin causes spots and speckles in eggs^{3,4}, and was mapped

only on fossil eggshell that yielded a protoporphyrin signal²; otherwise, only the nano-differential spectral background noise would have been detected^{2,6}. Both pigments were targeted for the depth profiles². Analysing only samples with a protoporphyrin and/or biliverdin signal present was determined by a technical constraint⁶, rather than a sampling decision that we made². The wavelengths chosen to map out protoporphyrin (1,350 cm⁻¹) and protoporphyrin and biliverdin $(1,160 \text{ cm}^{-1})$ were selected to avoid the pigment fingerprint region affected by the signal produced by protein fossilization products (PFPs) and by background fluorescence associated with pigment peaks localized on top of the rather broad PFP spectral shoulder². Figure 1c shows the pigment peak loadings in a chemospace principal component analysis including all the eggshells²; the peak loadings (including background noise) represent their significance for pigment concentration $(PC1)^2$ and identification $(PC2)^2$. The two chosen peaks are diagnostic of functional groups unique to protoporphyrin $(1,350 \text{ cm}^{-1})^{2,7}$ and tetrapyrrol pigments $(1,160 \text{ cm}^{-1})^{2,7}$; they are not affected by a PFP background (Fig. 1c), and represent the most reliable indicator of pigment concentrations and types² (Fig. 1c). Our protoporphyrin maps and pigment depth profiles do not represent noise, but provide evidence of original egg spots and the distribution of pigment across fossil eggshell² (Extended Data Fig. 1). However, it is not possible to reconstruct the original colour of fossil dinosaur eggs in detail, as pigments are lost and/or transformed during diagenesis².

Diagenetic transformation affects not only eggshell pigments^{2.5} but also the eggshell organic matrix⁸, which is originally composed of mucopolysaccharides. These compounds, like all proteinaceous matter in hard tissues exposed to oxidative conditions, form N-heterocyclic polymers, which are responsible for the brown discolouration of eggs during fossilization⁸. This fossilization process affects proteinaceous material in every eggshell (and other vertebrate hard tissue)⁸, regardless of the presence or absence of pigments⁸. The more abundant dark brown protein fossilization products overprint the weak colour generated by minor amounts of unaltered eggshell pigment preserved^{2.8}. The preservation of original colour in fossil dinosaur eggs is practically impossible^{2,8}, and chemical evidence such as that presented in our Letter² is necessary to determine whether a fossil egg was once coloured or not.

Our parsimony-based ancestral state reconstruction² was based on the assumption that detectable pigment reflects originally coloured eggs (as demonstrated in Fig. 1a–c); we coded taxa as either uncoloured (0) or coloured (1). Our analysis revealed a single evolutionary origin of egg colour in eumaniraptoran dinosaurs. Shawkey and D'Alba¹ coded protoporphyrin traces detected by high sensitivity mass spectrometry in a white *Crocodylus siamensis* (crocodile) eggshell⁹ as equivalent to 'coloured' in our fossil eggs as detected by Raman spectroscopy². They added this taxon to our published dataset and ran a maximum likelihood-based ancestral state reconstruction⁹. However, traces of

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Fig. 1 | Raman spectroscopic and statistical analysis of an eggshell pigmentation versus an egg colour signal. a, Raman spectra (n = 5)over the wavelength range 300–3,000 cm $^{-1}\,\pm$ 2 cm^{-1} (6 accumulations (technical replicates), 20-s exposure) of Geoclemys hamiltonii (turtle), Crocodylus siamensis (Siamese crocodile), Struthio camelus (ostrich), Rhea americana (rhea), and white Gallus domesticus (chicken) eggshell (following our previous Letter²). All spectra are baselined and normalized². The colour below the spectral function represents the absence of a pigment signal (grey), the presence of protoporphyrin (orange), or biliverdin (blue). Pigment peak positions are depicted as transparently coloured dots: orange dots indicate a protoporphyrin band, blue dots a biliverdin peak. Increased saturation of dots represents the presence of a pigment peak in the spectrum below. b, Cluster analyses for extant coloured (n = 4) and uncoloured/white eggs (n = 4) based on Raman spectra. Eggshell spectra are clustered by UPGMA hierarchical clustering in the top topology, and by neighbour joining clustering in the bottom topology. The taxa are those we investigated in our Letter², with the additions in **a**. **c**, Loadings plots of all pigment peaks (n = 20) based on a principal component (PC) analysis of all eggshells in our Letter² and added in **a**. Orange bars indicate loadings on PC1 separating samples based on pigment concentrations; blue bars indicate loadings on PC2 separating samples based on pigment types. Brown asterisks label pigment peaks that are affected by PFP baseline noise and fluorescence. The blue asterisk labels the 1,128 $\rm cm^{-1}\pm 2~\rm cm^{-1}$ peak that is affected by the adjacent eggshell carbonate peak. d, Parsimony-based ancestral state reconstruction of egg colour (coded as: 0, uncoloured; 1, coloured) across an Archosauromorpha consensus phylogeny² based on the Raman spectroscopic characterization in our Letter², and the additions in **a** (n = 23). Grey dots represent uncoloured/ white eggs; orange-blue dots represent visibly coloured eggs. Egg icons on the terminal branches represent known egg colour and pattern for extant species, and the reconstructed egg colour and patterns for fossil eggs. A single evolutionary origin of egg colour is found for eumaniraptoran dinosaurs. AU, arbitrary units; br, brown eggshell; CH, Chinese; F, French; M, Mongolian; NA, North American; SA, South American; wh, white eggshell.

