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Authors	McNamara, Maria E.;Orr, Patrick J.;Kearns, Stuart L.;Alcalá, Luis;Anadón, Pere;Peñalver, Enrique
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Reconstructing carotenoid-based and structural coloration in fossil skin

Maria E. McNamara[1]*, Patrick J. Orr[2], Stuart L. Kearns[3], Luis Alcalá[4], Pere Anadón[5] and Enrique Peñalver[6]

[1] School of Biological, Earth and Environmental Sciences, University College Cork, Cork, Ireland (maria.mcnamara@ucc.ie)

[2]UCD School of Earth Sciences, University College Dublin, Belfield, Dublin 4, Ireland (maria.mcnamara@ucc.ie)

[3]School of Earth Sciences, University of Bristol, Queen's Road, Bristol BS8 1RJ, UK (stuart.kearns@bristol.ac.uk)

[4]Fundación Conjunto Paleontológico de Teruel-Dinópolis, Avda. Sagunto s/n, E-44002 Teruel, Aragón, Spain (alcala@dinopolis.com)

[5]Consejo Superior de Investigaciones Científicas, Institut de Ciències de la Terra 'Jaume Almera', Lluís Solé i Sabarís s/n E-08028, Barcelona, Spain (panadon@ija.csic.es)

[6]Museo Geominero, Instituto Geológico y Minero de España, C/ Ríos Rosas, 23, E-28003, Madrid, Spain (e.penalver@igme.es)

*Corresponding author. Email: maria.mcnamara@ucc.ie

Running title: fossil skin colour

Summary

Evidence of original coloration in fossils provides insights into the visual communication strategies used by ancient animals and the functional evolution of coloration over time [1-7]. Hitherto, all reconstructions of the colours of the plumage of fossil birds and feathered dinosaurs and reptile integument have been of melanin-based coloration [1-6]. Extant animals also use other mechanisms for producing colour [8] but these have not been identified in fossils. Here we report the first examples of carotenoid-based coloration in the fossil record, and of structural coloration in fossil integument. The fossil skin, from a 10 Ma colubrid snake from the Late Miocene Libros Lagerstätte (Teruel, Spain) [9, 10], preserves dermal pigment cells (chromatophores) – xanthophores, iridophores and melanophores – in calcium phosphate. Comparison with chromatophore abundance and position in extant reptiles [11-15] indicates that the fossil snake was pale-coloured in ventral regions; dorsal and lateral regions were green with brown-black and yellow-green transverse blotches. Such coloration most likely functioned in substrate matching and intraspecific signalling. Skin replicated in authigenic minerals is not uncommon in exceptionally preserved fossils [16, 17] and dermal pigment cells generate coloration in numerous reptile, amphibian and fish taxa today [18]. Our discovery thus represents a new means by which to reconstruct the original coloration of exceptionally preserved fossil vertebrates.

Results

The integument of vertebrates is a complex system with important functions in homeostasis, sensory reception and, via its coloration, visual signaling [18]. Recent studies have reconstructed the melanin-based [2-6] plumage colours of feathered dinosaurs and birds on the basis of preserved melanosomes [2-5] and feather chemistry as revealed by X-ray mapping [6]. Melanin-based pigmentation, however, is only one of several pigment-based mechanisms for producing colour [18]; evidence of other pigments has not been reported in fossil vertebrates. Examples of fossilized vertebrate skin are not uncommon and have yielded insights into the biology [19-23] of non-feathered dinosaurs and other fossil reptiles, but evidence of original coloration and patterning in fossil skin has, until now, been limited to rare instances of subtle monotonal patterning [5, 19]. Here we report the discovery of intact dermal chromatophores, the pigment cells responsible for coloration and patterning, in a 10 million year old colubrid snake. We use scanning electron microscopy to analyze the relative abundance and vertical position of the chromatophores from different body regions. By comparing these data to those from extant snakes, we reconstruct the original integumentary colour patterns of the fossil snake and reveal their ecological functions.

The fossil snake (Museo Nacional de Ciencias Naturales (CSIC) MNCN 66503) occurs within Vallesian (11.2–8.7 Ma) oil shales of the Libros Gypsum lacustrine sequence [9, 24], which outcrops 25 km SE of Teruel city, NE Spain (40º07’38”N 1º12’1”W). The specimen was recovered during mining operations in the early 20th century; stratigraphic data are not available. It is in lateral aspect and lacks a cranium (Figure 1A), and is assigned to the Colubridae. A more precise taxonomic determination is not possible in the absence of a cranium. The specimen is on permanent display at Dinópolis palaeontological museum in Teruel, Spain.

Ultrastructure and chemistry of the fossil snake skin

The fossil skin extends from the vertebrae to the ventral termini of the ribs (Figures 1A, 1B); overlapping scales are evident (Figure 1B). Scanning electron microscopy reveals that the fossil skin, as with many fossilized decay-prone tissues [25], is replicated in calcium phosphate. It exhibits a tripartite division into a thin (6–9 μm thick) outer layer that is structureless and nanocrystalline, a thicker (15–25 μm thick) central layer that contains mineralized fibres and oblate to spheroidal bodies, and a thick (100–180 μm) lowermost layer that comprises a plywood-like array of fibres (Figures 1C, 1D). These fossil skin layers correspond to the main layers of the skin in extant reptiles [8, 18], i.e. the epidermis (comprised of keratinized cells), upper dermal stratum spongiosum (loosely packed collagen fibres and chromatophores (pigment cells)) and lower dermal stratum compactum (a dense orthogonal array of collagen fibres). The stratum compactum in the fossil snake is locally underlain by a thin (8–13 μm thick) structureless layer (Figure 1C) that represents the remains of the basement membrane which in extant reptiles separates the skin from the underlying hypodermis [8, 18].

The most striking features of the stratum spongiosum in the fossil snake skin are abundant oblate to spheroidal bodies, consistently located immediately below the epidermal-dermal boundary (Figures 1D–K; Figure 2). These bodies fall into three types that are differentiated on their location, size, morphology and internal fill.

Type 1 bodies occur at the top of the array; they are small (1–5 μm x 0.4–2 μm) cryptocrystalline discs (Figures 1E, 1F) that can be organized into a layer up to four discs thick (Figures 2C, 2H,

2I, Figures S1E–H). As with other features of the skin, the discs are frequently separated from the surrounding matrix by a void (Figure 1F).

These discs are underlain by Type 2 bodies, which are larger (3–8 μm long) irregular spheroids to ovoids that comprise granules of two types: small (0.15–0.4 μm) subspherical granules with irregular to rounded outlines, and larger (0.8–1.2 μm) rounded granules with smooth outlines (Figures 1G, 1J). The relative proportions of the two granule types are similar and consistent among the Type 2 bodies (smaller vesicles: 44.2 ± 4.7%; n = 38).

The Type 2 bodies are underlain by larger (8–20 μm long) ovoid features with smooth outlines, and prominent lateral processes. These Type 3 bodies contain densely packed granules with a narrow size distribution (0.18–0.3 μm) (Figures 1H, 1I, 1K).

Elemental mapping of the fossil snake skin reveals that the bodies in the stratum spongiosum and dermal collagen fibres contain elevated concentrations of S, and lower concentrations of C and P, relative to other ultrastructures in the skin (Figure 3). No other elements show spatial partitioning among the various structures in the skin.

Discussion

Interpretation of the bodies as fossil chromatophores

The bodies preserved in the stratum spongiosum of the fossil snake are unlikely to be skin glands: in extant snakes, skin glands are restricted to a pair of anal scent glands [18]. Similarly, there is no evidence that the bodies (or their internal granular fill) represent fossilized decay

bacteria [see 26]. The disc-like morphology of the Type 1 bodies is not consistent with that of bacteria. The Type 2 and 3 bodies are too large to represent bacteria, which are usually 0.5–2 µm long [27]. Fossil bacteria would be expected to infest the entire tissue during decay (including the dermis), not just specific features such as the interior of the chromatophores. Bacteria could also generate a characteristic texture whereby they pseudomorph the gross geometry of the original tissue; if replicated in calcium phosphate this is termed a microbial microfabric [28]. Further, preserved bacteria are not associated with other fossils from Libros: recent geochemical analyses reveal that microbe-like microstructures associated with fossil amphibians from Libros can be convincingly identified as preserved melanosomes [29]. The bodies preserved within the uppermost stratum spongiosum of the fossil snake skin are therefore interpreted as fossil chromatophores, which are common components of the upper stratum spongiosum in extant snakes [18]; the three types of body are interpreted as three different chromatophore types. The skin of the Libros snake is thus preserved as a substrate microfabric [28] whereby nanocrystalline calcium phosphate has faithfully replicated the ultrastructure of the tissue.