protoporphyrin in a white eggshell⁹ do not equate to 'coloured' in our dataset² (and such traces could also derive from haem-containing chorioallantoic vascularity protruding into the basalmost layers of the *Crocodylus siamensis* eggshell, as shown in Extended Data Fig. 1). We analysed eggshells of *Crocodylus siamensis* using Raman spectroscopy under identical conditions to those used for our samples² (Fig. 1a). On this basis, we recovered *Crocodylus siamensis* eggshell as 'uncoloured' (Fig. 1b). We ran a new parsimony-based ancestral state reconstruction based on our original archosaur dataset², together with white eggs of *Geoclemys* (turtle), *Crocodylus siamensis* eggs, and various birds (Fig. 1d); this yielded the same result as before². (Shawkey and D'Alba¹ used maximum likelihood even though the dataset is rather small

owing to biases in the fossil record, and includes non-sequence characters: this method is commonly outperformed by parsimony-based and Bayesian inferences¹⁰.)

The ancestral state reconstruction of Shawkey and D'Alba¹ reveals a single evolutionary origin of 'eggshell pigmentation' (problematic owing to the non-synonymous codings) in archosaurs¹, and they infer that eggshell pigmentation preceded egg colour¹. Our revised parsimony-based approach yields an 'uncoloured' ancestral egg in archosauromorphs, archosaurs, dinosaurs and saurischians (Fig. 1d). Even with the inclusion of various white-shelled avian eggs, we confirm a single evolutionary origin of egg colour in eumaniraptorans (Fig. 1d).

Eggshell pigmentation may represent a more basal trait than true egg colour, but the Raman data on fossil egg colour^{1,2} cannot address this question. The fossil and modern eggshells in our study are reliably coded as coloured or uncoloured. Nonavian eumaniraptoran dinosaur eggs came in various colours and patterns². Incorporating these data into a phylogenetic analysis requires standardized datasets^{2,8}.

Data availability

All data supporting the findings of this study are available within the paper (Fig. 1), and its Extended Data (Extended Data Fig. 1, Extended Data Table 1).

- Shawkey, M. D. & D'Alba, L. Egg pigmentation probably has an Archosaur origin. *Nature* https://doi.org/10.1038/s41586-019-1282-4 (2019). 1.
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Author contributions J.W., T.-R.Y. and M.A.N. discussed Shawkey and D'Alba's concerns. J.W. designed and performed the experiments, analysed the data, and created the figure. J.W. wrote the manuscript, which was reviewed by all authors.

Competing interests The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41586-019-1283-3

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Extended Data Fig. 1 | *Crocodylus siamensis* outer and inner eggshell surfaces. **a**, The outer eggshell surface (n = 1), which contains the highest pigment concentrations in eumaniraptorans is uncoloured/ white. **b**, **c**, The inner eggshell surface (n = 2) shows a reddish tint (**b**), which reflects chorioallantoic tissues and vascularity (**c**) protruding into the innermost layers of the eggshell. The inner eggshell surface in **b** is

manually cleaned of adjacent chorioallantoic tissue, whereas the inner eggshell surface in **c** is left untreated. Chorioallantoic tissues (**c**) are commonly vascularized, and therefore saturated in blood-derived haem, which represents, when dechelated, protoporphyrin. In this case, traces of protoporphyrin recovered from *Crocodylus siamensis* eggshell would not be homologous with eumaniraptoran eggshell protoporphyrin.

Extended Data Table 1	Information on added taxa (n :	= 4), catalogue numbers, ages	localities, and egg colours
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Assigned Taxon	Catalogue No.	Age	Locality	Colour
Geoclemys hamiltonii	uncatalogued	Extant	Unknown	white
Crocodylus siamensis	YPM HERR 018977	Extant	Myanmar, South East Asia	white
Struthio camelus	YPM ORN 141976	Extant	Egypt, Africa	beige
Gallus domesticus	uncatalogued	Extant	New Haven, CT, USA	white