Certain pigments, including melanins, pteridines and carotenoids are known to have an affinity for metal cations [30-32]. Elevated levels of sulfur in the dermal chromatophores and collagen fibres may reflect the presence of sulfur-bearing moieties in the original tissue structures [33, 34] or the incorporation of sulfur (in the form of sulfate) into the replacement phosphate during mineralization [35]. There is no evidence, however, for partitioning of trace elements among the various chromatophores in the fossil snake skin (Figure 3). This may reflect concentrations below detection limits (< 100 ppm) or overprinting of the original trace element chemistry during the mineralization process. The fossil chromatophores are therefore interpreted on the basis of

their size, geometry and, in some examples, internal structure compared with those in extant reptiles [8, 11-15] (Figures 1E-K, Figures S1-S5). Some of the chromatophores in the fossil skin are present as external moulds; their affinities are resolved by their shape and study (at high magnification) of the surface texture of the mould (see insets in Figure S1D).

In extant reptiles, dermal melanophores are readily identified by their position at the base of the chromatophore array, their large size (10–30 μm wide), prominent lateral processes, and infill of small granules of melanin (melanosomes) with a narrow size distribution [8, 12]. Dermal melanophores typically exhibit ovoid geometries when in the contracted state (whereby melanosomes are restricted to the main body of the melanophore [36]) and have few lateral processes [36] and a low packing density (Figure 1 in [12], Figure 7 in [36], Figure 8 in [37]). The melanosomes vary in size among modern taxa (0.15–0.8 μm long x 0.25–0.5 μm wide) but for a given taxon have a small size range (Figure 2a in [11], Figure 1 in [12], Figure 3 in [13], Figure 7 in [14], Figure 1 in [15], Figure 1 in [35], Figure 5 in [37]). The Type 3 bodies in the fossil snake skin share all the main characteristics of, and are thus best interpreted as, dermal melanophores.

Iridophores are small chromatophores (usually 5–10 μm wide [12, 14, 18]) that have irregular to flattened or disc-like morphologies. They can form vertical stacks up to four cells thick [12] and can occur at the top of the chromatophore array [11, 12] or below an upper layer of xanthophores [12, 14, 15]. The Type 1 bodies in the fossil snake also have a flattened geometry and occur in stacks in some body regions; these features are consistent with an interpretation as iridophores but not as any other ultrastructural feature of the skin. The small size of the fossil iridophores (1–

5 μm) may reflect taxonomic factors (as with melanophores, above) or degradation during the fossilization process. In extant reptiles, iridophores contain angular crystalline platelets of the purines guanine, hypoxanthine or adenine [18]. These platelets are not preserved in the fossil snake, but this is not unexpected: guanine is soluble in dilute acids [38], which are typical products of decay [25].

Xanthophores in extant snakes are typically 3–10 μm long and have irregular to spheroidal or ovoid geometries [12, 18]. They have been defined as chromatophores that contain abundant granules of carotenoids and pteridines [12, 18]; others differentiate between primarily carotenoid-bearing xanthophores, and primarily pteridine-bearing erythrophores [12, 18]. The former definition is used herein. Granules of pteridines – pterinosomes – are vesicles (0.3–1 μm) with a smooth rounded surface, spherical to elongate geometry and internal concentric laminae [13] (Figure 2A in [11], Figure 10 in [12], Figure 10 in [14], Figure 5 in [37], Figure 4 in [39]). Carotenoid granules are smaller (0.15–0.45 μm) and have smooth (Figure 1 in [36]) or irregular (Figure 2 in [12]) outlines, i.e. subrounded to angular geometries [12]. The Type 2 bodies in the fossil snake occur below the iridophores and above the melanophores, and have irregular spheroidal to ovoid outlines. The internal granules fall into two discrete types: small subspherical granules with irregular outlines, and larger rounded granules with smooth outlines; these most likely correspond to fossil carotenoid and pterinosome vesicles, respectively. The similar proportions of the two granule types in the Type 2 bodies is not consistent with an interpretation as erythrophores [12]. The most parsimonious interpretation is therefore that the irregular spheroidal to ovoid chromatophores in the fossil snake represent xanthophores filled with a combination of large pterinosomes and smaller carotenoid granules.

Relating chromatophores to visible hue

In extant reptiles, the visible hue of the integument is produced by a combination of dermal chromatophores, epidermal melanocytes and epidermal diffraction gratings. In the fossil snake, the epidermis is poorly preserved and thus the former presence of epidermal melanocytes and surficial diffraction gratings cannot be determined. The contribution of these features to visible hue and patterning, however, would have been minimal [12, 18, 36, 40]. Epidermal melanocytes are not involved in creating colour patterning [12, 18]; they typically occur only in skin regions of dark brown to black hue, enhancing the effect of a thick dense layer of dermal melanophores [41]. Epidermal diffraction gratings generate weak spectral iridescence that is superimposed on colour patterns generated by dermal chromatophores, which are the primary contributors to visible hue [40].

Our interpretation of the original colour of the fossil snake is therefore based entirely on the dermal chromatophores. Samples of skin from different body regions of the fossil snake exhibit systematic differences in the type, and relative abundance, of chromatophores (Figures 1C, 2, Figures S1-S5; Table S1); these differences are statistically significant ($\chi^2 = 42.6$; df = 3, 5; $\chi^2_8 = 20.09$, $p < 0.01$). There is no evidence that this variation reflects taphonomic factors. The fidelity of preservation of the chromatophores does not vary with chromatophore abundance, i.e. the chromatophores are equally well preserved (in terms of definition of external margins and nature of internal fill) where rare and abundant (compare Figures 2A and 2F). Further, the overall fidelity of preservation of the skin does not vary among different body regions, e.g. collagen fibres are preserved with equal fidelity throughout. There is thus no evidence that certain regions

of the skin were subjected to more extensive decay than others and that the preserved abundance of melanosomes is a taphonomic artefact.

Synthesis of published literature on reptile chromatophores (Table S2) and primary observations (Figure S4) reveal that in extant reptiles, specific combinations of chromatophores correspond to different hues (Table S2). The colours of the fossil snake can thus be reconstructed based on the relative abundance and stratigraphy of the chromatophores. In extant reptiles, iridophores scatter light from crystals of guanine and other purines through thin film interference [8]. Xanthophores are capable of producing a range of yellow, orange and red hues, depending on the relative proportions of carotenoid granules and pterinosomes present [12, 18]. Xanthophores with equal amounts of both granule types – as in the fossils – produce yellowish hues [12]. Melanophores produce brown to black hues as their melanosomes absorb most, or all, wavelengths of light [12].

Samples 1, 2, 3, 5 and 7 are from lateral body regions; 4 is dorsal, and 6, ventral. Patterning in snakes is typically repeated along the length of the body [8] and thus our colour reconstruction, based on comprehensive sampling of one body region, can be extrapolated to the remainder. There is no evidence that the fossil snake skin exhibited white, red, blue, or grey hues. All skin regions studied preserve chromatophores, eliminating the possibility of white hues [12]. There is no evidence that any xanthophores comprised primarily pterinosomes, eliminating the possibility of red hues [12, 18, 41]. No skin regions exhibited only iridophores and melanophores, eliminating the possibility of structural blue [11], structural green (Figure S4) and grey hues. Iridophores can reflect specific, or all, visible wavelengths depending on the thickness and

organization of the internal purine platelets [8]. Given that the latter are not preserved in the fossils, we cannot comment on their potential contribution to the original colour.

Iridophores and xanthophores are abundant and melanophores common in two samples from lateral body regions (samples 5 (Figure 1E) and 7 (Figures 2A, S2)). In extant reptiles, similar chromatophore architectures (in particular, the presence of carotenoid-bearing xanthophores and the position of iridophores at the top of the chromatophore array) are associated with green hues [11]. In other lateral body regions (sample 2) melanophores are more abundant and iridophores and xanthophores, less abundant (Figures 2B, S2), suggesting darker, less saturated green hues. Skin samples from other lateral body regions (sample 1) exhibit stacks of iridophores up to four cells thick (Figure 2C), indicating brighter green hues: layering of iridophores markedly increases integument albedo [42]. Conversely, other lateral regions (sample 3) exhibit abundant melanophores; xanthophores are common and iridophores, rare to absent (Figure 2D, S3), characteristic of dark brown/black tones [11]. In dorsal regions (sample 4) xanthophores are abundant, iridophores, common and melanophores, rare (Figures 2E, S3), indicating yellowish to pale brown hues [18]. In ventral regions (sample 6), iridophores and xanthophores are abundant, and melanophores, rare to absent (Figures 2F, S1), corresponding to cream-coloured hues [40].

The fossil snake can therefore be reconstructed as green with brown/black blotches on its dorsal and lateral surfaces, and pale ventrally (Figure 4). Similar coloration characterizes some extant Colubrid snakes, e.g. *Nerodia floridana* and *Dispholidus typus*.

Broader implications

Green coloration is an effective adaptation for substrate matching in foliage [43]. This cryptic visual signal was enhanced by two pattern elements. The superimposition of brown/black tones on the green background formed a disruptive pattern to conceal the body contours [43]. Countershading via dark and light colours on dorsal and ventral surfaces, respectively, decreases apparent relief [44]. Complex patterning indicates a diurnal lifestyle and strong selection for substrate matching to reduce visibility to visual predators [45]. Patterning in extant reptiles often comprises a mosaic of elements reflecting antagonistic selective pressures relating to homeostasis and signalling [11]. Bright hues may impact negatively on survival but are implicated in social interactions [46]. Thus the patterning in the fossil snake probably served dual functions in camouflage and intraspecific signalling.

Until now, reconstructions of the original coloration of fossil vertebrates have been of melanin-based mechanisms and from soft tissues preserved as carbonaceous remains. Reconstructions of the original colours of vertebrates preserved via this pathway have not been able to incorporate contributions from non-melanin-based coloration mechanisms [3]. Maturation experiments simulating aspects of the organic preservation process have shown that non-melanin-based coloration mechanisms have a lower preservation potential than those that are based on melanin [47]. Our discovery confirms that direct evidence for diverse coloration mechanisms can be preserved in fossils preserved via an alternative preservation pathway, namely replication of tissues in authigenic minerals, and that the high fidelity of preservation allows original coloration to be reconstructed. The various factors that control phosphatization of soft tissues are known [25] and fossil examples of phosphatized skin are not uncommon; importantly, they have been reported from various taxa and fossil localities [16, 17], suggesting that our discovery has broad

applications in the fossil record. Our discovery should prompt a search for other examples, and is likely to be the first example of a recurrent phenomenon. Integuments replicated in calcium phosphate are obvious targets for further attempts to reconstruct colour patterns derived from melanin and, critically, other pigments and structural coloration mechanisms, across diverse vertebrate groups.

Experimental Procedures

Electron microscopy

Samples of fossilised skin were prepared for scanning and transmission electron microscopy as in [7]. Samples of skin from the extant snake *Ahaetulla prasina* were frozen with liquid N_2 and fractured with a scalpel. Samples were examined using a FEI XL-30 ESEM-FEG SEM, a FEI Quanta 650 FEG SEM and a Hitachi S-3500N variable pressure SEM at accelerating voltages of 5–15kV and a JEOL 2100 TEM at an accelerating voltage of 200 kV.

Electron probe microanalysis

Samples of fossilised skin were embedded in resin, polished, and examined using a JEOL JXA 8530F Electron Microprobe. All maps were produced in wavelength dispersive X-ray spectroscopy mode at an accelerating voltage of 15 kV, current of 10 nA and dwell time of 500 ms per pixel.

Histology

Samples of skin from the extant snakes *Ahaetulla prasina*, *Crotalus scutulatus* and *Thamnophis sirtalis* were fixed and dehydrated as in [7] and embedded in paraffin wax. 30 µm thick sections were stained using haematoxylin and eosin.

Author contributions

MMN designed the study and wrote the manuscript with input from all other authors. SK carried out EPMA analyses, and MMN, all other analyses.

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Data Accessibility

Data are available at the Cork Open Research Archive (CORA) (http://cora.ucc.ie). Requests for specimens and samples should be addressed to MMN.

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Figure legends

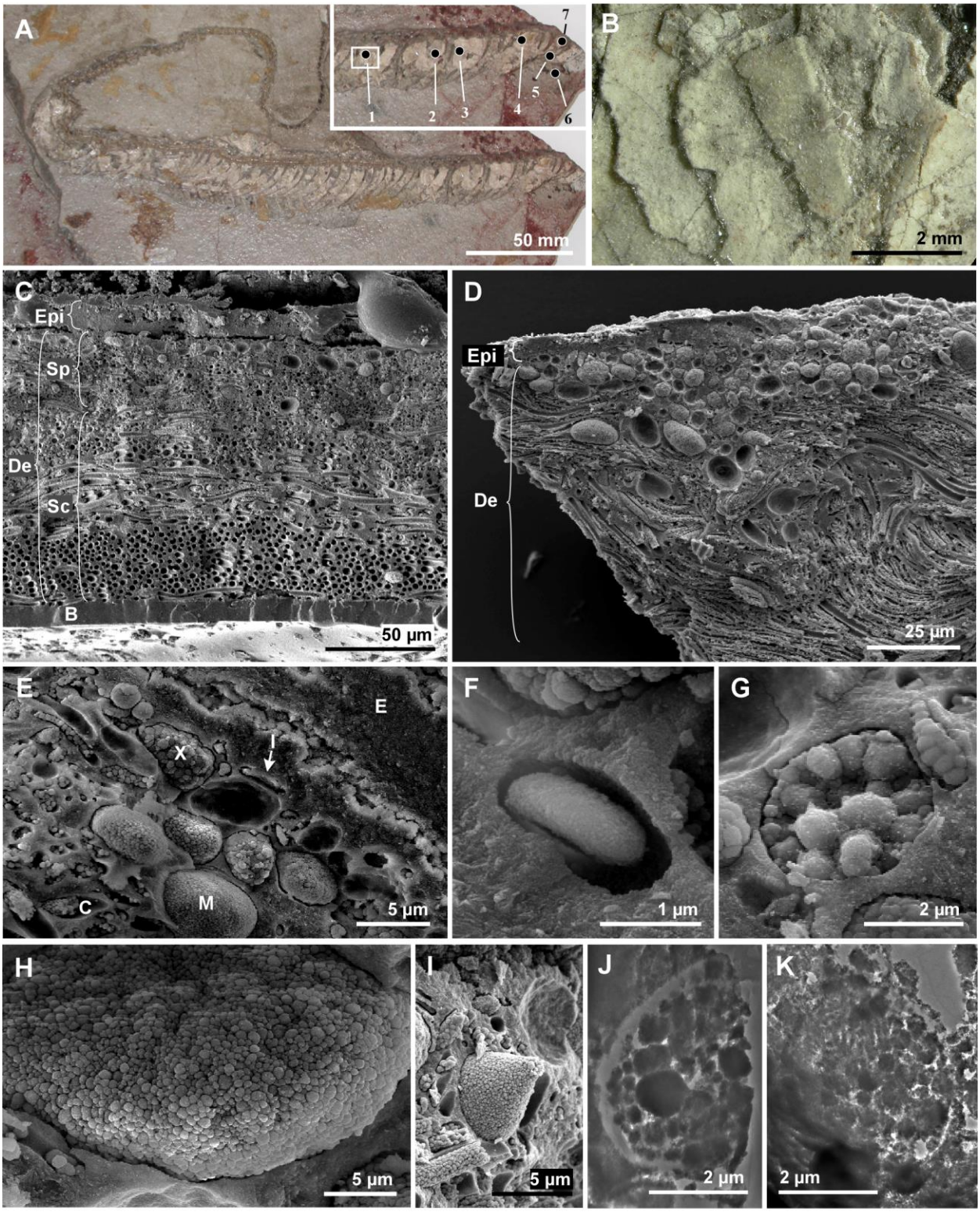
Figure 1. Preserved skin in a fossil colubrid snake (MNCN 66503). (A) Entire specimen; inset, anterior. Cream-coloured material is fossil skin. Numerals 1–7 indicate sample locations. (B) Overlapping scales. (C–E) Scanning electron micrographs (SEMs) of fractured vertical sections through the skin, showing epidermis (Epi), dermis (De), basement membrane (B), chromatophores (iridophores (I), melanophores (M), xanthophores (X)), stratum spongiosum (Sp), stratum compactum (Sc), and collagen fibres (C). (F–I) Details of iridophore (F), xanthophore (G), melanophores (H, I). (J, K) Transmission electron micrographs of xanthophore (J), melanophore (K). The voids in SEM images typically represent structures that have separated into the counterpart of the sample during preparation.

Figure 2. SEMs of vertical sections through the fossil skin showing variation in the relative abundance of different chromatophores (A–C, G–I) with interpretative drawings (D–F, J–L). Encircled numerals correspond to sample numbers in Figure 1(A). (A) Abundant xanthophores, common iridophores and melanophores. Epi., epidermis. (B) Common iridophores and xanthophores, occasional melanophores. (C) Abundant iridophores, common melanophores and xanthophores. (G) Abundant melanophores, common xanthophores, rare iridophores. (H) Abundant xanthophores, occasional iridophores, rare melanophores. (I) Abundant xanthophores and iridophores, rare melanophores. See also Figures S1–S4.

Figure 3. Electron probe microanalysis X-ray maps of a polished vertical section through the skin of the fossil snake MNCN 66503. Areas mapped in (A) and (B) show the uppermost stratum spongiosum; the upper surface of the skin is to the left in (A) and associated elemental maps for C, Mg, Al, P, S, Cl, K, Ca, Mn, Fe and Cs, and to the top of (B) and associated maps for Co, Cu

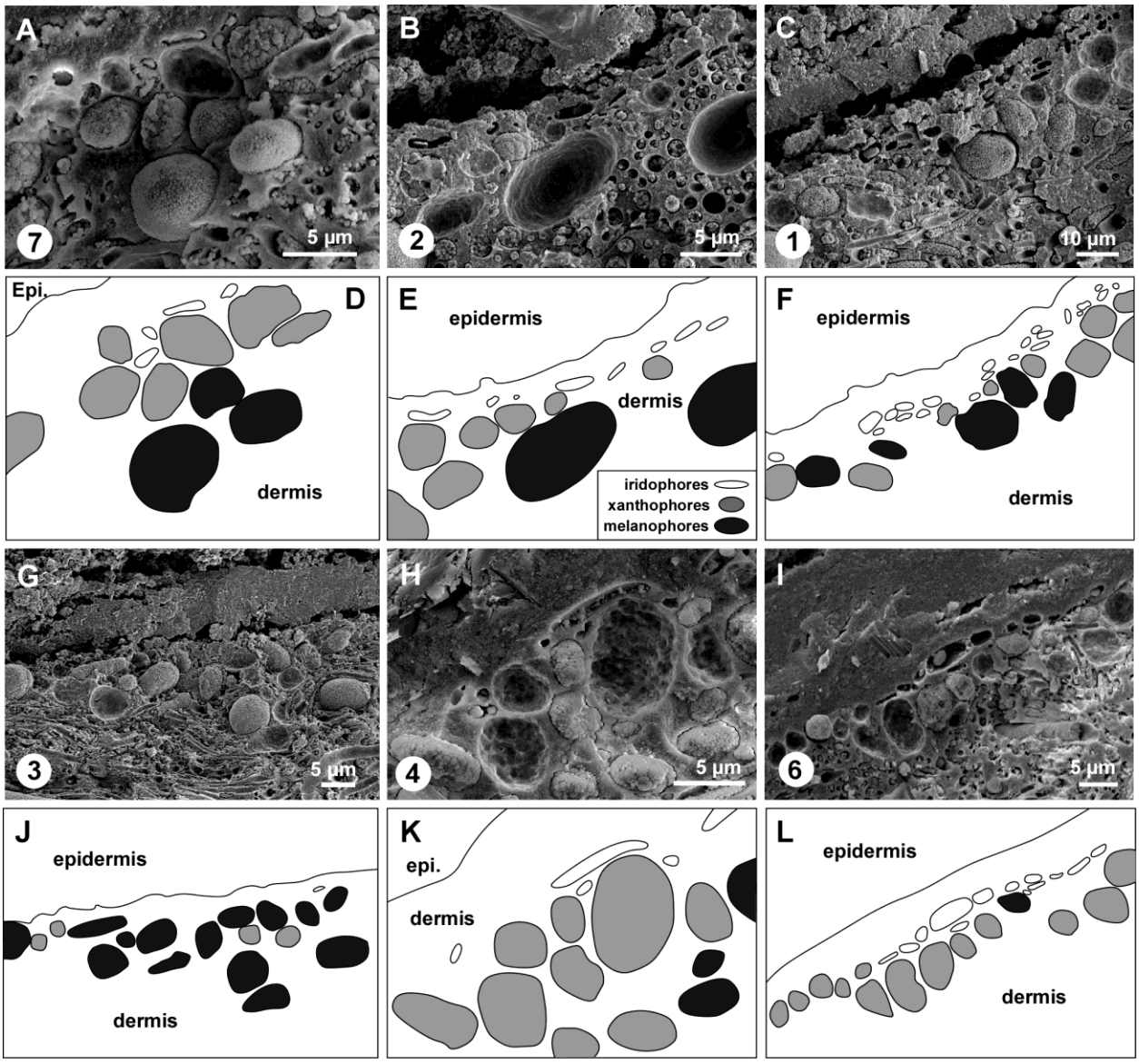
and Zn. C, collagen fibre; M, melanophore; X, xanthophore. Limited variation in tone in maps for Cu, Co and Zn indicate consistently low concentrations of these elements over the area analyzed; colour scale for all other images ranges from blue (low values) to red (high values). Scale bars: 10 µm.

Figure 4. Colour reconstruction of the fossil snake MNCN 66503. (A) Schematic representation of the relative abundance and position of chromatophores in samples of skin from different body regions. Numbers denote samples discussed in the text. See also Tables S1, S2. (B) Colour plate is by Jim Robbins.



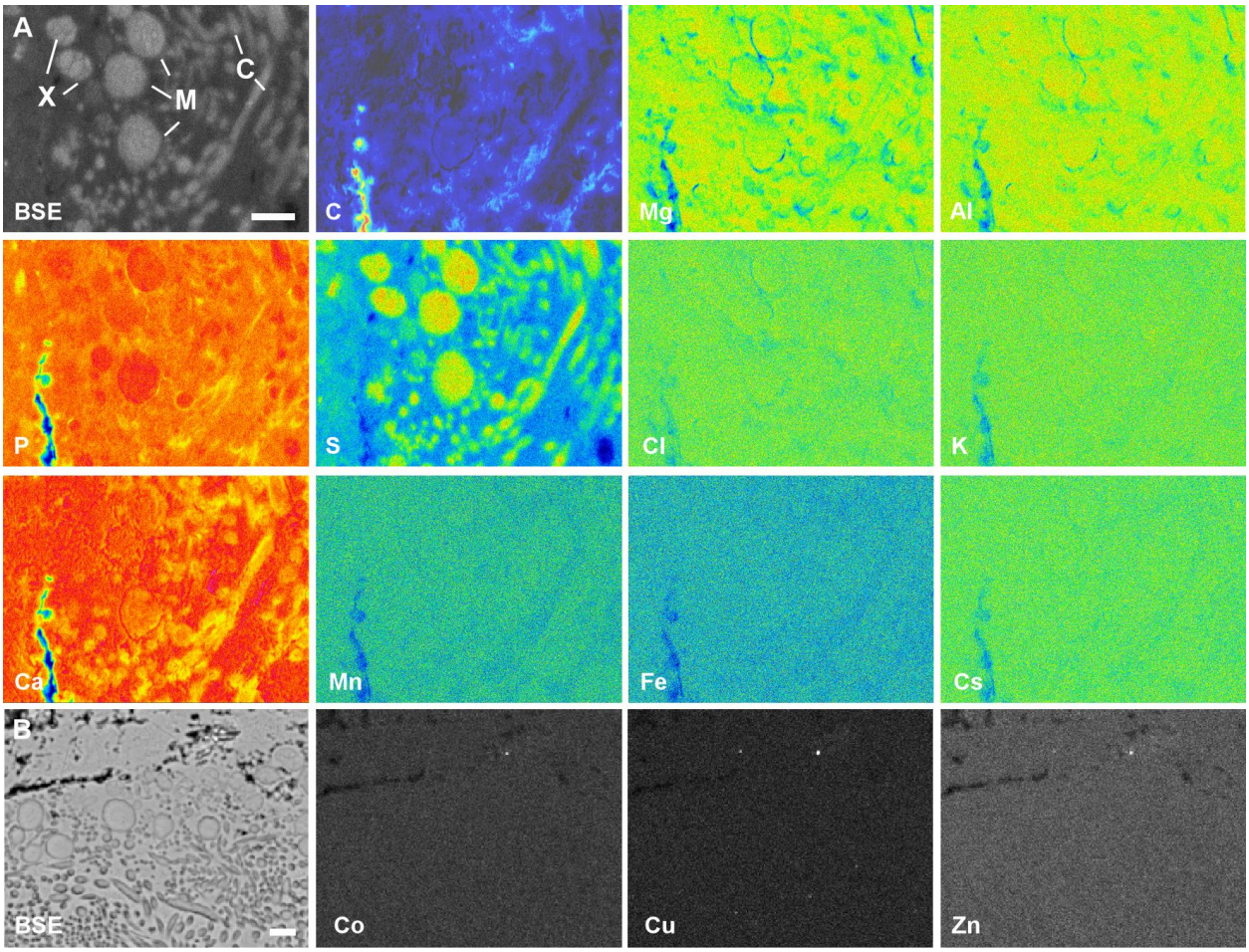
McNamara et al. Figure 1

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